PRINCIPLES OF MEDICAL BIOLOGY

Edited by

E. EDWARD BITTAR

NEVILLE BITTAR

MICROBIOLOGY

Microbiology

PRINCIPLES OF MEDICAL BIOLOGY
A Multi-Volume Work, Volume 9A

Editors: **E. EDWARD BITTAR,** *Department of Physiology, University of Wisconsin, Madison*
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Principles of Medical Biology
A Multi-Volume Work

Edited by **E. Edward Bittar**, *Department of Physiology, University of Wisconsin, Madison and*
Neville Bittar, *Department of Medicine, University of Wisconsin, Madison*

This work provides:

* A holistic treatment of the main medical disciplines. The basic sciences including most of the achievements in cell and molecular biology have been blended with pathology and clinical medicine. Thus, a special feature is that departmental barriers have been overcome.
* The subject matter covered in preclinical and clinical courses has been reduced by almost one-third without sacrificing any of the essentials of a sound medical education. This information base thus represents an integrated core curriculum.
* The movement towards reform in medical teaching calls for the adoption of an integrated core curriculum involving small-group teaching and the recognition of the student as an active learner.
* There are increasing indications that the traditional education system in which the teacher plays the role of expert and the student that of a passive learner is undergoing reform in many medical schools. The trend can only grow.
* Medical biology as the new profession has the power to simplify the problem of reductionism.
* Over 700 internationally acclaimed medical scientists, pathologists, clinical investigators, clinicians and bioethicists are participants in this undertaking.

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Microbiology

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PREFACE

There is a need in small group teaching for a readable module that provides a balanced treatment of the four main areas of medical microbiology–bacteriology, mycology, virology and parasitology. It need not be encyclopedic in scope nor didactic, but it should emphasize principles and concepts. Any existing gaps in this type of presentation are, of course, left for the student to fill.

Some subject material has been excluded. An example is a chapter on laboratory procedures including PCR for rapid bacterial and viral diagnosis. The discussion of bacterial sexually transmitted diseases does not cover gonococcal infections. This is not a serious matter because the tutor can assign the topic to the students. Moreover, we have reluctantly omitted a separate chapter on anaerobic bacteria. The subject of nosocomial pathogens is touched upon but not in sufficient detail (e.g., control). These bacteria (e.g., *S. aureus*, *E. coli* and pseudomonas) are found in hospitals and are resistant to disinfectants and antibiotics. A new but serious problem is the emergence of resistance to antiviral agents.

Without question, molecular biology owes more to the study of viruses than bacteria. The fact remains, however, that effective therapy against most viral diseases is not yet available. Perhaps one of the most dramatic examples of this situation is the fight against the AIDS virus and the search for a vaccine. The public health challenge of AIDS remains formidable in spite of the recent encouraging results obtained with protease inhibitor therapy. At the moment at least six receptors for HIV are known to be present in human cells. One of them is the CCR5 receptor

in the absence of which cells fail to get infected with the virus. Drugs that can interrupt CCR5 binding sites on the virus envelope are being vigorously sought. Thus, Volume 9B gives a large place to HIV disease.

The last group of chapters highlight several features of microbiology which are also of clinical importance and heuristic value. The chapter on fever of unknown origin provides fertile soil for problem based learning.

We would like to thank the various contributors for their cooperation and forbearance. Our thanks are also due to Mr. Fred Verhoeven and the staff members of JAI Press for their skill and courtesy.

E. EDWARD BITTAR
NEVILLE BITTAR

Chapter 1

Ultrastructure of Bacteria

SPENCER A. BENSON

INTRODUCTION

The most obvious structural feature of bacteria is their small unicellular size. Bacteria are customarily 0.5 to 2.0 μm in diameter and have lengths of 0.5 to 10 μm or more. A μm equals 10^{-6} meters. For comparison a red blood cell is approximately seven μm in diameter. An important attribute of their small cell size

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is a high surface to volume ratio. This beneficial surface to volume ratio allows simple diffusion to be sufficient for many cellular processes. Small size also allows for rapid growth and lessens the need for internal organelle systems. While the dimensions of a bacterial cell are indicative of its genera it is not a fixed criterion. Bacteria naturally undergo a twofold doubling in size during reproduction. In addition physiological factors such as nutrient concentration, environment, and growth phase all affect cell size.

A second distinguishing feature is their shape. Bacteria have characteristic shapes that are used to identify and classify bacteria. These shapes are shown in Figure 1. They include: round (cocci), rod (bacillus), vibrio (a curved rod), spirilla (corkscrew shaped), and spirochete (a flexible wavy shape). As with size, not all cells within a population necessarily have identical shapes. This type of variation is called pleomorphism. The degree of pleomorphism is affected by growth conditions and culture age. Extreme degrees of pleomorphism are seen for L-form bacteria and mycoplasmas due to the absence of cell walls.

A second unusual cell morphology is the spore body. Spores are specialized dormant forms of the organism able to withstand severe environmental conditions, including dehydration, heat, salt, and pressure. Spores are easily identified as refractory bodies in light microscopy. Often they are present within the mother cell.

A third morphological characteristic is cell grouping. Certain species of bacteria are typically found in specific cell–cell arrangements (see Figure 1 for repre-

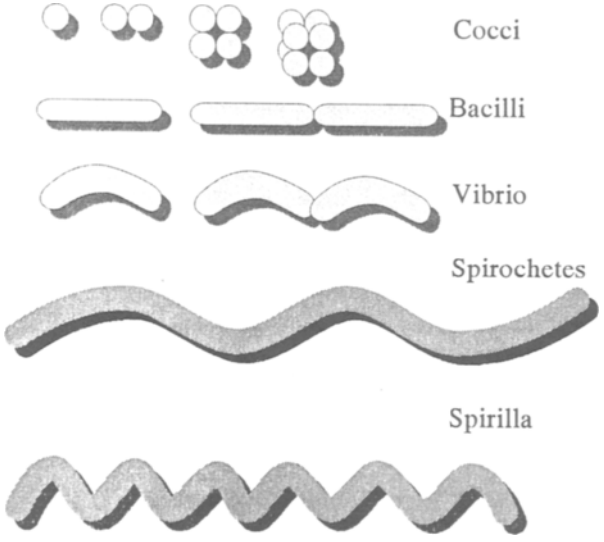

Figure 1. Shapes of bacterial cells. The various common shapes of bacterial cells and common cell grouping are shown. Cells drawn in approximate relative scale to each other.

sentative grouping). These arrangements are useful in identifying the species. They result from the division planes used during replication. Examples of common arrangements for cocci are, paired cocci (two cells), tetrads (four cells), or cubic clusters called sarcina of eight, sixteen, or more cells, and long linear chains of cocci. For bacillus shaped organisms cell grouping is confined to chains of end to end cells. These cells divide across the long axis of the cell.

THE CYTOPLASM

The minimal cell requirements are a cytoplasm surrounded by a membrane termed the cytoplasmic membrane. Nearly all cells are more complex than this. Bacteria are typical cells containing approximately 70 percent by weight water. Most of this water is in the cytoplasm where it serves as the solvent for the concentrated solution of ions, proteins, nucleic acids, metabolic precursors, and other small molecules. Although bacteria generally lack internal structures and do not have an internal cytoskeleton it is inappropriate to think of them as a loose membrane enclosed bound bag of macromolecules. The cytoplasm appears to be highly organized (Goodsell, 1991). This organization is presumably necessary in order for the cell to efficiently carry out the multitude of reactions that must occur simultaneously. Cytoplasmic organization allows for coordination of linked events and for sequentially ordered processes to occur efficiently. An example of metabolic coordination is the coupling of transcription (DNA $\rightarrow$ RNA, information flow) to translation (RNA $\rightarrow$ Protein, informational flow).

Details of the internal organization of the cell are emerging through the use of high resolution electron microscopy. Thin sections through a bacterial cell typically show a dense area that is the chromosome. The bacterial chromosome is the largest molecular structure within the cytoplasm. Typically it contains three to five million base pairs of DNA arranged in a single double stranded circular molecule complexed with protein and RNA. This complex is termed the chromosomal nucleoid. In addition to their chromosome, bacteria often carry ancillary DNA molecules called plasmids. Plasmids are small circular DNA molecules containing a few thousand to several hundred thousand base pairs. These ancillary genomes encode genes whose products are involved in resistance to detrimental agents such as antibiotics or heavy metals, the degradation of unusual compounds, and in the exchange of genetic material between cells. Plasmids are the primary vehicles by which genetic engineers manipulate genes.

A few bacterial species have organelle-like structures in their cytoplasm. None of the internal structures are enclosed by a membrane. Thus, they are not true organelles. The internal structures include storage bodies, gas vacuoles, and membrane associated structures called mesosomes. Mesosomes are membrane-containing structures thought to play a role in segregation of genetic material to daughter cells and energy coupling. They are seen only in Gram positive bacteria and their specific relevance remains to be learned.

The cytoplasm contains many thousands of ribosomes. Ribosomes are biomolecular machines that carry out protein synthesis. Ribosomes are seen in high magnification electron micrographs as specks dispersed throughout the cytoplasm, often as chains called polysomes. They are often associated with membranes and mesosome structures. Bacterial ribosomes are composed of two subunits, a 50s subunit and a smaller 30s subunit. Each unit is composed of proteins and RNA, which is called ribosomal RNA (rRNA). During protein synthesis the two subunits form a 70S complex. Svedberg (S values) are units of molecular size used to describe cellular components. They are determined by ultracentrifugation sedimentation rates. Bacterial ribosomes are sufficiently different from eukaryotic ribosomes. As such, they are important target sites for many antibiotics. A second identifiable cytoplasmic structure is the F1/F0 ATPase. These protein complexes are seen in high magnification electron micrographs as lollipop shaped structures on the inner surface of the cytoplasmic membrane. They are involved in the production of ATP via electron transport.

THE CELL ENVELOPE

The cytoplasm is surrounded and contained by the cell envelope. The cell envelope can be as simple as a single membrane, as is the case for mycoplasmas and L-form bacteria. Usually the cell envelope is a multi-layer structure composed of the cytoplasmic membrane, a cell wall, and one or more layers exterior to the cell wall. The cell envelope is the primary means for identifying and classifying bacteria based on their staining by dyes. The ability to classify bacteria on this basis was recognized by Dr. Christian Gram, a Danish microbiologist, nearly a century ago. It is called the Gram stain. Bacteria fall into three general classes, gram positive, gram negative, and acid fast bacteria (Figure 2). The envelopes of Gram positive organisms are composed of a cytoplasmic membrane surrounded by a thick cell wall containing teichoic, teichuronic and lipoteichoic acids (Figure 2). In gram positive organisms the cell wall forms the exterior of the organism (Figure 3a). Gram negative bacteria have a cytoplasmic membrane, a thin cell wall, and a second membrane, the outer membrane, that forms the exterior of the cell (Figures 2 and 3b). Acid fast bacteria have a cytoplasmic membrane, a thin cell wall, and a thick exterior lipid layer (Figure 2). This unusual lipid layer is composed of mycolic acids, waxes, and other glycolipids. It makes these bacteria impermeable to many substances, notably antibiotics.

Many bacteria produce a bound or loosely associated polysaccharide capsule that surrounds the cell's exterior. This layer is termed the glycocalyx. It is seen in light microscopy as a large non-staining region surrounding the cell when they are placed in colloidal suspensions such as India ink.

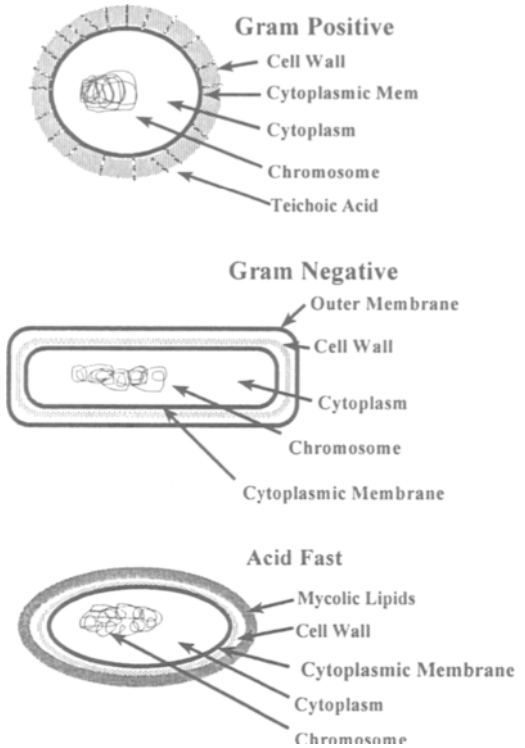

Figure 2. Cell types. The compartments and layer of the three most common bacterial cell classes are shown. The layers are not drawn to scale.

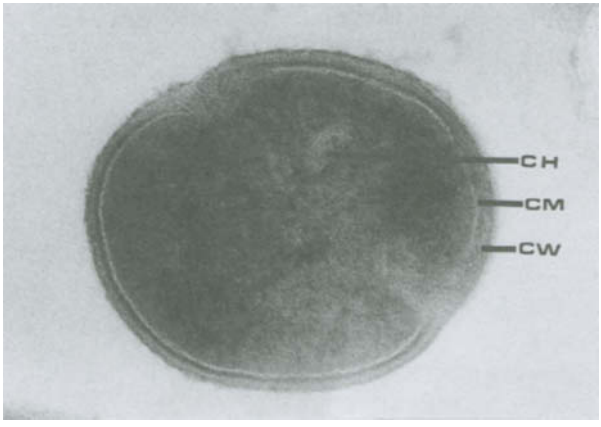

Figure 3. **A**. Thin section through a Gram positive organism (*Staphylococcus*). CH = chromosome, CM = Cytoplasmic membrane, CW = Cell wall. The region that is migrating is the initial stage of septum formation.

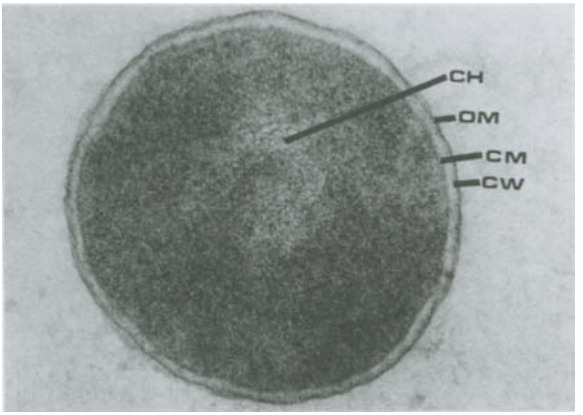

Figure 3. **B**. Thin section through a Gram negative organism (*Escherichia coli*). CH = chromosome, OM = Outer membrane, CM = Cytoplasmic membrane, CW = Cell wall. The periplasm is seen as the space between the OM and CM. Cells were plasmolyzed to enhance the region.

THE CYTOPLASMIC MEMBRANE

Disruption of the cytoplasmic membrane results in cell death; thus, it is essential for cell survival. The cytoplasmic membrane is a thin (4 nm) elastic membrane composed of lipids, (60–70 percent) and proteins (30–40 percent). It contains approximately 20 percent of the cell's protein and most of the cell's lipids. The cytoplasmic membrane contains many different protein species that function in many metabolic processes. These include proteins involved in active transport of molecules into and out of the cytoplasm, electron transport and oxidative phosphorylation, protein synthesis, DNA replication and segregation, secretion of macromolecules, and synthesis of cell wall components. The proteins involved in these processes including proteins that span the membrane one or more times (integral membrane proteins), are associated with one face of the bilayer (peripheral membrane proteins), and proteins that are loosely attached via hydrophobic or protein–protein interactions to either face. The exact protein-lipid composition of the cytoplasmic membrane is influenced by environmental and physiological conditions. However, the ratio of proteins to lipids tends to remain constant reflecting the cell's need to maintain a certain degree of membrane fluidity. To maintain this fluidity the cell regulates the amount and types of cytoplasmic membrane lipids and proteins in response to temperature and other environmental conditions.

The barrier created by the cytoplasmic membrane is sufficiently tight to allow formation of an H^+ gradient across it. This gradient is one of the driving forces behind ATP production via chemiosmosis and the F0/F1 inner membrane structure. All substances that enter the cytoplasm must cross the cytoplasmic membrane by

means of specific transport systems which are energy-dependent. Similarly proteins, polysaccharides, and precursor molecules destined for an extra-cytoplasmic location have overlapping mechanisms that export them across this barrier.

THE CELL WALL

Bacterial shape is determined by the architecture of the cell wall. Bacterial cell walls are made up of a mesh work of interconnected strands of unique polysaccharides cross-linked by small peptides often containing unusual D isomer amino acids. The unique nature of the cell wall components and its structure, which are not present in animal cells, makes it an important target for antibiotics. The cell wall can be thought of as a single huge peptidoglycan molecule, the murine sacculus, the murine layer or simply the peptidoglycan. An important function of the cell wall is to protect the cell from osmotic lysis. The cytoplasm, due to its high concentration of macromolecules, is hypo-osmotic to most environments. The osmotic differential results in several atmospheres of pressure being applied outward against the flexible cytoplasmic membrane and the cell wall. Disruption of the cell wall results in rupture of the cytoplasmic membrane—a lethal event—unless the osmotic pressure is equilibrated by placing the cells in an isotonic solution. Under these conditions the cells form spherical structures called spheroplast (Gram negative bacteria) or protoplast (Gram positive bacteria).

Despite its ridged nature, the cell wall is a dynamic structure. Growth of the cell requires the synthesis of new cell wall components and a restructuring of the existing cell wall (Park, 1987). Consequently, assembly of new cell wall is concomitant with the restructuring of the existing cell wall. Growth proceeds by the coordinate insertion of new peptidoglycan strands concomitant with the cleavage of cross-links between existing strands. Following which new peptide cross-links are formed between the newly synthesized and old polysaccharide strands.

The cell walls of Gram positive bacteria are many layers thick (see Figure 1) and contains several unusual diagnostic molecules such as teichoic, teichuronic, and lipoteichoic acids. These molecules help to anchor the cell walls to the cytoplasmic membrane. They are highly immunogenic and are used as taxonomic markers for the identification of several species (e.g., *Staphylococcus* and *Enterococcus* species). Often there are proteins associated with the surface of the Gram positive cells. An example is the M protein of *Staphococcus*. It extends from the cytoplasmic membrane to the surface of the cell. These surface proteins can be decorated with lipoteichoic acid. In Gram positive bacteria, the cell wall forms the outer surface of the cell. As such, it is the site of primary interactions with host tissues and the environment. Gram positive bacteria are more sensitive to detergents, dyes, and many antibiotics than Gram negative bacteria. This results from the absence of an outer membrane and the mesh work like nature of the cell wall.

The cell wall of Gram negative bacteria is much thinner than that of Gram positive bacteria. It is only one to three layers thick and surrounded by a second exterior

membrane, the outer membrane. The presence of this second membrane protects the cell wall and delineates a specialized compartment, the periplasm or periplasmic space between the cytoplasmic (inner) and outer membrane. The cell wall lies within the periplasm. The cell wall is attached to both the outer membrane and the cytoplasmic membrane. Attachment to the cytoplasmic membrane occurs at sites of synthesis while attachment to the outer membrane occurs via outer membrane proteins that are covalently linked to the peptidoglycan.

Acid fast bacteria such as *Mycobacterium tuberculosis* have a limited amount of peptidoglycan and thus a thin cell wall. The cell surface is covered by a thick layer of specialized lipid molecules. This specialized lipid layer makes the cell resistant to drying, and limits diffusion of molecules into the cell. To a large degree it is responsible for the slow growth characteristics of these strains and the inherent resistance to many antibiotics that are effective against Gram positive and Gram negative bacteria.

THE PERIPLASM

When Gram negative bacteria are plasmolyzed a space between the cytoplasmic and outer membrane is seen (Figure 3b). This area (the periplasm) is on average about 4 to 10 nm wide and often not of uniform width at all points around the cell. The periplasm makes up 1 to 20 percent of the cell's volume. The consistency of the periplasm is gel-like due to the very high concentration of soluble components and the presence of structural linkages that span the periplasm (Foley et al., 1989). The soluble components include specific binding proteins involved in the uptake of sugars, amino acids, vitamins, and phosphate, salvaging enzymes, detoxifying enzymes, and degradation enzymes (Oliver, 1987). The structural links include cell wall synthesis sites, linkages between outer membrane proteins and the cell wall, and Bayer's patches. Bayer's patches are specialized structures connecting the inner and other membranes that are visible in electron micrographs. They are thought to be conduits for the movement of components from the cytoplasm to the outer surface of the cell.

An important function of the periplasm is to protect the cell against environmentally induced osmotic changes. The periplasm is iso-osmotic with the cytoplasm due to the presence of high concentrations of proteins, membrane derived oligosaccharides (MDOs), and the peptidoglycan. It is hypo-osmotic to the environment. Consequently, it serves as an osmotic stabilizer for the cytoplasm. The soluble components can be released by spheroplasting or osmotic shock. These procedures rupture the outer membrane. There are specialized regions at the poles of the cell due to the presence of periseptual annuli that connect the cytoplasmic and outer membranes in these regions. Periseptual annuli are sites of cell wall synthesis involved in septum formation (MacAlister et al., 1983). The priseptal annulus is a structure associated with cell division in Gram-negative bacteria. Septums are the structures that form and separate daughter cells during fission.

THE OUTER MEMBRANE

The outer membrane in thin section electron micrographs appears as a 7 to 10 nm thick double track membrane structure at the outer edge of the cell (Figure 3b). It is a typical membrane bilayer composed of proteins, phospholipids, and lipopolysaccharides (Nikaido and Vaara 1987). The outer membrane contains 3 to 5 percent of the cell's proteins. In contrast to the cytoplasmic membrane, where there are many protein species, the outer membrane has relatively fewer protein species. However, many outer membrane proteins are present in high concentrations (i.e., more than 250,000 molecules per cell). Outer membrane proteins function as general diffusion pores, specific transport proteins, and structural proteins that stabilize outer membrane structure.

An unusual feature of the outer membrane is the asymmetric distribution of the phospholipids and lipopolysaccharides. Phospholipids are found exclusively in the inner leaflet while lipopolysaccharides are found exclusively in the exterior leaflet. Lipopolysaccharides form an effective barrier to hydrophobic compounds and prevent them from integrating into the bilayer. This protects the membrane from being solubilized by detergents and bile salts. The composition of lipopolysaccharides varies widely across species, among members of the same species, and even within a single cell. Due to their tremendous structural diversity and immunogenicity, they are the basis for clinical serotyping of many bacteria. Their outward facing location in the exterior leaflet makes them accessible to the environment and host responses. They are highly immunogenic and endotoxic.

SURFACE APPENDAGES

Many bacteria have surface appendages. These include stock structures, flagella, pili, fimbriae, and axial filaments. These structures are observed in Gram positive, Gram negative, and spirochete species. Bacterial stocks or hold-fasts are stiff rod shaped structures involved in anchoring cells to surfaces. They are thought to be specialized extensions of the peptidoglycan. Bacterial species have different flagella arrangements. These arrangements include a single polar flagellum (monotrichous), multiple flagella at one pole of the cell (lophotrichous), flagella at both poles (amphitrichous), and many flagella distributed along the cell's axis (peritrichous) (Figure 4). Flagella are long, up to ten times the length of the cell, whip-like structure (Figure 5). Flagella are anchored in the envelope structure by a series of proteinous ring structures (Figure 6) (DePamphil and Adler, 1971). These ring structures in combination with the shaft form the basal body. The long filament that makes up the bulk of the flagellum is constructed from flagella protein subunits that are assembled outward from the tip of the flagella. Flagella play important roles in cell movement and chemotaxis.

For bacteria to colonize surfaces the cells must adhere. Adherence to the surfaces of medical instruments, plastic tubing, catheters, and epithelial cells is promoted

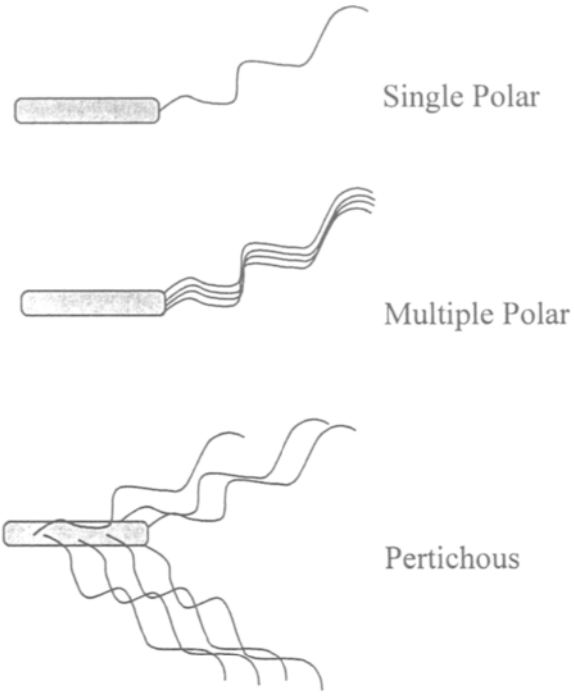

Figure 4. Common flagella arrangements. The common arrangements of flagella for bacillus organisms are shown.

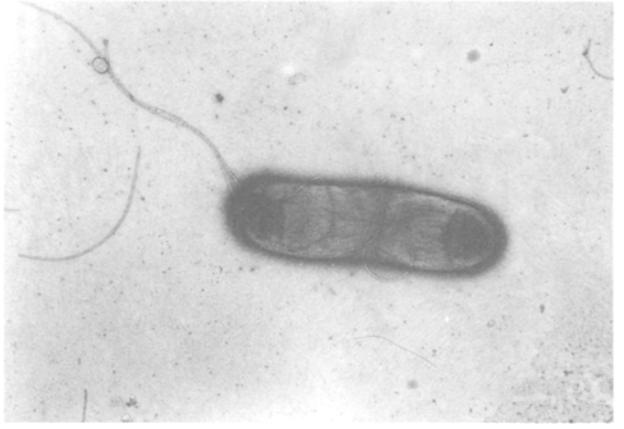

Figure 5. Negative strain of bacteria showing flagella (*Escherichia coli*).

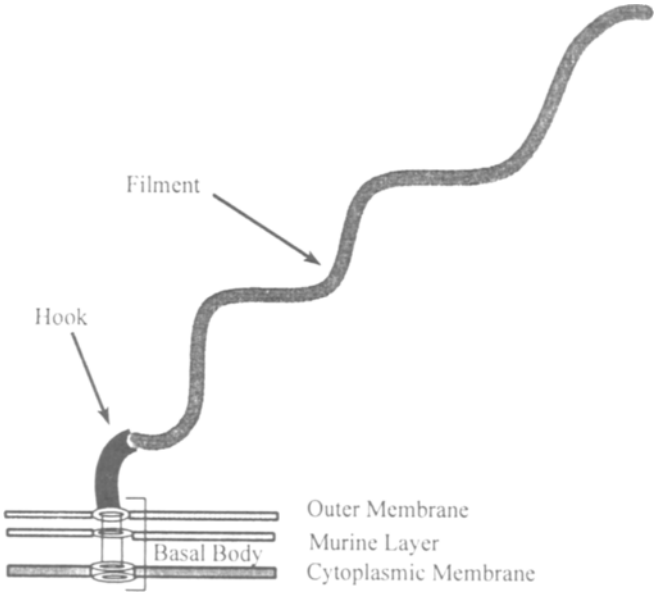

Figure 6. Structure of the flagella. The basic structure of a flagella in a Gram negative organism is diagrammed. The representation is based on that presented by Macnab (1987).

by specialized structures called fimbriae or pili. For clarity, we will use the term fimbriae to refer to appendages involved in adherence and pili to refer to appendages involved in transmission of genetic material. Fimbriae are generally short bristle-like or corkscrew shaped structures visible in electron micrographs as projections radiating from the cell. There are hundreds of fimbria per cell. Fimbria promote adherence by binding to specific host cell receptors, to each other, and presumably innate surfaces. Their actions facilitate cell clumping and adherence. Many pathogenic bacteria require fimbria to establish infections.

Sex pili are long appendages that facilitate the generic exchanges between cells. Unlike fimbria and flagella there is generally a single sex pili per cell. Pili are structurally similar to flagella in that they are long filamentous appendages. They are necessary for the rapid and efficient spread of genetic information within and across bacteria strains and to other organisms such as fungi and plants. Sex pili are encoded by fertility or resistance plasmids; strains carrying these factors are termed males. Mating generally occurs predominantly to recipients (females) that lack the fertility or resistance factor although mating to an other male type strain does occur under certain conditions. Pili facilitate the formation of mating pairs. They function to help bring the two cells next to each other prior to the transfer of genetic material to the recipient cell.

Spirochetes have sheathed axial filaments that originate from the cell poles and wrap around the cell in a helical fashion passing each other at the center of the cell. These filaments play an important role in the movement of the spirochete and are believed responsible for the variety of movements observed for spirochetes.

CAPSULES AND SLIME

The outer surface of many bacteria is covered by a polysaccharide layer called the glycocalyx. This external polysaccharide (EPS) layer is visualized as a non-staining halo surrounding the cell when cells are placed in colloidal suspensions of India ink or methylene blue. An equally striking phenotype conferred by EPS is the mucoidy, slimy, or smooth appearance of colonies on solid media. EPS can be attached to the outer surface where it is referred to as a capsule or it may be shed into the media, in which case it is referred to as slime. Capsules and slimes are composed of repeating units of a sugar polymer containing various xylans, glucans, and other heteropolysaccharides. The biochemical structures of EPS are known for many bacterial capsules and slimes. Physical analyses suggest the polymers are fibrillar in nature. Capsules and slimes protect cells from dehydration and attack from bacteriophages (viruses that attack bacteria), help in evading hosts immune responses, and increase the pathogenicity of the organism. The medical importance of EPS has long been recognized for Gram positive *Pneumoccal* and *Strepococcal* species. It was the capsular nature of virulent *Pneumococci* that assisted, more than fifty years ago, in proving DNA was the genetic material. Capsules are important pathogenic determinants for many Gram negative plant and animal pathogens. Of special concern is the EPS of pathogenic *Pseudomonades*. It plays an important role in plant–microbe interactions, and in respiratory infections of cystic fibrosis patients.

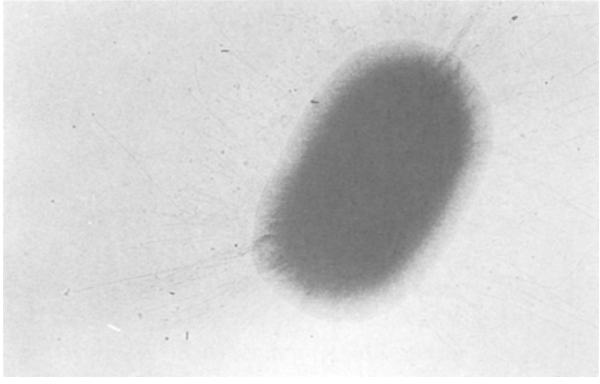

Figure 7. Negative stain electron micrograph showing fimbriae of *Escherichia coli.*

SUMMARY

Bacteria are unicellular organisms that have a variety of sizes, shape, and envelope structures. The minimal requirements are cytoplasm, a cell membrane that surrounds the cytoplasm, and a DNA chromosome. A few have internal structures such as vacuoles and storage bodies but none have true organelles. The cell envelope can be as simple as a single membrane. However, it is generally a multilayered structure that includes a cytoplasmic membrane, a cell wall, and additional structures exterior to the cell wall. The nature of the bacterial envelope determines whether the strain is a Gram positive, Gram negative, or acid fast organism. Gram positive bacteria have thick cell walls, Gram negative bacteria thin cell walls plus a second exterior membrane, and acid fast bacteria have a thin cell wall plus a thick layer of specialized lipids. Cell walls function as an exoskeleton that define the overall cell shape. Outer membranes protect Gram negative cells from detergents and enzymes but limit permeability. They contain specialized channel-forming proteins that provide the means for small molecules to diffuse across this barrier. The outer leaflet of the outer membrane contains lipopolysaccharides. Lipopolysaccharides are unique glycolipids that form a barrier that protects the cell from hydrophobic agents. They are endotoxins, highly immunogenetic, and the surface components recognized by serotyping antibodies. Bacteria can have a variety of surface appendages. These include flagella for cell movement, fimbriae for adherence, and pili for genetic exchanges. Many bacteria surround themselves with a thick polysaccharide coat (the glycocalyx) in the form of a capsule or as slime. This layer helps protect the cells from dehydration and contributes to the pathogenicity of many pathogens.

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Chapter 2

The Bacterial Outer Membrane and Surface Permeability

SPENCER A. BENSON

INTRODUCTION

Gram negative bacteria are distinguished from Gram positive bacteria by the presence of a second membrane, the outer membrane at the exterior of the cell. The outer membrane is the first permeability barrier of the cell. The outer membrane provides both advantages and disadvantages to the cell. It helps protect cells against a variety of chemical and biological agents and, as a result, Gram negative bacteria

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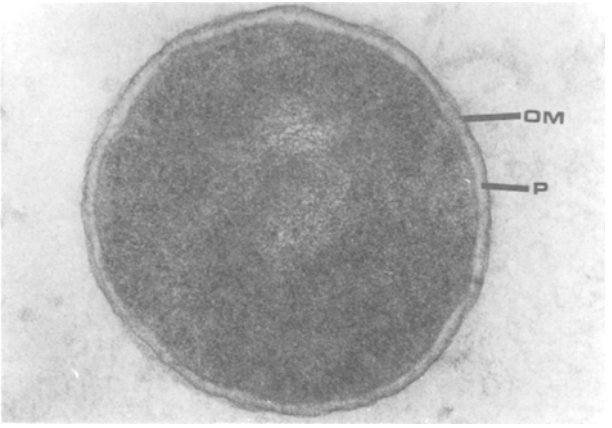

Figure 1. Thin section electron micrograph through a Gram negative organism (*Escherichia coli*). OM = Outer membrane, P = periplasm. The periplasm is seen as the space between the OM and CM. Cells were plasmolyzed to enhance the region.

are more resistant to antibiotics, chemicals, detergents, and degradatative enzymes than are Gram positive organisms. It however also limits the flow of substances into the cell and provides important surface components for recognition by bacteriophages, antibodies, and other detrimental biological molecules such as colicins. Colicins are small proteins produced by certain bacterial strains that are detrimental to other bacterial strains. The outer membrane is not essential for the cell's survival since spheroplasting, which strips away large sections of the outer membrane, yields spheroplasts that are viable and can regenerate an intact structure.

In electron micrograph thin sections, the outer membrane is seen as a 5–10 nm thick double-track structure at the exterior of the cell (see Figure 1). Scanning, negative-staining, and thin sectioning electron micrograph procedures suggest that the outer surface of Gram negative cells is corrugated with many invaginations and surface extrusions (blebs). This rough appearance in part results from the spheroplasting and drying steps used in the preparation of specimens. When Gram negative cells are subjected to freeze-fracture techniques, a different picture emerges. In freeze-fracture micrographs, the outer membrane surface appears smooth with scattered mounds.

COMPOSITION

The outer membrane is composed of phospholipids, lipopolysaccharides, and proteins arranged in a typical fluid mosaic model. The structure of the outer membrane is typical in its organization, but unusual in the asymmetric arrangement

of the phospholipid and lipopolysaccharide components. Phospholipids are present only in the inner leaflet while lipopolysaccharides are present only in the outer leaflet (Nikaido, 1996). The asymmetric distribution of the lipid moieties coupled with the covalent attachment of the outer membrane to the cell wall enables researchers to separate cytoplasmic and outer membranes based on differences in their density and detergent solubility (Figure 2). The outer membrane is more dense than the inner, and is more resistant to detergent solubilization. The phospholipids in the inner leaflet are similar in composition and distribution to those found in the cytoplasmic membrane. The presence of sugar moieties distinguishes lipopolysaccharides from phospholipids. The proteins of the outer membrane include both integral and peripheral membrane proteins. They function in the uptake of molecules, as structural components of the bacterial envelope, as receptors for bacteriophages, and in the secretion of molecules such as colicins, hemolysins, and toxins into the environment.

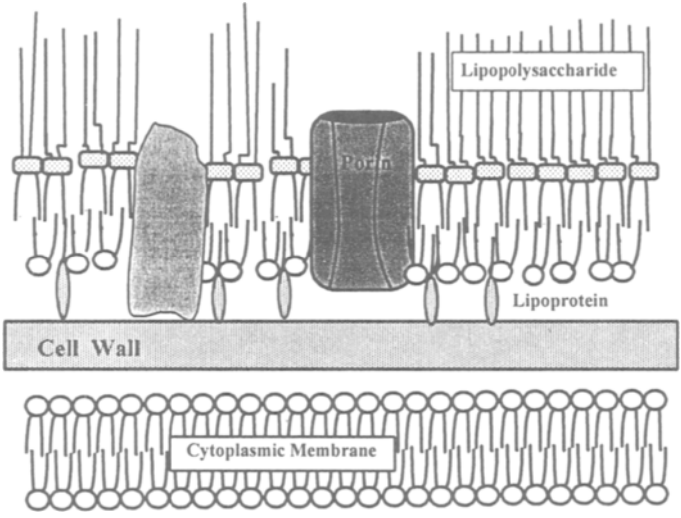

Figure 2. Diagram of the outer membrane. The outer membrane is shown at the top of the figure. The various components, porin, lipoprotein, and lipopolysaccharide are labeled. The asymmetric nature of the two outer membrane leaflets is represented by the differences in the representation of the phospholipid molecules. The cell wall is shown between the cytoplasmic and outer membranes. The cytoplasmic membrane is represented by the phospholipid bilayer at the bottom of the diagram. The cytoplasmic membrane proteins are not shown. The sizes of the various components are not drawn to scale.

OUTER MEMBRANE PROTEINS

The outer membrane contains some 30–50 different protein species. These proteins are present in widely varying amounts. Several outer membrane proteins are extremely abundant cellular proteins present in numbers exceeding 100,000 copies per cell. An example is the Braun's lipoprotein of *Escherichia coli*. This protein can be present at >700,000 molecules per cell, making it the most abundant protein (in number of copies) in the cell. Other examples are the porins and OmpA type proteins. Only a few of the proteins in the outer membrane have known enzymatic activities.

The roles of the various outer membrane proteins reflect the functions of the outer membrane: protection and uptake. Proteins that help maintain the structural integrity of the outer membrane do so by covalent attachment to the cell wall. This linkage stabilizes the outer membrane and serves to make the cell envelope an integrated structure. These proteins, while necessary to maintain the structural integrity of the outer membrane, are not essential, such as the genes that encode these proteins can be destroyed without killing the cell. Braun's lipoprotein which is an unusual structural protein, has a lipid moiety at its amino terminal end. This lipid tail integrates into the inner leaflet of the outer membrane, attaching the protein to the outer membrane. The other end of the protein is attached (bound) to the peptidoglycan. The OmpA protein spans the outer membrane and may be bound to the peptidoglycan. It serves as a structural component that stabilizes the outer membrane and helps in the formation of mating pairs for the transmission of genetic information during plasmid-mediated conjugation. Recent work shows that it also has a channel-forming activity. Like many outer membrane proteins it functions as a receptor for numerous bacteriophages.

The best understood proteins of the outer membrane are the porins. Porins are specialized proteins that form channels through the outer membrane. They are found not only in all Gram negative bacteria but also in mycobacteria and eukaryotic organelles such as mitochondria and chloroplasts (Benz and Bauer, 1988). They are the primary means by which small molecules cross the barrier of the outer membrane. As such, changes in the number or composition of the porins directly influence the cell's permeability to small molecules such as antibiotics. Consequently, they are an important component in determining resistance to antibiotics and other compounds.

The structure of the porins has been extensively studied mainly because they are a model system for structural studies of transmembrane channel proteins. They are one of the few membrane proteins that are amenable to X-ray crystallographic studies. Porins are extremely robust, protease resistant, and have a tight association with lipopolysaccharides. They are generally found in a stable trimeric structure. Physical and crystallographic studies show that they are composed of a series of anti-parallel β-sheets, arranged like the staves of a barrel to form an angled channel that passes through the center of the protein. Approximately midway through the

channel is a short peptide segment, or arm, that extends into the channel (Cowan et al., 1992). This helps to determine the size of the channel. Porin channels are sufficient to allow molecules of 400 to 600 daltons to pass.

Bacterial porins can be divided into two categories based on the type of molecules they take up. They can be nonspecific, i.e., the channel selectively screens on the basis of size, or they can be specific and mediate the uptake of a particular compound or class of compounds. Both types of porins are regulated in response to environmental signals. For the nonspecific porins the signals include osmolarity, heat, and the presence of certain chemicals. The specific porins are expressed when there is a need for the compound for which they have specificity. Examples of specific porins are those that facilitate the uptake of phosphate and other anions, porins involved in the uptake of glucose and glucose polymers, and porins involved in the uptake of nucleosides.

Molecules larger than 600 daltons generally require specific outer membrane proteins to transfer them across the outer membrane barrier. These can be porin type-proteins or other non-porin channel forming proteins. Of particular interest are proteins involved in the uptake of iron. Bacteria, like all cells, need iron to grow. Although iron is an abundant element, it is not readily accessible to bacteria. Iron in the environment is mostly tied up in insoluble oxide forms. More importantly, there is very little iron, in vertebrates and that which is present is complexed with protein such as hemoglobins, cytochromes, and lactoferrins. To compete for iron bacteria have evolved extremely efficient iron salvaging systems. In Gram negative bacteria these systems include chelators, called siderophores, that bind iron and toxins (hemolysins) that lyse red blood cells. To get iron across the outer membrane Gram negative strains have specialized outer membrane proteins for the recognition and uptake of the iron-siderophore complexes.

LIPOPOLYSACCHARIDES

The outer membrane is an unusual membrane in that it confers resistance to hydrophobic agents. Unlike typical membranes, e.g., the cytoplasmic membrane, it is not dissolved by detergents. This allows Gram negative organisms to withstand high detergent concentrations. This resistance is illustrated by the finding that *Pseudomonas* strains can colonize many types of soaps. Detergent resistance is conferred by lipopolysaccharide components in the outer leaflet.

Lipopolysaccharides are unique to Gram negative bacteria and are found only in the outer leaflet. Lipopolysaccharide is composed of three adjoining structural units (Figure 3). The first is the glycolipid, lipid-A, which is buried in the outer membrane bilayer. It anchors the lipopolysaccharide to the membrane through the attached fatty acid moieties. The general structure of the Lipid-A moiety is conserved among Gram negative species. This is not surprising, since Lipid-A is an essential cellular component. Mutations and compounds that block synthesis of lipid-A result in cell

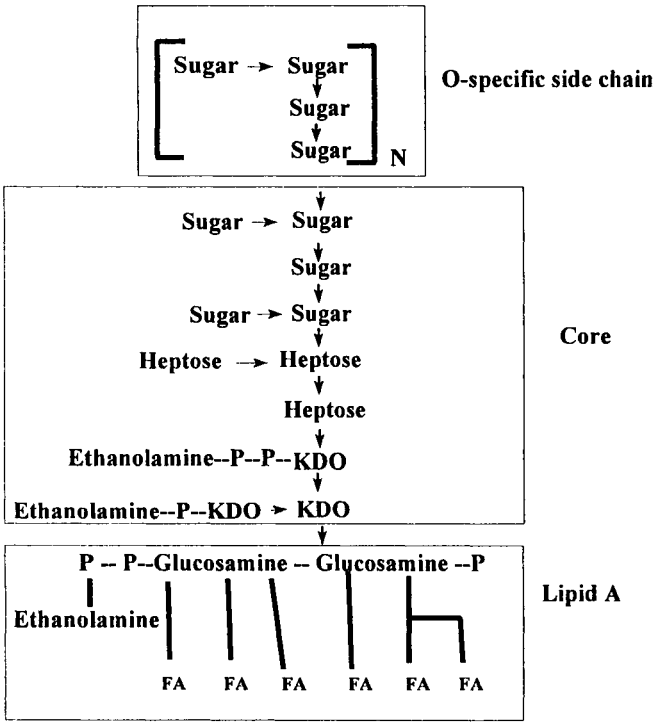

Figure 3. General structure of lipopolysaccharide. The structure is based on the lipopolysaccharide of a Gram negative species such as *Salmonella*. The three regions of lipopolysaccharide are boxed. The O-side chain is generally larger than the core region and is composed of repeating units of tri- tetra- or penta- saccharides. The number (N) of O-specific side chain repeating units can be as large as 20–40. FA designates the fatty acid chains, which include dodecanoic, tetradecanoic and hexadecanoic acids. Phosphate residues are represented by the letter P. Adapted from Neidhardt et al. (1990).

death. Lipid-A is a potent endotoxin responsible for the fever, malaise, and other symptoms often associated with bacterial infections.

Extending outward from the lipid-A unit is the second unit, the core. The core contains sugar units linked by glycosidic bonds. These units can be in either straight or branched chain configurations. They characteristically include heptose and ketodeoxyoctonoic (KDO), as well as hexoses and hexosamine sugars. Often there are attached phosphate and ethanolamine residues. Mutations that result in a truncated core confer upon the cell a greatly increased sensitivity to detergents and hydrophobic antibiotics. There is enormous variation in the primary structure, e.g., the type and sequences of sugar residues, of the core region in different bacterial strains.

The third unit of the lipopolysaccharide structure is the O-antigen. This highly variable layer is composed of repeating units, as many as 40 units, of defined sugar groups. The resulting long carbohydrate chains cover the cell's outer surface. This carbohydrate layer together in conjunction with the core, helps to exclude detergents and hydrophobic compounds from the cell. There are thousands of different permutations of the repeating units found in O-antigen. O-antigen is highly immunogenic. Due to its immunogenic nature it is a primary target for the host immune system. This, coupled with its highly variable nature, allows researchers and health officials to easily identify specific strains using antibodies to this surface component. This is called serotyping. For enteric pathogens, such as, *Escherichia* and *Salmonella*, there are hundreds of different serotypes. O-antigen is not essential for lipopolysaccharide structure. Some bacterial strains naturally lack O-antigen. Examples include *Escherichia coli* K12, the world's most-studied and best understood organism, and the pathogen *Neisseria gonorrhoea*, the casual agent of the sexually transmitted disease Gonorrhoea.

PERMEABILITY

The permeability of Gram negative bacteria is, to a large measure, determined by the outer membrane. The transit of most material across this membrane barrier is predominantly via diffusion-driven processes. Diffusion processes are dependent upon the concentration of substrates on either side of the membrane, the physical nature of the substrate, and the nature of the membrane channel. Large substrates will have slower diffusion rates than small substrates if both pass through the same nonspecific channel. The number of channels affect the permeability of the outer membrane, i.e., there is increased permeability when there are more channels. Presumably the cell has an abundance of nonspecific porins so that uptake of necessary metabolites does not become growth-limiting. In addition to number, the size of the channels influences permeability, i.e., if a cell's channels are small then permeability is less than it would be for a cell which has a similar number of channels but of larger diameter. Some strains have nonspecific porins with different channel diameters, and regulate relative expression of these porins in response to environmental signals. Channels that have specificity for certain compounds bypass limitations of channel size and/or level of expression. Lastly, factors that decrease or increase accessibility to the cell surface and channels through the outer membrane alter permeability.

The bulk permeability of the outer membrane is determined by many factors. Foremost is the composition of the membrane itself. Mutations that alter the structure of individual components such as the porins, lipopolysaccharides, and lipid components can result in increased or decreased permeability. Physiological conditions such as temperature and pH, and physical conditions, such as freezing and treatment with local anesthetics can alter the permeability of the outer membrane. Lastly, genetic mechanisms that regulate expression of outer membrane

proteins, lipopolysaccharides, and the glycocalyx are used by the cell to modulate permeability. In combination these internal and external factors allow bacteria to adapt to changing environmental conditions and successfully establish populations in many different ecological niches.

SUMMARY

The outer membrane forms the exterior layer of the cell envelope of Gram negative bacteria and differentiates these from Gram positive and acid fast bacteria. It serves as the first selective permeability barrier of the cell by limiting access of components to the periplasm. It also serves as the site of recognition for many agents including bacteriophages, colicins, and antibodies. The membrane bilayer is asymmetric with phospholipids present in the inner leaflet and lipopolysaccharides in the outer leaflet. Proteins in the outer membrane link the outer membrane to the cell wall and function as channels through which substances cross the lipid bilayer. These channels can be specific or nonspecific. Large molecules and those with charges use specific mechanisms to move across the outer membrane. Lipopolysaccharides are glycolipid macromolecules that are unique to Gram negative bacteria. They protect the cell from hydrophobic substances such as detergents. They are highly immunogenic, used to taxonomically classify bacterial strains, and endotoxins. The bulk permeability of Gram negative bacteria is largely determined by the composition of the membrane proteins and the structure of the lipopolysaccharides.

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Chapter 3

Bacterial Metabolism

HARRIS BERNSTEIN and CAROL BERNSTEIN

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INTRODUCTION

When life emerged about 4 billion years ago, the earliest protocellular organisms were probably primitive bacteria. Furthermore, bacteria were probably the only existing cellular life form for more than half of the total period of evolution of life on earth. A high proportion of the genetic information in extant bacteria is devoted to metabolism. For instance, about 35 percent of the 1400 known genes in *Escherichia coli* encode metabolic enzymes (Bachmann, 1990). This finding, plus the fact that there is great similarity among divergent bacterial species in their metabolic pathways, suggests that the perfection of enzyme-catalyzed metabolic pathways was a high priority during the early evolution of life. Many of the metabolic processes that emerged in early bacteria were passed on to eukaryotic descendants when eukaryotes diverged from their prokaryotic ancestors about 1.5 billion years ago. These descendants include mammals and, in particular, humans who share many basic metabolic pathways with extant bacteria. A knowledge of bacterial metabolism therefore is helpful in understanding basic aspects of human metabolism. Familiarity with this subject is also helpful in understanding bacterial infection processes and in the identification of pathogenic bacteria. Bacterial metabolism has been comprehensively reviewed by Gottschalk (1986), whose book should be consulted for detailed information.

CARBOHYDRATE METABOLISM AND ATP PRODUCTION

An energy source is needed to fuel most of the life processes of bacteria. For instance, replication of the genetic material, considered by some to be the most basic life process, requires energy. Methods for extracting energy from molecules in the environment probably emerged in protocellular organisms in order to drive genome replication and other processes, such as protection from environmental hazards and the gathering of substrates used as cellular building blocks.

In general, those metabolic pathways employed in the synthesis of cellular constituents are referred to as *anabolic*. These pathways generally require energy input. Degradative pathways are referred to as *catabolic*. These catabolic pathways include energy yielding processes such as fermentation and respiration. Fermentation is a rearrangement of the atoms of an organic substrate (without net oxidation) which yields useful energy. Respiration ordinarily involves the net oxidation of substrates at the expense of molecular oxygen (although some bacteria use an inorganic electron acceptor other than O_2 such as nitrate, sulfate, or CO_2). Respiration is a much more efficient process than fermentation for obtaining useful energy from organic substrates. Despite the greater efficiency of respiration, fermentative bacteria persist in the biota because many species are adapted to grow in the depths of liquids or in tissues of a host organism where oxygen is not ordinarily available.

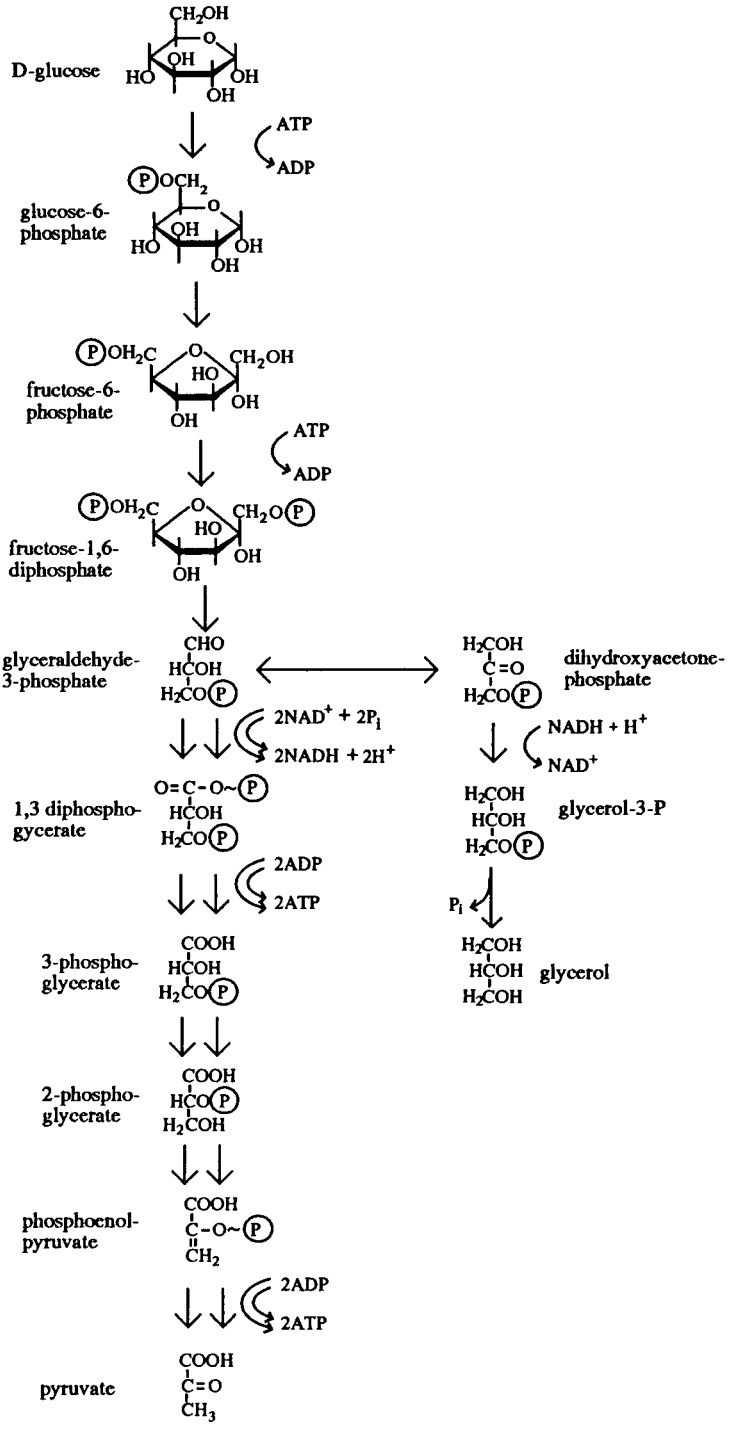
Bacteria which use organic compounds, rather than inorganic ones, as a source of carbon and energy, whether by fermentation or respiration, are known as chemoheterotrophs. Other broad classes of bacteria are phototrophs which obtain energy from light, and chemoautotrophs which obtain energy by the oxidation of inorganic substances such as ammonia, sulfides and ferrous compounds. We will confine our further discussion of energy metabolism to chemoheterotrophs since they include all the bacteria of medical importance. Although chemoheterotrophic bacteria can utilize numerous different organic substrates as a source of energy (e.g., fatty acids, lipids, and amino acids), carbohydrates, and particularly glucose, are a preferred source. Glucose is a very widely distributed sugar, being the monomer of both starch and cellulose, and most bacteria can use it as a source of energy. In order for the energy contained in an organic molecule to be useful it must ordinarily be transferred to the form of the high energy bond of a nucleotide triphosphate (primarily adenosine triphosphate, ATP). ATP is so widely used for energy transfer that it is regarded as the universal coin of energy exchange in living systems. The study of bacterial carbohydrate metabolism is thus, to a large extent, the study of ATP production.

Glycolysis and Fermentations

The process of trapping metabolic energy in the form of ATP occurs by either of two basic mechanisms: (1) substrate level phosphorylation, discussed in this section, or (2) oxidative phosphorylation, which will be discussed below in the next section. Substrate level phosphorylation occurs during the glycolytic conversion of glucose to pyruvate, referred to as the Embden-Myerhof-Parnas (EMP) pathway (Figure 1). By this pathway, the six-carbon sugar glucose is cleaved into two three-carbon compounds, each of which is converted to pyruvate.

In the EMP pathway substrate level phosphorylation occurs at two points. The first is the uptake of inorganic phosphate by glyceraldehyde-3-phosphate to form 1,3 diphosphoglycerate resulting in the conversion of one of the phosphate bonds to a high energy state (Figure 1). The transfer of this phosphate to ADP then generates a molecule of ATP. The second involves conversion of 3-phosphoglycerate to 2-phosphoglycerate and then to phosphoenolpyruvate. This again generates a high energy phosphate bond and provides for the transfer of energy to ATP. The energy transfer occurs in the reaction of phosphoenolpyruvate to pyruvate (Figure 1). Overall in the EMP pathway, for each molecule of glucose used, four ATPs are formed and two are used for a net yield of two ATPs.

Under anaerobic conditions, nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH during the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Figure 1). (Another name for NAD^+ is DPN^+ which is the abbreviation for diphosphopyridine nucleotide.) The NADH needs to be oxidized back to NAD^+ in order to allow the recycling of this hydrogen carrier. The regeneration of NAD^+ can be accomplished in the absence of oxygen through


D-glucose
ATP
ADP
glucose-6-phosphate
fructose-6-phosphate
ATP
ADP
fructose-1,6-diphosphate
glyceraldehyde-3-phosphate
dihydroxyacetone-phosphate
2NAD+ + 2Pi
2NADH + 2H+
NADH + H+
NAD+
1,3 diphospho-gycerate
glycerol-3-P
2ADP
2ATP
Pi
3-phospho-glycerate
glycerol
2-phospho-glycerate
phosphoenol-pyruvate
2ADP
2ATP
pyruvate

further reactions of pyruvate (the final product of the EMP pathway). The further reaction of pyruvate with NADH can occur by different pathways. These different pathways, together with the prior steps of EMP glycolysis, are types of fermentations. Since the particular fermentation(s) used by a bacterial species is characteristic of that species, determination of fermentation end-products is often used to identify bacteria. These further reactions of pyruvate may, or may not, generate additional ATPs beyond the two generated by the EMP pathway.

Several, but not all, bacterial fermentations involve the EMP pathway. In these fermentations there are usually two successive stages to be considered as shown in Figure 2: (1) The EMP pathway leading to the formation of pyruvate (shown in abbreviated form at the top of the figure), and (2) the further reactions which convert the pyruvate into the specific fermentation end-products. Some of these fermentations are reviewed next.

Fermentation pathways, in general, are named after their end products. The lactate fermentation (see [1] in Figure 2), also called the homolactate fermentation, occurs in many lactobacilli and in some pathogenic streptococci as well as in mammalian muscle. In this fermentation, pyruvate is reduced in one step to lactate. In the process NADH is oxidized to NAD^+.

The ethanolic fermentation (see [2] in Figure 2) occurs in yeast and is vital to the baking and brewing industries. Carbon dioxide is released from pyruvate with the formation of acetaldehyde. This in turn is reduced in a reaction utilizing NADH. Although characteristic of yeasts, this fermentation is uncommon as a major pathway in bacteria.

The mixed acid fermentation (see [3] in Figure 2) is used by most Enterobacteriaceae (a family of bacteria whose members grow in the vertebrate intestinal tract). This group includes organisms belonging to the genera *Escherichia, Salmonella*, and *Shigella*, some of which are pathogens. These organisms convert part of the available pyruvate to lactate by the lactate fermentation, but most pyruvate is split to form acetyl CoA and formate; the acetyl CoA undergoes further reactions to yield acetate and also an ATP molecule. Since these reactions do not cycle NADH back to NAD^+, fermentation balance requires that an equal amount of pyruvate be reduced by pathways taking up (H). Two pathways which accomplish this are the conversion of acetyl CoA to ethanol (shown in Figure 2) and the conversion of pyruvate to succinate (not shown). As the pH of the medium drops due to the acids produced, an enzyme is formed by some species that converts formic acid (HCOOH) to H_2 and CO_2 gas. Gas formation is an important diagnostic test for

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Figure 1. The glycolytic formation of pyruvate (EMP pathway). Overall reaction: Glucose + 2ADP + $2P_i$ + $2NAD^+ \rightarrow$ 2 pyruvate + 2ATP + 2NADH + $2H^+$. The double arrows indicate two molecules reacting per molecule of glucose. The phosphate group $-PO_3H_2$ is symbolized by (P). The symbol ~(P) indicates a high energy phosphate. The pathway from dihydroxyacetone-P to glycerol is also indicated.

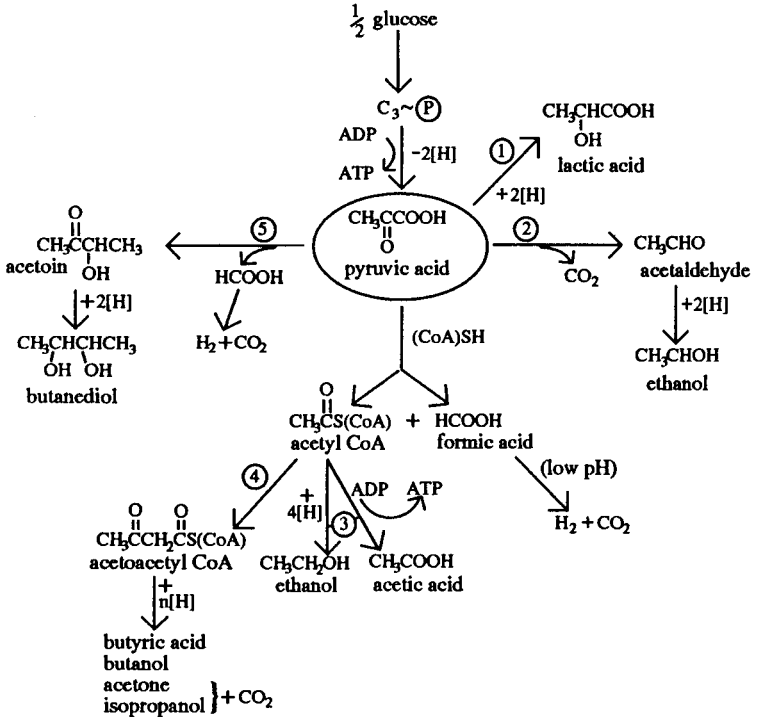


Figure 2. The central role of pyruvate in some important fermentations. 1. Homolactate. 2. Ethanolic. 3. Mixed acid. 4. Butyrate acid, butanol, acetone. 5. Butanediol.

these organisms. Also, in humans, intestinal gas accumulates when these bacteria produce H_2 and CO_2 faster than they can be absorbed by the colon.

The butyrate, butanol, acetone fermentation (see [4] in Figure 2), in general, is carried out only by obligate anaerobes. These include pathogens of the genus *Clostridium*. For instance, gas gangrene is caused by a number of clostridial species, principally *Clostridium perfringens*. The gas that accumulates is predominantly H_2 which is less soluble than the CO_2 also produced by this fermentation. This fermentation has historical significance. Chaim Weizmann developed its use for the industrial production of acetone which solved a critical problem in England during World War I related to explosives manufacture. It is considered that this contribution promoted the Balfour Declaration leading eventually to the State of Israel and also to the election of Chaim Weizmann as the first president of Israel.

The butanediol fermentation (see [5] in Figure 2) occurs in certain Enterobacteriaceae and some members of the genus *Bacillus*. Exposure to air oxidizes the butanediol to acetoin. Acetoin is readily recognized by a specific laboratory color reaction called the Voges-Proskauer test. This widely used test is of considerable diagnostic value. For instance, it is used in sanitary engineering to discriminate

between bacteria which reach bodies of water primarily by fecal contamination and those which originate primarily from vegetation.

Oxidative Metabolism: Tricarboxylic Acid Cycle and Oxidative Phosphorylation

Oxidative phosphorylation, the second process for trapping metabolic energy in the form of ATP, occurs as electrons are released during substrate oxidation and then passed through a series of electron carriers to an ultimate electron acceptor, usually oxygen.

Bacteria are placed into four groups according to how they respond to oxygen; obligate aerobes, obligate anaerobes, facultative organisms, and aerotolerant anaerobes. This classification is of significance in pathogenesis and in the identification of bacteria. The obligate aerobes require oxygen for growth and lack the capacity for substantial fermentation. *Mycobacterium tuberculosis*, the most common cause of tuberculosis, is an example of this type of bacterium. The next group, the obligate anaerobes, can grow only in the absence of oxygen. *Clostridium perfringens* (which, as mentioned above, causes gas gangrene) and *Clostridium tetani* (which causes tetanus) as well as other clostridial species are in this group. Facultative organisms, the third group, can grow without air using fermentation, but switch to respiration in the presence of air. This group includes many enterobacteria and yeasts. Organisms of the fourth group, the aerotolerant anaerobes, can grow with or without oxygen, but in contrast to facultative organisms their metabolism remains fermentative in air. Many of the lactic acid bacteria (those that form large amounts of lactic acid as a fermentation end-product) are in this group.

Most obligate anaerobes are rapidly killed by air. The cause of this lethality is a highly reactive form of O_2, referred to as the superoxide free radical (O_2^-). Superoxide is neutralized by superoxide dismutase. This enzyme is present in aerobes, facultative and aerotolerant organisms but is lacking in strict anaerobes. The reaction catalyzed by superoxide dismutase is:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The clostridial species which cause gas gangrene are examples of bacteria which lack superoxide dismutase and are thus subject to the toxic effect of O_2^-. This has clinical significance, since it is beneficial to treat some gangrene patients (e.g., those in which the infection has spread to regions that cannot be amputated, such as the trunk) with hyperbaric oxygen in a compression chamber.

In those bacteria possessing superoxide dismutase, the hydrogen peroxide (H_2O_2) formed is also toxic, but this can be destroyed by the catalase normally present by the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

When glucose is available as substrate, respiration involves its oxidation by molecular oxygen to carbon dioxide and water in the overall reaction:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

This reaction releases 688,000 calories per mole, equivalent to the amount of energy released as heat upon burning glucose in air. In bacteria this process occurs by over 20 enzyme-catalyzed steps during which much of the energy that is released is captured in the form of the high energy bond of ATP (although some is still lost as heat).

In the commonest pattern, respiration involves the conversion of glucose to pyruvate by the EMP pathway. The pyruvate is then oxidized to acetyl CoA and CO_2, and the acetyl CoA is further oxidized via the tricarboxylic acid (TCA) cycle (Figure 3). Electrons (from the substrate hydrogen atoms) released during the oxidation steps of this cycle are transferred through the electron transport chain to oxygen, resulting in the generation of substantial energy. To initiate this reaction a substrate hydrogen atom (proton + electron) plus another electron from a second hydrogen atom is transferred to NAD^+ to form the reduced derivative NADH plus a proton (H^+). As indicated in Figure 3, electrons are passed to NAD^+ to form NADH at three stages in the TCA cycle as well as at one stage in the conversion of glucose to pyruvate and in the conversion of pyruvate to acetyl CoA. Also electrons are transferred directly from succinate in the TCA cycle to flavoprotein without going through a NADH intermediate. Altogether, in the conversion of glucose to H_2O and CO_2 by respiration, two NADH molecules (or their equivalent) can be produced from a glucose molecule at six stages. Since each NADH releases enough energy to generate three high energy bonds of ATP the theoretical yield of ATP by oxidative phosphorylation per molecule of glucose is 36 ($2 \times 6 \times 3$). Also, as discussed above, an additional two ATP's per glucose are produced by substrate level phosphorylation. Thus the overall theoretical yield is 38 ATP's per fully oxidized molecule of glucose, although the actual yield for any particular organism is ordinarily somewhat less. This contrasts with the overall yield of 1–3 ATP's per molecule of glucose typically produced by a fermentation pathway.

The components of the electron transport chain (which include flavoprotein, cytochromes and cytochrome oxidase) are located in the cytoplasmic membrane of bacteria. A widely accepted explanation for the coupling of electron transport to the generation of ATP is referred to as the chemiosmotic model. This model proposes that the energy released, as electrons are passed from one electron transport carrier to the next, is used to extrude protons (H^+) from the cell. This establishes an electrochemical gradient (protonmotive force) across the proton impermeable cytoplasmic membrane. The protons can return into the cell through a channel in ATP synthase (also referred to as ATPase) which traverses the membrane. This inward flow of protons provides the energy for ATP synthase to catalyze oxidative phosphorylation by the reaction:

$$\text{ADP} + \text{inorganic phosphate} \rightarrow \text{ATP}$$

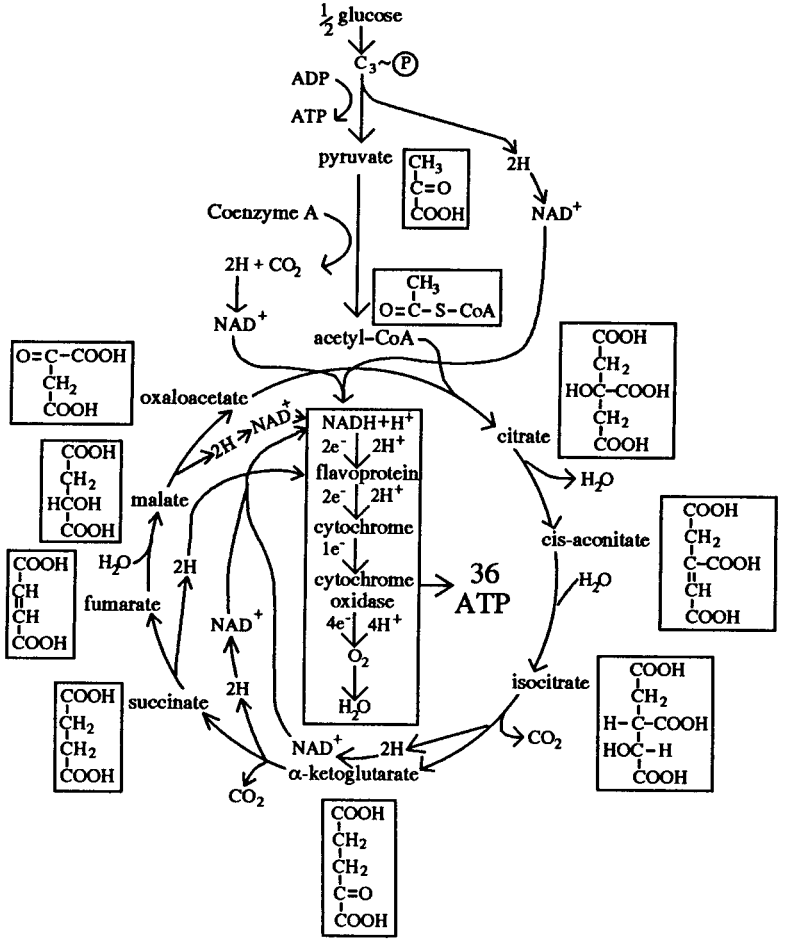


Figure 3. Outline of the tricarboxylic acid (TCA) cycle and its relationship to glycolysis and electron transport.

Other Pathways for Carbohydrate Utilization

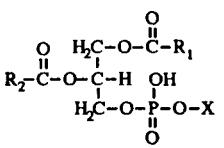
Another important pathway for glucose utilization is the hexose monophosphate shunt (also called the pentose phosphate pathway). This shunt pathway involves the conversion of glucose to glucose-6-phosphate as in the first step of the EMP pathway, but it subsequently diverges. By a series of reactions the glucose-6-phosphate is converted to pentose phosphate and carbon dioxide; and in the process two molecules of nicotinamide adenine dinucleotide phosphate ($NADP^+$) are reduced to NADPH. In contrast to NADH which functions primarily in energy

metabolism (catabolism), NADPH is required for reductive steps in biosynthesis (anabolism). The NADPH produced by this shunt pathway provides a driving force for many biosynthetic reactions. The pentose phosphate also formed in this pathway is used in the synthesis of the ribonucleotides of RNA and the deoxyribonucleotides of DNA, as well as in several other biosynthetic pathways. The pentose phosphate may also be cleaved to glyceraldehyde-3-phosphate and acetyl phosphate. The glyceraldehyde-3-phosphate is converted to lactate via a series of reactions identical to those in the EMP pathway (Figures 1 and 2). The acetyl phosphate may be reduced to acetaldehyde and then to ethanol. The generation of lactate and ethanol via this route is referred to as the heterolactic fermentation.

LIPIDS

Membranous structures such as the cytoplasmic membrane, mesosomes and the outer membrane of Gram negative organisms are the major lipid containing components of bacteria. The bimolecular leaflet structure of bacterial membranes is comprised mainly of phospholipids (also called phosphoglycerides). Among the most common lipids in bacteria are phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerol and phosphatidic acid. These structures are shown in Figure 4.

The fatty acid components of phospholipids are synthesized by a series of reactions starting with acetyl-CoA. These reactions ultimately lead to the formation of long-chain fatty acids of the forms indicated in Figure 4, and where the saturated form is most common. The glycerol-3-phosphate component of phosphoglycerides can be synthesized from dihydroxyacetone phosphate (an intermediate in the EMP pathway, Figure 1) by the reaction:



GENERAL PHOSPHOLIPID STRUCTURE

X = ETHANOLAMINE in phosphatidyl ethanolamine
= SERINE phosphatidyl serine
= GLYCEROL phosphatidyl glycerol
= H phosphatidic acid

R_1 and R_2= fatty acids of the form

$CH_3(CH_2)_{2n}COOH$ (saturated; where 2n is predominantly 14)

or

$CH_3(CH_2)_pCH{=}CH(CH_2)_qCOOH$ (mono-unsaturated; where p+q is 12, 14 or 16)

Figure 4. Phospholipid structures.

$$\text{Dihydroxyacetone phosphate} + \text{NADH} + H^+ \rightarrow \text{glycerol-3-phosphate} + NAD^+$$

Glycerol-3-phosphate can also be derived from glycerol (obtained from the EMP pathway as in Figure 1) by the reaction:

$$\text{Glycerol} + \text{ATP} \rightarrow \text{glycerol-3-phosphate} + \text{ADP}$$

The succeeding reactions of glycerol-3-phosphate to form phospholipids are illustrated in Figure 5.

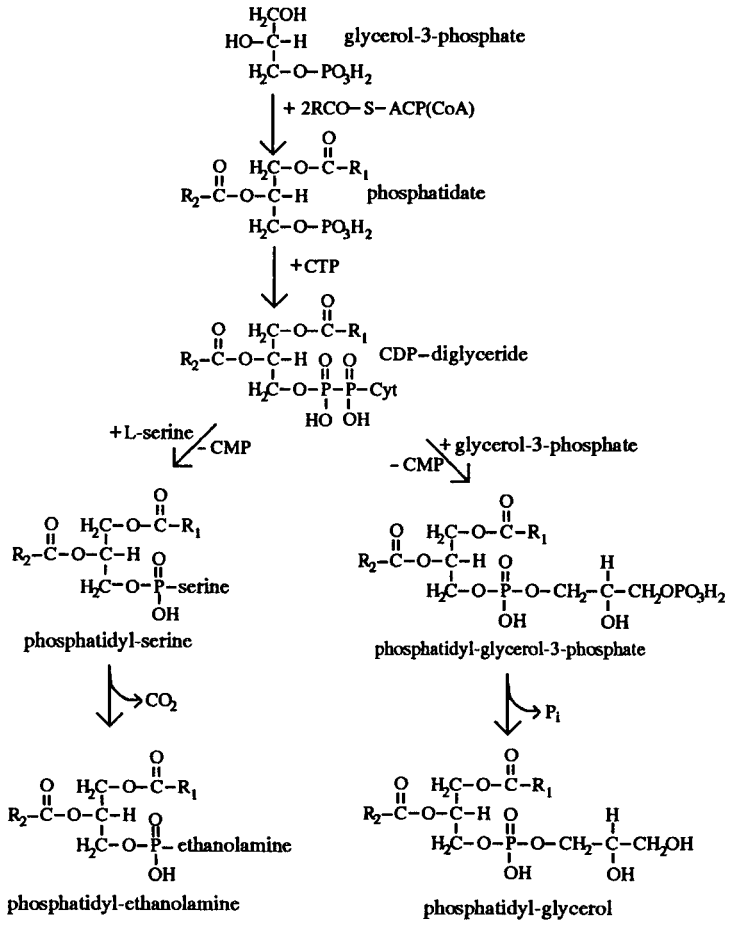


Figure 5. Biosynthesis of phospholipids. R_1 and R_2 = fatty acids; ACP = acylcarrier protein; Cyt = cytidine.

NITROGEN METABOLISM

Animals require the majority of their nitrogen intake in the form of organic compounds, whereas plants depend on fixed nitrogen in the form of ammonia (NH_3) or nitrate (NO_3^-). In contrast, bacteria display wide variation in their nitrogen requirements. Some types of bacteria require preformed compounds such as amino acids, purines, and pyrimidines. Other bacteria, in a manner similar to green plants, can use ammonia or nitrate for the synthesis of nitrogen-containing organic compounds. In addition, certain species of bacteria are able to fix gaseous nitrogen (N_2) from the atmosphere into ammonia.

Bacteria, along with other microorganisms, play an important role in the Earth's nitrogen cycle because of their ability to convert atmospheric nitrogen into ammonia and to assimilate this into organic molecules. Bacteria also degrade many organic compounds in dead animal and plant material and return the nitrogen in these compounds to the nitrogen cycle.

The assimilation of ammonia into organic compounds is ordinarily initiated by its replacement of the keto group of a keto acid to yield an amino acid. Although there are 20 encoded amino acids in protein, only a few amino acids are produced directly by ammonia assimilation. The amino acid most commonly produced by this process is glutamate. Figure 6 shows an important reaction by which this assimilation occurs. In this reaction the alpha-ketoglutarate is derived from the TCA cycle (Figure 3) and the NADPH is derived from the hexose monophosphate shunt (or pentose phosphate pathway) described previously.

BIOSYNTHESIS OF AMINO ACIDS

Many bacteria are able to synthesize all the amino acids required for protein synthesis. The pathways for biosynthesis of amino acids branch off from various intermediates in the major energy-yielding pathways. Thus, the amino acids are usually grouped on the basis of the point of departure of their biosynthesis pathway from the EMP pathway, the TCA cycle, or the pentose phosphate pathway as shown in Figure 7. The amino acid biosynthesis pathways, although elucidated mainly in bacteria, appear, with some exceptions, to be largely universal.

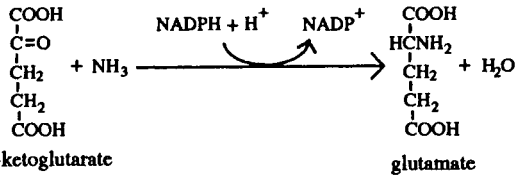


Figure 6. Formation of the amino acid glutamate by the addition of ammonia to α-ketoglutarate.

The Glutamate Family

Glutamate is formed from the TCA cycle intermediate, α-ketoglutarate, as shown in Figure 6. Glutamate provides the carbon skeleton of proline and arginine which are each synthesized by a separate series of enzymatic reactions (Figure 7). Glutamine is synthesized from glutamate using ammonia as the nitrogen donor to form the amide group of glutamine. Glutamate also acts as the main source, by transamination via pyridoxal-phosphate (vitamin B_6), of the alpha amino group of all other amino acids.

The Pyruvate Family

Pyruvate is derived from the EMP pathway. As shown in Figure 7, the pyruvate family of amino acids includes alanine, valine, and leucine. Alanine is produced in most bacteria by transamination of pyruvate. Pyruvate can also condense with a two carbon intermediate to form alpha-ketoisovalerate which is a branch-point in the further biosynthesis of valine and leucine.

The Aspartate Family

Aspartate is produced by transamination of oxaloacetate derived from the TCA cycle (Figure 7). Aspartate gives rise to asparagine, threonine, methionine, and lysine via different pathways. Threonine then gives rise to isoleucine by a pathway that is unusual in that it shares the same enzymes that catalyze parallel reactions in the synthesis of valine from pyruvate. (Aspartate is also a precursor of the pyrimidine ring and of nicotinamide, a component of NAD^+.)

Serine-Glycine or Triose Family

The serine-glycine family of amino acids is also referred to as the triose family because the triose phosphate sugar 3-phosphoglyceraldehyde from the EMP pathway (Figure 1) is the starting point for the synthesis of the amino acids serine, glycine and cysteine. First, serine is formed from 3-phosphoglyceraldehyde. Formation of glycine involves transfer of the CH_2OH from serine to tetrahydrofolate (THF), which gives rise to glycine plus hydroxymethyl-THF. (Several important antibacterial agents are THF inhibitors. Bacterial THF synthesis is inhibited by sulfonamides and THF function in one-carbon transfer reactions is impaired by trimethoprim.) Cysteine is also formed from serine.

Aromatic Amino Acid Family

The aromatic amino acids (those containing a benzene ring) are tyrosine, phenylalanine, and tryptophan. These are all derived from the carbohydrate precursors phosphoenolpyruvate (EMP pathway) and erythrose-4-phosphate (tetrose phosphate, from the pentose phosphate pathway) as indicated in Figure 7. These two

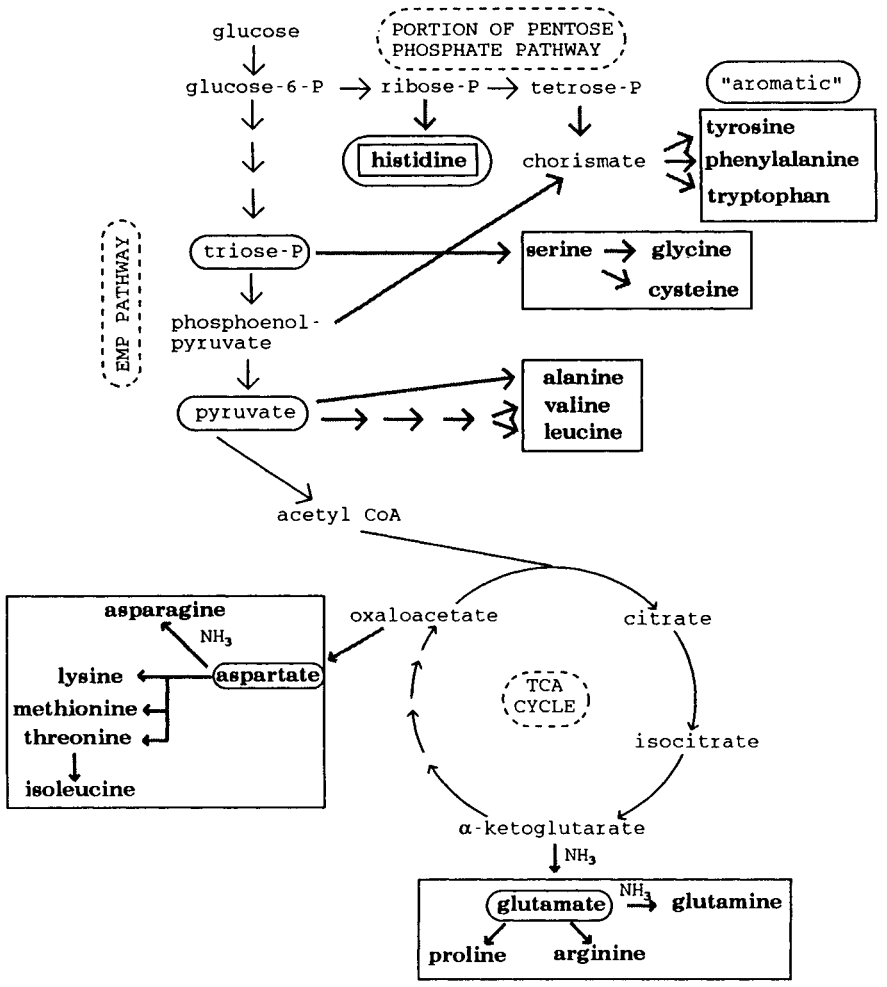


Figure 7. The relationship of the biosynthesis pathways of amino acids to the intermediates of the energy pathways from which they originate: the EMP pathway, the pentose phosphate pathway and the TCA cycle. These energy pathways are indicated within dashed ovals. The six "family names" of groups of amino acids are shown within continuous ovals. The energy pathways are shown with thin arrows and the amino acid synthesis pathways are shown by thicker arrows. Amino acids are shown in bold print. The "families" of amino acids are enclosed within boxes.

compounds condense to form a seven carbon compound which is an intermediate in the formation of chorismate, a common intermediate in the formation of the three aromatic amino acids.

Histidine

This amino acid derives its 5-carbon backbone from 5-phosphoribosyl-1- pyrophosphate (PRPP) which in turn is derived from ribose-5′-phosphate of the pentose phosphate pathway (Figure 7). Other components of the histidine structure are derived from ATP, glutamine, and glutamate.

PURINE AND PYRIMIDINE NUCLEOTIDE BIOSYNTHESIS

Purine and pyrimidine ribonucleoside triphosphates are precursor building blocks in the synthesis of RNA. In addition, ATP is a source of high energy phosphate in numerous kinase (phosphate transfer) reactions, GTP is the energy source in protein synthesis, and ATP is utilized in amino acid or fatty acid activation or in sugar activation for polysaccharide synthesis. Similarly, cytidine triphosphate (CTP) is used in phospholipid biosynthesis. Ribonucleotides also are the precursors of the deoxyribonucleotide triphosphates and, therefore, of DNA.

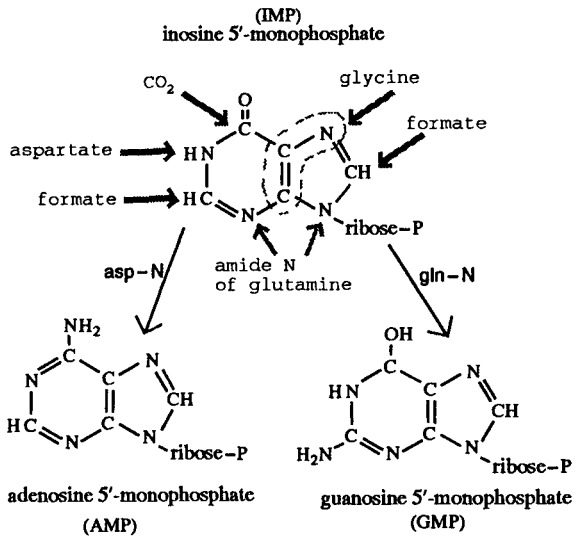


Figure 8. Origin of the atoms of the purine ring of inosine-5′-monophosphate (IMP). The purine biosynthesis pathway branches at IMP with one path leading to adenosine-5′-monophosphate (AMP) and the other leading to guanosine-5′-monophosphate (GMP). gln-N = amide nitrogen of glutamine; asp-N = amino nitrogen of aspartate.

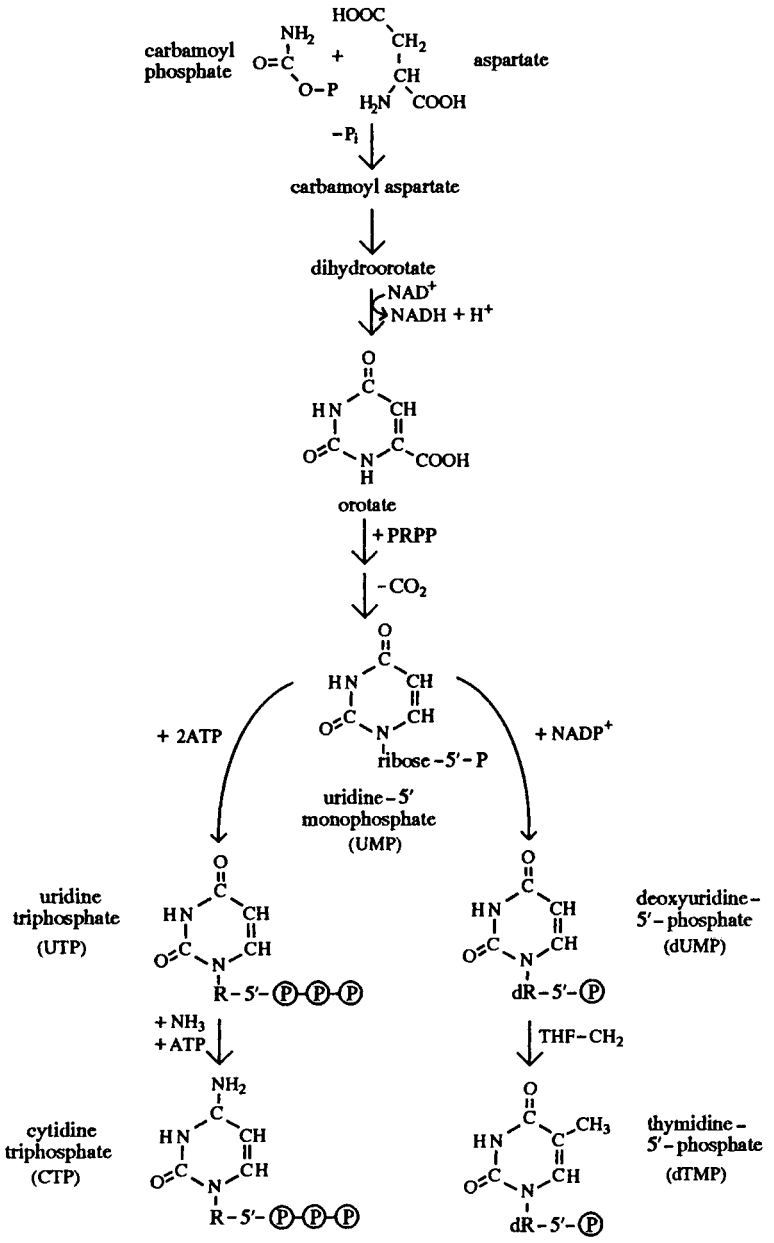


Figure 9. Pyrimidine biosynthesis (an abbreviated version). THF-CH_2 is the methyl group transferring agent N^5,N^{10}-methylene tetrahydrofolate.

Purine Nucleotide Biosynthesis

Purine nucleotide synthesis, like histidine biosynthesis, starts with 5-phosphoribosyl-1-pyrophosphate (PRPP). Subsequently there are eleven sequential enzyme-catalyzed reactions leading to the purine nucleotide inosine 5′-monophosphate (IMP). The reactions of the pathway involve addition of substituents derived from glutamine, formate, glycine, CO_2 and aspartate as indicated in Figure 8. Additionally, as Figure 8 indicates, further reactions take IMP to AMP and GMP. AMP can be phosphorylated by a kinase to adenosine diphosphate (ADP) and from this ATP can be formed, as described above, by oxidative phosphorylation or substrate level phosphorylation. GMP can be phosphorylated by kinases to GDP and then to GTP.

Pyrimidine Nucleotide Biosynthesis

The initial reaction in pyrimidine nucleotide biosynthesis is the condensation of carbamoyl phosphate with aspartate to yield carbamoyl aspartate (Figure 9). Succeeding reactions generate a ring compound, dihydroorotate, then orotate, and, with the use of PRPP to add ribose-5′-phosphate, orotidine-5′-phosphate. This is then decarboxylated to yield uridine-5′-monophosphate (UMP). UMP can be converted to uridine triphosphate (UTP) and, in the presence of ammonia, to CTP. UMP can also be converted to the deoxyribose derivative dUMP, and in the presence of a methyl group transferring agent, to thymidine-5′-phosphate (dTMP).

SUMMARY

During the first two billion years of evolution of life on Earth, single-celled organisms (bacteria) emerged that were capable of complex enzyme catalyzed pathways of metabolism. Some of these metabolic pathways were adaptations for generating useful energy from organic molecules available in the environment. Other pathways were adaptations for synthesizing the building blocks (amino acids, lipids, nucleotides, and so on) needed for making the macromolecules and cellular structures that were employed in maintenance and reproduction. Much of this metabolic capability was passed on to evolutionary descendants, including humans. Descendent bacterial species developed characteristic metabolic features related to the peculiarities of their ecological niches, and, in some cases, to their pathogenic ability. Thus knowledge of bacterial metabolism is useful for understanding human metabolism, for identifying particular species and for understanding aspects of pathogenic behavior.

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Chapter 4

Bacterial Genetics

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INTRODUCTION

Bacterial genetics involves the isolation and characterization of mutants and the transfer of mutations between cells. Why study bacterial genetics? Bacterial genetics has played a critical role in elucidating the mechanisms of basic cellular processes such as DNA replication, transcription, translation, and the regulation of gene expression. Understanding these processes is much easier if you understand the genetic approaches used to determine the mechanism. But bacterial genetics also has many practical uses in current medical science. The growing problem with antibiotic resistant bacteria is one clear example (Culotta, 1994; Davies, 1994). More and more pathogenic bacteria are found to be resistant to a wide variety of antibiotics, some resistant to virtually every useful antibiotic known. Solving this problem will require an understanding of how antibiotic resistance is acquired and how the resistance genes are transferred between bacteria. Bacterial genetics also provides a powerful approach for dissecting the molecular mechanisms of bacterial pathogenesis which may allow the development of alternative treatments.

MUTANTS AND MUTATIONS

Mutations are the raw material of evolution, providing the changes on which selection can act. Mutations can also be used as genetic tools to construct a strain with desired properties, to determine the role of the normal gene product in the cell, or to localize the position of a gene in the genome.

A mutation is any change in the DNA sequence of an organism compared to a parental wild-type strain. A strain with a mutation is called a mutant. The mutation may or may not change the observable properties of the mutant compared to the parental strain: that is, not all changes in the genotype defined by the DNA sequence of an organism result in changes in the phenotype defined by the observable properties of an organism. Furthermore, different mutations in a single gene (called different "alleles" of the gene) may produce different phenotypes.

Mutations can be classified in several ways. One classification is based on the conditions in which the mutant phenotype is expressed. A nonconditional mutation displays the mutant phenotype under all conditions. Nonconditional mutations that completely eliminate activity of a gene product are called null mutations. In contrast, a conditional mutation does not always show the mutant phenotype; the phenotype observed depends either on environmental conditions or on the presence of other mutations. An example of a conditional mutation is a temperature-sensitive mutation, which has the wild-type phenotype at one temperature (typically 30°C), and has a mutant phenotype at another temperature (typically at 42°C). (The gene itself is not altered at 42°C; rather, the *product* of the gene is inactive at 42°C.) Another common type of conditional mutations are suppressor-sensitive mutations, which exhibit the mutant phenotype in some bacterial strains but not in others.

Another way of classifying mutations is based on the number of changes that have occurred in the genetic material (that is, the number of DNA base pairs that have changed). If only one base pair has been changed, the mutation is called a point mutation (Figure 1a). If all or part of a gene is physically removed, the mutation is called a deletion (Figure 1b). If genetic material is added without removal of any other material, the mutation is called an insertion mutation (Figure 1c).

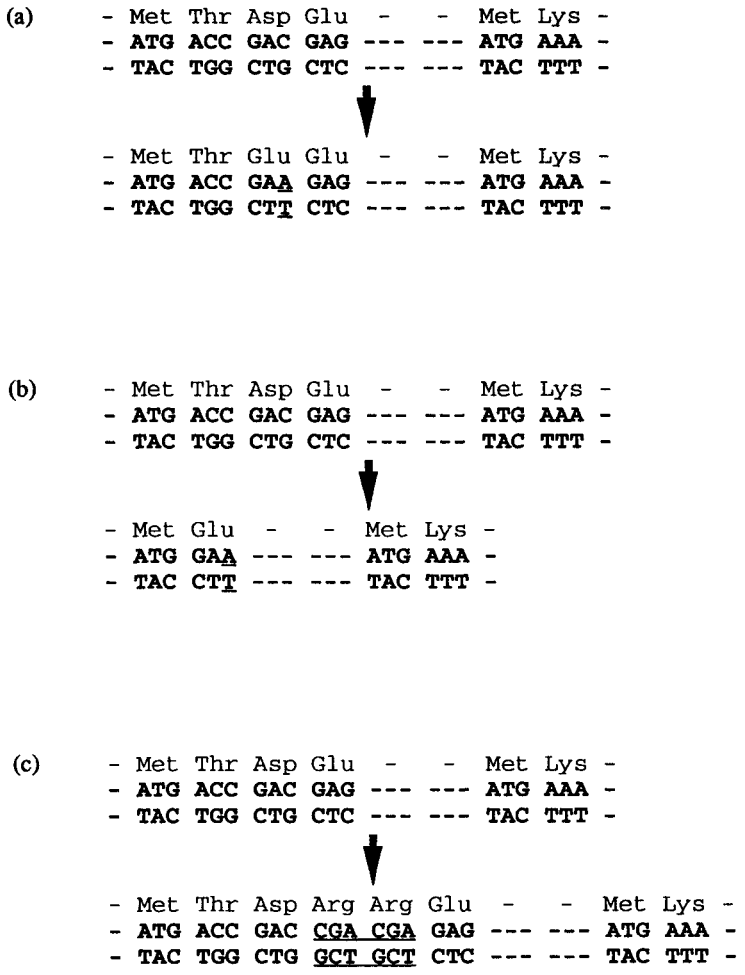

Figure 1. Types of mutations: (a) a point mutation; (b) a deletion mutation; (c) an insertion mutation.

The change in DNA sequence caused by a mutation can affect a gene encoding a protein, a gene encoding a structural RNA (for example, ribosomal RNA), or a regulatory site. Although mutations in each of these different targets will have different properties, the way that mutations change the phenotype of a cell can be understood by considering the effects of a mutation on a gene encoding a protein. The chemical and physical properties of proteins are determined by their amino acid sequence. An amino acid substitution can change the structure and, hence, the biological activity of a protein. Even a single amino acid change is capable of

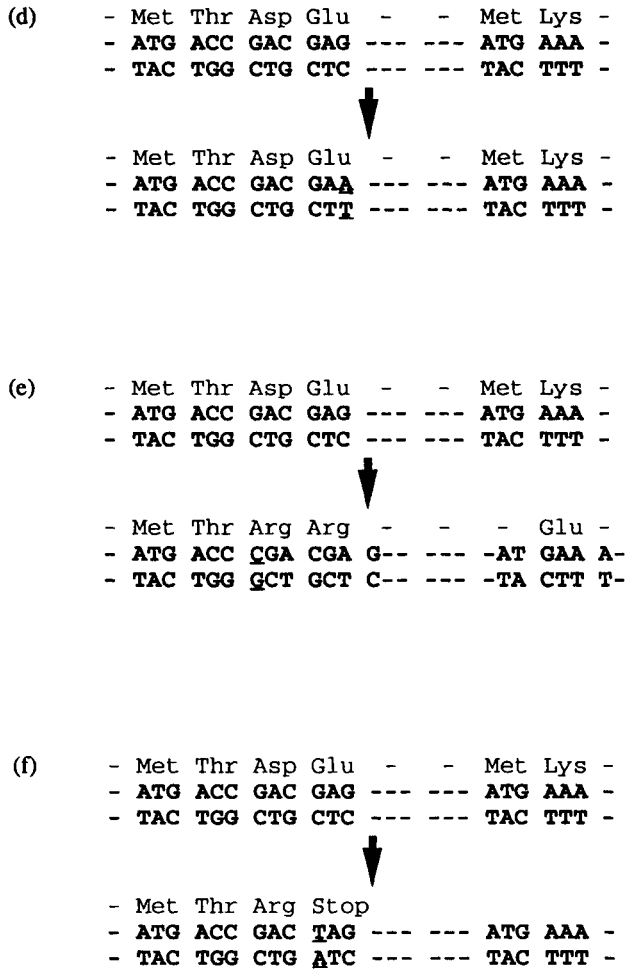

Figure 1. Types of mutations: (**d**) a silent mutation; (**e**) a frameshift mutation due to the insertion of a single base pair; (**f**) a chain termination mutation. The base pairs changed by the mutations are underlined.

altering the activity of, or even completely inactivating, a protein. For example, consider an enzyme with a histidine residue that is essential for its catalytic function. A single base change could substitute proline for the histidine and thus inactivate the enzyme. Other mutations may not change specific residues at the active site of the protein, but may inactivate the protein by disrupting its three-dimensional structure.

When a base change has no detectable effect on phenotype, it is called a silent mutation. How come some base substitutions do not produce a phenotype? Because the genetic code is redundant, many base changes do not result in an amino acid substitution (Figure 1d). Furthermore, some amino acid substitutions do not have much effect on the structure and function of a protein. For example, a protein might be virtually unaffected by a point mutation that replaces a leucine with another nonpolar amino acid such as isoleucine. Sometimes an amino acid substitution is only partially disruptive. This could cause a reduction, rather than a complete loss, of an enzyme activity. For example, a bacterium with such a mutation in an enzyme that synthesizes an essential substance might grow, but would grow very slowly unless the substance is provided in the growth medium. Such a mutation is called a leaky mutation.

In addition to simple amino acid substitutions, other types of mutations may also eliminate activity of a protein: (1) a deletion which causes one or more amino acids to be absent in the completed protein; (2) a deletion or insertion that causes shift in the reading frame such that all the codons after the mutation are changed (Figure 1e); (3) a chain termination mutation, in which a base change generates a stop codon resulting in a shorter polypeptide that lacks the carboxy terminus of the protein (Figure 1f).

Spontaneous Mutations

Rare mutations arise spontaneously in a population of cells. The two most common causes of spontaneous mutagenesis are errors occurring during replication and random alteration of a nucleotide (Drake, 1991). One reason for such incorporation errors is tautomerism or deamination of the nucleotide bases. This may allow the bases to form incorrect base pairs. If this happens during DNA replication, an incorrect base will be correctly hydrogen-bonded to the template strand (Figure 2). Errors in nucleotide incorporation during DNA replication occur with sufficiently high frequency that the information content of a daughter DNA molecule would differ significantly from that of the parent were it not for two mechanisms for correcting such errors: proofreading by DNA polymerase (Kornberg and Baker, 1992) and mismatch repair (Modrich, 1991). Both proofreading and mismatch repair are very efficient; however, they are not perfect so sometimes mutations escape the repair system.

Most mutations are caused by random errors, not by some adaptive advantage the mutation may confer on the organism in its environment. However, every gene

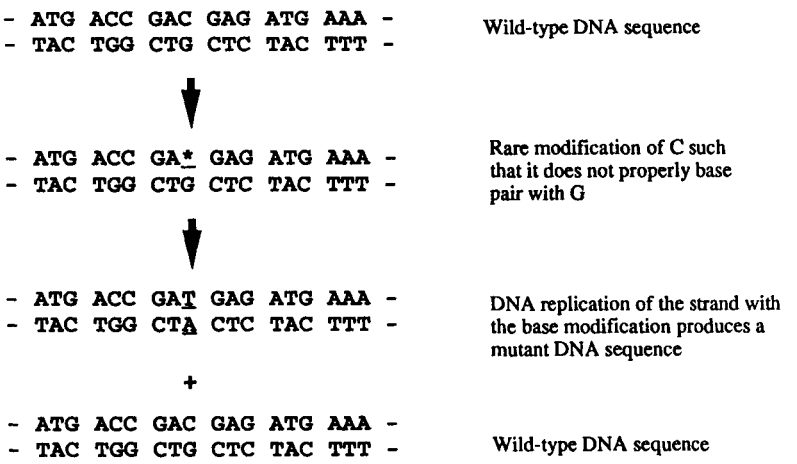

Figure 2. An example of a point mutation caused by mispairing and subsequent DNA replication.

mutates spontaneously at a characteristic rate which depends on the size and nucleotide sequence of the gene and on the amino acid sequence and three-dimensional structure of the gene product. Thus, it is possible to estimate the probability that a given gene will mutate in a particular cell, and the corresponding probability that a mutant allele of the gene will occur in a population of a particular size.

Isolation and Characterization of Mutants

Genetics depends on the analysis of rare mutants that affect the process of interest. Because mutations are very rare, some means is needed to identify the mutant cells from a large population of wild-type cells. Detection of mutants requires a selection or screen. A selection is a condition that allows specific mutants to grow but not the parental cells. Genetic selections are very powerful because they allow isolation of rare mutations from a population of cells. For example, antibiotic resistant mutants can be selected by simply plating a large number of bacteria on solid medium containing the antibiotic—only resistant mutants can form colonies. If the mutation is relatively common and there is no direct selection for mutants, it is often necessary to resort to screening for mutants on media where both the mutant and parental cells grow but the phenotype of the mutant can be distinguished from the phenotype of the parental cells. For example, rare antibiotic sensitive mutants present in a population of antibiotic resistant cells can be identified by replica-plating. The cells are plated on nutrient agar such that there are several hundred colonies on a plate. This master plate is then replicated onto two different agar plates, one containing the antibiotic and the other lacking the antibiotic. Antibiotic

resistant cells will form colonies on both plates, but any antibiotic sensitive mutants will only form colonies on the plate without the antibiotic.

The practical difference between a selection and a screen can be seen in the following examples. Compare the selection for a streptomycin resistant (Str^r) mutant with a screen for a mutant which requires histidine (His^-). Isolation of a Str^r colony is a positive selection because only Str^r cells can grow on an agar plate containing streptomycin. Thus, as many as 10^{10} cells can be spread on the plate allowing a mutant as rare as $1/10^{10}$ to be detected. However, isolating a His^- mutant requires screening for the rare mutant. Usually the incidence of a mutation in a given gene is 10^{-6} or less. That is, at most only one in a million cells will have the desired mutation. No more than a few hundred colonies can be examined on a single plate, so thousands of plates would be needed to find a single His^- mutant.

Mutagens

Often selections are not strong enough to isolate very rare mutants and, as described in the example above, screening methods are very inefficient for isolating rare mutants. For these reasons, the bacteria are often treated with a mutagen before trying to isolate a mutant. This can be a chemical mutagen (for example, nitrous acid), radiation (for example, UV), or a transposon. Bacteria may be mutagenized by treatment of cell suspensions with the mutagen or by treatment of isolated DNA with the mutagen before it enters the bacteria.

How do mutagens induce mutations? Different types of mutations are induced by specific mutagens (Kornberg and Baker, 1992; Maloy et al., 1994). A few mechanisms of chemical and physical mutagens include: (1) stimulating tautomerization of a base, resulting in a base substitution upon subsequent DNA replication, (2) inhibiting the cell from removing an incorrectly inserted base, (3) chemically altering a base, resulting in a different base-pairing specificity, (4) promoting the insertion or deletion of one or more extra bases by an error during DNA replication.

GENETIC ANALYSIS OF MUTANTS

Two of the most important tools for analysis of mutations are genetic recombination and complementation. These two methods are often confused, but the actual methods and the conclusions they reveal are quite different. Recombination allows you to make new combinations of genes and determine the positions of genes on a chromosome with respect to one another. Complementation allows you to determine the number of genes responsible for a particular phenotype and to distinguish regulatory genes from regulatory sites.

Genetic Recombination

Genetic recombination is the process of physically exchanging two genetic loci, initially on two different DNA molecules, onto a single DNA molecule (Stahl,

1987). The molecular mechanisms are very complex and are not yet fully understood. However, most genetics was done long before any understanding of the molecular mechanism of recombination. For many genetic experiments recombination can be visualized quite simply: two DNA molecules align with one another, then a cut is made in both DNA molecules at random but matching points, and then the DNA molecules are joined together to form two new combinations of genes (Figure 3). The recombinant DNA that results from this process may be stably inherited by the progeny cells.

Complementation

A particular phenotype often due to a pathway encoded by many genes. To understand any genetic system it is essential to know the number of genes and regulatory elements that constitute the system. Since recombination can occur within genes and different genes which affect the same function may map very close to each other, it is not possible to determine if two mutations are in the same or different genes by genetic recombination. Instead, complementation analysis is used to determine if mutations are in different genes.

Complementation analysis requires that two copies of the genes are present in the same cell. In bacteria this can be done by constructing a partial diploid or merodiploid—that is, a cell containing one complete set of genes and a duplicated copy of part of the genome. The basic idea behind complementation is that if both copies of a gene are mutant then no functional gene product will be made, but if the two mutations are in different genes and one wild-type copy of each gene is present then both functional gene products will be made (Figure 4). That is, if two mutations complement, they are in different genes. (The exceptions to this rule are rare examples of intragenic complementation which occur in proteins with multiple, identical subunits.) However, the converse is not always true if two mutations do

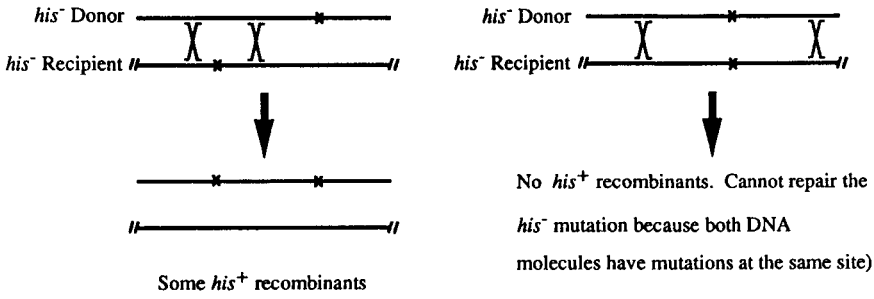

Figure 3. Two examples of genetic recombination between DNA molecules with specific point mutations. The DNA is cut and rejoined by cellular enzymes. After recombination both DNA molecules are covalently joined to give a new combination of genes.

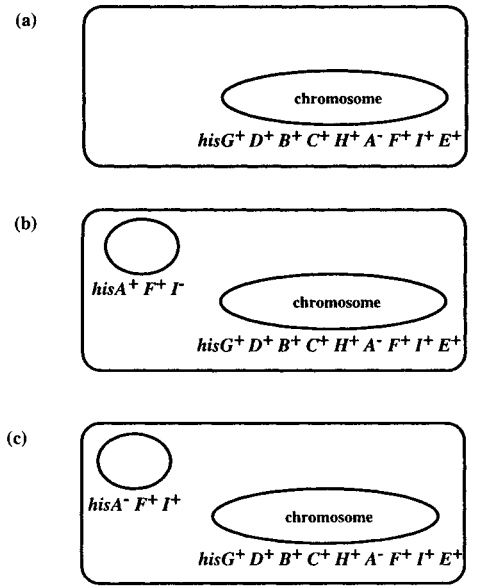

Figure 4. An example of complementation analysis. Nine genes are required for biosynthesis of histidine. (**a**) If one of the chromosomal genes is mutant (*hisA*), the cell will require histidine. (**b**) If a second copy of the wild type gene is provided in the cell (*hisA*$^+$ on the plasmid), the cell will not require histidine. (**c**) If the second copy of the wild type gene is also mutant, the cell will require histidine.

not complement, they are not necessarily in the same gene. Failure to complement may also occur because one of the mutations may affect expression of the other gene or because one of the mutations may make a gene product that inhibits the other gene product.

Reversion Analysis

A mutant can sometimes revert to the wild-type phenotype by acquiring an additional mutation which restores the affected function. Reversion may occur in two ways: (1) "true reversion" is due to a back-mutation that exactly repairs the original DNA sequence; (2) "pseudoreversion" or "suppression" is due to an additional mutation at a second site that restores the original phenotype.

Reversion can result from spontaneous mutagenesis and, like the formation of all spontaneous mutations, is a nearly random process. Unless there is some selective pressure for revertant phenotype, the frequency of reversion is so rare that an appropriate selection or screening method is required to isolate revertants from a population of mutant cells. The probability of obtaining a revertant depends upon the nature of the original mutation. Thus, the reversion frequency is sometimes used

as a criterion for identifying the type of mutation present in a mutant. The reversion frequency is the fraction of cells in a population of mutants that regain the original phenotype per generation. Point mutations revert at the highest frequency because a single base change in the DNA is sufficient to restore the original sequence, so spontaneous mutations that yield true revertants occur at some measurable frequency (typically about 10^{-8}). In contrast, deletion mutations cannot revert by repairing the original DNA sequence, because the probability of replacing the missing DNA with an equivalent DNA sequence is virtually zero.

Many reversion events can be selected by simply measuring the ability of a population of bacteria to form colonies on solid growth medium. For example, if 10^9 cells of a *his*$^-$ bacterial strain are placed on a solid medium lacking histidine, about 10 colonies arise; these colonies are formed by spontaneous His$^+$ revertants (cells able to grow without an external supply of histidine). The reversion frequency in this case is $10/10^9 = 10^{-8}$.

Detecting Mutagens and Carcinogens by Reversion Analysis

With the increased number of chemicals used in foods and cosmetics and accumulated as environmental contaminants, it is important to have quick and simple tests to determine if a chemical is a carcinogen. Most carcinogens are also mutagens, so assaying mutagenicity can be used for the initial screening for these hazardous agents. One simple method for screening large numbers of substances for mutagenicity is a reversion test using auxotrophic mutants of bacteria. In the simplest type of reversion test a compound that is a potential mutagen is added to solid growth media, known numbers of a mutant bacterium are spread on the plate, and the number of revertant colonies that arise is counted. A significant increase in the reversion frequency above that obtained in the absence of the compound tested indicates that the substance is a mutagen. However, simple tests of this type fail to demonstrate the mutagenicity of a number of potent concentration of the substance being tested and correlates well with the potency of known carcinogens and mutagens.

The Ames test has now been used with thousands of substances and mixtures (such as industrial chemicals, food additives, pesticides, hair dyes, and cosmetics), and numerous unsuspected substances have been found to stimulate reversion in this test. A high frequency of reversion does not mean that the substance is definitely a carcinogen, but only that it has a high probability of being so. As a result of these tests, many industries have reformulated their products: for example, the cosmetic industry has changed the formulation of many hair dyes and cosmetics to render them nonmutagenic. Ultimate proof of carcinogenicity is determined by testing for tumor formation in laboratory animals. The Ames test and several other microbiological tests are used to reduce the number of substances that have to be tested in animals since to date only a few percent of more than 300 substances known from animal experiments to be carcinogens failed to increase the reversion frequency in

the Ames test. Thus, this test uses the power of bacterial genetics to greatly reduce the number of animals used for this type of research.

GENE TRANSFER

Thus far, our discussion has concentrated on rare mutations that arise spontaneously in a population of bacteria. However, genes can also be transferred between bacteria. Gene transfer is a useful tool in the laboratory because it can be used to construct new strains with desired properties. However, gene transfer in nature may have some serious consequences, such as spread of antibiotic resistance genes to pathogenic bacteria (Davies, 1994). The three most common methods of gene transfer are transformation, conjugation, and transduction.

Transformation

Uptake of naked, exogenous DNA is called transformation. Some species of bacteria are naturally transformable. However, many bacteria that are most useful for genetic engineering are not naturally transformable. These bacteria may be induced to take up exogenous DNA by treatment with specific chemicals or electrical shock, but the mechanism of transformation is different from natural transformation.

The discovery of bacterial transformation provided the initial evidence that DNA is the genetic material and is a good example of the potential importance of transformation in nature (McCarty, 1985). Bacterial pneumonia in mammals is caused by certain strains of *Streptococcus pneumoniae*. Cells of these strains are surrounded by a polysaccharide capsule that protects the bacterium from the immune system of the infected animal and thereby enables the bacterium to cause disease. When a pathogenic strain of *S. pneumoniae* is grown on nutrient agar, the capsule gives the bacterial colony a glistening, smooth (S) appearance. Some mutant strains of *S. pneumoniae* lack the enzyme activity required to synthesize the capsular polysaccharide, and these bacteria form colonies that have a rough (R) surface. R strains do not cause pneumonia, because without their capsules the cells are rapidly inactivated by the host immune system. Both R and S phenotypes are stable genetic traits, but rare mutations in R strains can produce the S phenotype and rare mutations in S strains can produce the R phenotype.

In 1928, Griffith noticed that mice injected with either live cells of the R strain or with heat-killed S cells remained healthy, but mice injected with a mixture containing a small number of R cells and a large number of heat-killed S cells died of pneumonia. Bacteria isolated from blood samples of the dead mice produced pure S cultures with a capsule typical of the heat-killed S cells (Figure 5). Evidently, dead S cells could in some way provide the living R bacteria with the ability to withstand the immunological system of the mouse, multiply, and cause pneumonia.

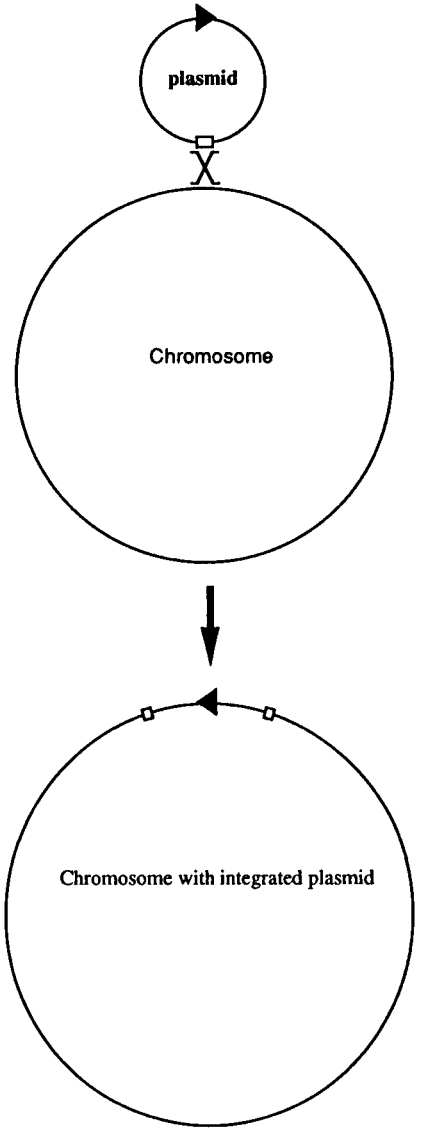

Figure 5. **A.** Integration of a conjugative plasmid into the chromosome.

Furthermore, cultures derived from these changed or "transformed" bacteria retained the ability to cause pneumonia.

In 1944 Avery, MacLeod, and McCarty purified DNA from S cells and found that addition of minute amounts of this DNA to growing cultures of R cells consistently

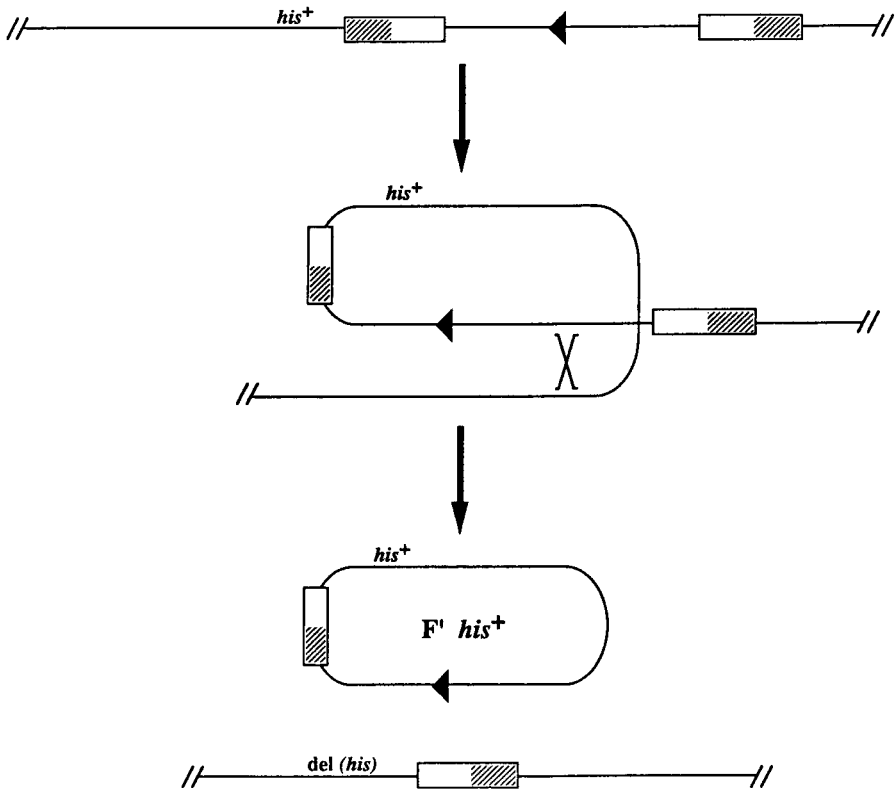

Figure 5. **B.** Aberrant excision of a conjugative plasmid from the chromosome, forming a new recombinant plasmid that carries the chromosomal *his*$^+$ genes.

resulted in production of smooth colonies whose cells contained S type capsular polysaccharide. The transforming activity was not altered by treatment with enzymes that degrade proteins or by treatment with RNase, but was completely destroyed by DNase. These experiments demonstrated that the substance responsible for genetic transformation was the DNA of the donor cells and, hence, that DNA is the genetic material.

Like most methods of gene transfer, transformation is a relatively rare event. Transformation is usually detected by selection for recipient cells that acquire a specific phenotype from the donor DNA. For example, purified DNA obtained from an erythromycin resistant (Ery^r) culture of *S. pneumoniae* is mixed with cells from an Ery^s culture, and the cells are spread on agar plates containing erythromycin. Formation of Ery^r colonies at a frequency greater than the frequency of spontaneous mutagenesis from Ery^s to Ery^r (about 10^{-8}) indicates that transformation has

occurred. For *S. pneumoniae* the maximum frequency of transformation for most antibiotic resistance markers is 0.1–1%.

The ability of most bacteria to take up DNA efficiently is limited (Stewart and Carlson, 1986). Even in a species capable of transformation, DNA can penetrate only a very small fraction of the cells in a growing population. However, incubation of cells of these species under certain conditions yields a population of cells in which the uptake of DNA is greatly enhanced, by a factor of 10^4–10^6. A culture of such cells is said to be "competent." The conditions required to produce competence and the fraction of the cells that are competent vary from species to species. The ability of a cell to take up DNA can also be artificially elicited in a wide variety of bacterial strains by chemical techniques. However, the state of the cells produced by this treatment is quite different from that of naturally competent cells.

How common is transformation in nature? For transformation to occur in nature DNA must be released from some cells and other cells must be competent for DNA uptake. Many Gram positive bacteria, such as *B. subtilis*, spontaneously lyse and release their DNA when cells are starved for nutrients or experience harsh growth conditions (Dubnau, 1991). Thus, there is a ready supply of DNA in the environment. Low frequencies of transformation have been observed between genetically marked cultures of *B. subtilis* that are under these conditions, indicating that a small fraction of the cells are competent for DNA uptake. Furthermore, transformation has been observed when different strains of genetically marked bacteria are placed in the gut of a mouse. Thus, although transformation in nature may occur at a very low frequency, it can and does occur and, provided an environmental selection, it may play an important role in the transfer of genes between bacteria.

Conjugation

DNA can also be transferred between cells by conjugation (Clewell, 1993). Conjugation requires functions encoded on certain plasmids. Plasmids are DNA molecules that can replicate independent of the host chromosome. Plasmids are present in most species, but not all strains, of bacteria. Most plasmids are small, from about 0.2 to 4% the size of the bacterial chromosome. Under most conditions of growth, plasmids are dispensable to their host cells. However, many plasmids contain genes that have a selective benefit in particular environments. For example, R plasmids render their host cells resistant to certain antibiotics, so in nature a cell containing such a plasmid can survive better in environments in which the antibiotic is present.

Conjugation requires four activities: (1) interaction between specific donor and recipient cells, (2) sites on the plasmid that allow mobilization of the plasmid DNA, (3) transfer of the plasmid DNA into the recipient cell, and (4) re-formation of a functional plasmid in the recipient cell (Figure 6). Some plasmids can carry out all four steps of this process, but many plasmids lack the genes needed to carry out some subset of these processes. However, in many cases the conjugative functions

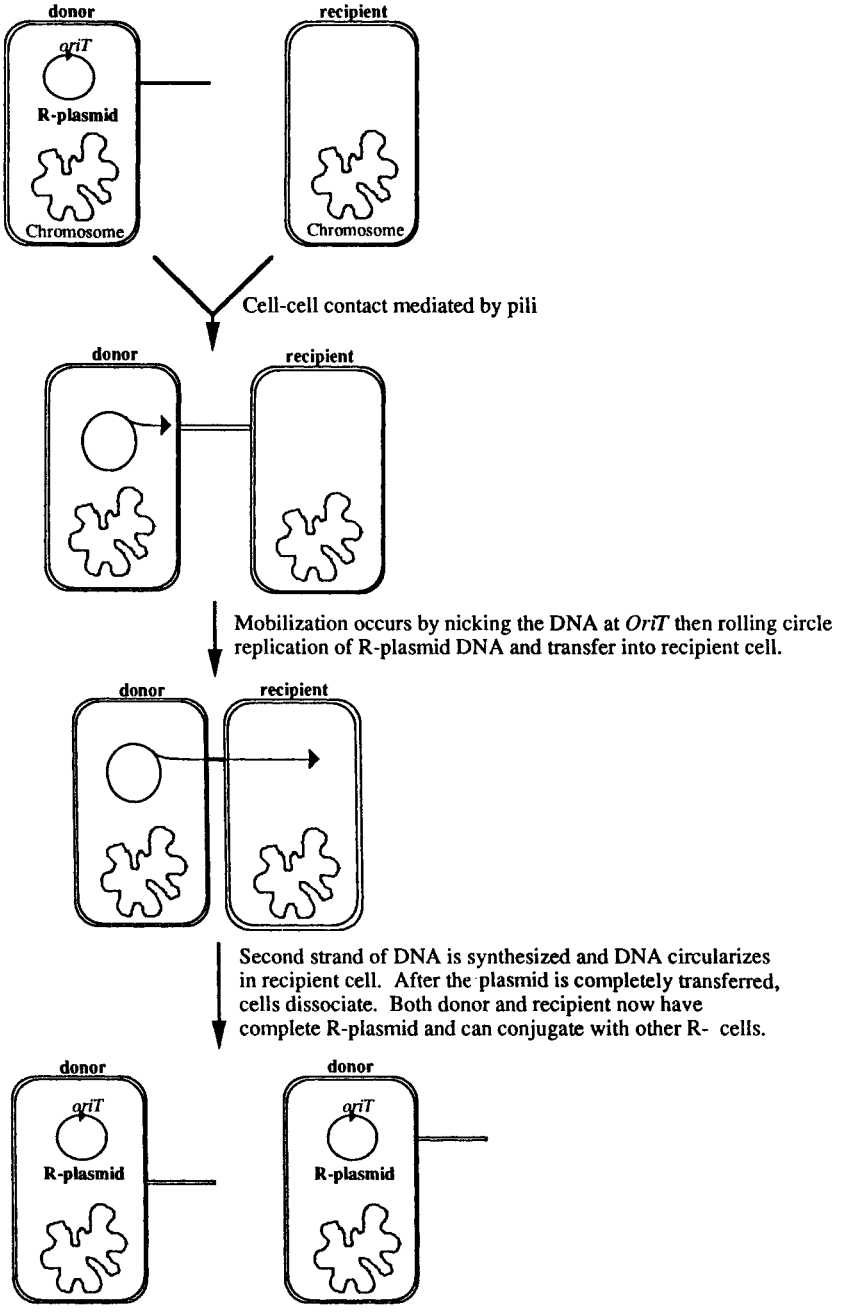

Figure 6. Conjugation between a donor bacterium carrying an R-plasmid and a plasmid-free recipient strain.

are not plasmid-specific, so one plasmid can assist transfer of a second. For example, a single cell may contain both an F plasmid and a ColE1 plasmid. F can carry out each of the four functions needed for conjugation. In contrast, ColE1 has the sites needed for mobilization, but lacks the other functions needed for conjugation, so a cell containing only ColE1 cannot transfer the plasmid. In a cell containing both plasmids, F can provide the missing conjugative function to ColEl, so ColE1 can be transferred to a recipient that lacks both plasmids.

Unlike transformation, conjugation requires intimate contact between a donor and a recipient cell. For most gram negative bacteria, the cell–cell contact requires a hairlike protein appendage, called a sex pilus, on the donor cell. The pilus seems to bring the cells into initial contact and then to draw the cells together to allow DNA transfer between the cells. The way that DNA transfer between the cells actually occurs is not yet clear. In contrast, gram positive bacteria do not require pili for conjugation. In this case, cell–cell contact seems to be due to a mating protein (called a pheromone) secreted by the plasmid-free recipient cells but not made in plasmid-containing donor cells. The pheromone induces the donor cells to synthesize a protein that coats the cell surface of donor cells and causes donor-recipient pairs to clump together. Once the plasmid is transferred, synthesis of the pheromone is inhibited.

Conjugative plasmids may also integrate into the bacterial chromosome (Figure 5a). When this happens, the plasmid can transfer the entire chromosome to a recipient cell. Furthermore, the integrated plasmid can occasionally excise out of the chromosome. Excision is often imprecise resulting in an excised circular plasmid which contains genes that were adjacent to the integrated plasmid in the chromosome (Figure 5b). The resulting recombinant plasmid can then transfer those chromosomal genes to recipient cells at a high frequency.

Virulence Plasmids

Transfer of certain plasmids to non-virulent plasmid-free recipients confers pathogenic properties to the bacteria (Salyers and Whitt, 1994). Several examples include the Ent plasmids of *Escherichia coli* that synthesize enterotoxins responsible for travelers' diarrhea, a large plasmid that encodes virulence functions in *Salmonella typhimurium*, and a penicillinase producing plasmid from *S. aureus*.

Drug-Resistance Plasmids

The drug-resistance, or R plasmids, were originally isolated from the bacterium *Shigella dysenteriae* during an outbreak of dysentery in Japan and have since been found in *E. coli* and many other bacteria. R plasmids confer resistance on their host cell to a variety of fungal antibiotics and are usually self-transmissible. Most R plasmids consist of two contiguous segments of DNA. One of these segments is called RTF (resistance transfer factor); it carries genes regulating DNA replication and copy number, the transfer genes, and sometimes the gene for tetracycline

resistance (Tet). The other segment, sometimes called the R determinant, is variable in size and carries other genes for antibiotic resistance. R plasmids commonly carry resistance to the drugs ampicillin (Amp), chloramphenicol (Cam), streptomycin (Str), kanamycin (Kan), and sulfonamide (Sul), in a variety of combinations. The drug-resistance genes on the R-plasmids are acquired from transposons that carry antibiotic-resistance genes.

R plasmids are of considerable medical interest since they can be transferred between bacteria that cause major epidemics, such as *Salmonella typhimurium* and *Shigella dysenteriae*, and to strains that cause infections in hospitals (various enterobacteria, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). In fact, it has become clear that since the beginning of the "antibiotic era," R plasmids have increased dramatically in nature (Levy, 1993). For example, penicillin was introduced to general use in the early 1940s. By 1946, 14% of *S. aureus* strains isolated in hospitals were Pen^r. The fraction was 38% in 1947, 59% in 1969, and nearly 100% by the 1970s. The majority of these resistant strains either carry an R plasmid or a Pen^r gene of the type found in R plasmids. Transfer of bacteria that carry R plasmids also commonly occurs from farm animals to humans. R plasmids are rampant in farm animals due to the extensive use of penicillin and tetracycline in animal feed. (Use of low levels of antibiotics in animal feed leads to more rapid growth of the animals and hence are economically valuable.) For example, poultry is frequently contaminated with *E. coli* and *S. typhimurium*, which can colonize the human intestine. Handling of raw meat is a common route for transmission to humans; bacteria from the meat gets on utensils and kitchen surfaces, eventually transferring to humans. Numerous epidemics of drug-resistant salmonellosis caused by transmission from farm animals to humans have occurred since 1960. Because the bacteria were resistant to multiple antibiotics, the antibiotics commonly used for *S. typhimurium* infections were not effective. To make matters worse, treatment with broad spectrum antibiotics often kills off some of the normal bacteria in the host allowing the pathogenic bacteria to proliferate even more, thus exacerbating the infection.

Transposons

As described for R-plasmids above, new functions acquired by gene transfer are often due to transposons. Transposons are mobile genetic elements with several important features (Cohen and Shapiro, 1980; Berge and Howe, 1989). (1) Most transposons can insert at many different sites in the DNA. (2) Specific enzymes act on the two ends of the transposon DNA and promote the movement of the transposon to a new site (a process called transposition). The enzymes required for transposition are encoded on the transposon itself, so transposition does not require the host recombination system. (3) When a transposon inserts within a particular gene, it creates a mutation in that gene. (4) Any genes located between the ends of a transposon can move together with the transposon. Transposons carry a wide

variety of genes, including genes encoding antibiotic resistance, resistance to heavy metals, and even toxin genes. The antibiotic-resistance genes present in transposons are usually quite different from antibiotic-resistance genes that arise by simple mutation of bacteria lacking transposons.

Transduction

Transduction is the transfer of host DNA packaged in a phage particle. Although the extent of transduction in nature is not known, phage are quite ubiquitous in natural environments suggesting that this may be a significant method of gene exchange. Understanding transduction requires some background on the biology of phage. Phage are viruses that replicate within a bacterial cell. There are many different types of phage based upon their physical structures and specific features of their life cycles. Usually the phage particle consists of a protective protein capsid surrounding a single nucleic acid molecule—which may be single- or double-stranded, linear or circular DNA, or single-stranded, linear RNA.

Although different phage utilize many different strategies, two common methods that phage reproduce are by lytic or lysogenic growth.

Lytic Growth of Phage

Lytic growth of a phage converts an infected cell into a "factory" which efficiently produces many progeny phage. Lytic growth of phages containing double-stranded DNA typically occurs in several sequential steps (Hendrix et al., 1983).

1. *Adsorption of the phage to specific receptors on the bacterial surface.* Many different types of phage receptors exist. Typically phage receptors are proteins or carbohydrates on the surface of the bacteria that normally serve purposes other than phage adsorption.
2. *Injection of the DNA from the phage into the cytoplasm of the recipient cell.*
3. *Synthesis of phage nucleic acid and proteins.* Often the phage encodes proteins that coerce the cell to specifically synthesize the phage nucleic acids at the expense of the host nucleic acids. Expression of phage genes is regulated such that phage proteins are only synthesized at the time they are needed. Usually there is a fairly distinct difference in the time of synthesis of phage-specified enzymes (made early in the life cycle) and the structural proteins of the phage particle (made late in the life cycle).
4. *Assembly of phage particles* (morphogenesis). Two types of proteins are needed for the assembly of new progeny phage: structural proteins which form the phage particle, and catalytic proteins which participate in the assembly process but do not become part of the phage particle. Usually 50–100 phage particles are produced per cell, the number depending on the particular phage and the physiology of the host cell.

5. *Release of newly synthesized phage.* Late in the infection cycle, most phages synthesize enzymes that lyse the host cell. These enzymes disrupt the cell membrane and cell wall causing the cell to burst (lysis), and phage are released to the surrounding medium.

Generalized Transduction

Generalized transduction occurs when a phage accidentally packages a piece of DNA from the bacterial host instead of phage DNA (Figure 7). The resulting phage particle contains host DNA but no phage DNA (Masters, 1985). This phage particle can then adsorb to a new recipient and inject the bacterial DNA into the cell. This DNA may recombine with the recipient chromosome, yielding recombinants with the acquired phenotype.

Lysogenic Growth of Phage

Instead of rapidly reproducing and lysing the host cell, certain phages can enter the cell and remain quiescent, replicating once each cell division and segregrating into each daughter cell. This life style is called lysogeny. Lysogenic growth of the well studied *E. coli* phage λ involves the following steps (Ptashne, 1992); (1) The linear phage DNA molecule is injected into a bacterium. (2) After a brief period of mRNA synthesis needed to synthesize a repressor protein (which inhibits the synthesis of the mRNA species that encode the lytic functions) and a site specific recombination enzyme, phage mRNA synthesis is turned off by the repressor. (3) Recombination between the phage DNA molecule and the DNA of the bacterium inserts the phage DNA into a specific site on the bacterial chromosome. (4) The bacterium continues to grow and multiply, and the phage genes replicate as part of the bacterial chromosome. (5) Under appropriate conditions, the phage DNA may be excised from the chromosome and begin to grow lytically. The phage may remain quiescent for many cell generations before it becomes induced to begin lytic growth.

Most phages form lysogens by insertion into the bacterial chromosome as described for λ. However, not all lysogenic phage insert into the chromosome. For example, lysogeny with *E. coli* phage P1 is markedly different because the prophage is not inserted into the chromosome: it remains as a free supercoiled plasmid DNA molecule, roughly one or two per cell. Once per bacterial life cycle the P1 DNA replicates and this replication is coupled to chromosomal replication (the coupling is controlled by a phage gene). When the bacterium divides, each daughter cell receives one copy of the P1 plasmid.

A phage capable of entering either a lytic or a lysogenic life cycle is called a temperate phage. A bacterium containing a complete set of phage genes is called a lysogen. Whether integrated into the chromosome or present as a plasmid, the phage DNA in lysogens is called a prophage. More than 90% of the thousands of known phages are temperate. The advantage of this life style may be that it allows the phage to survive in environmental conditions that are not suitable for lytic growth. For

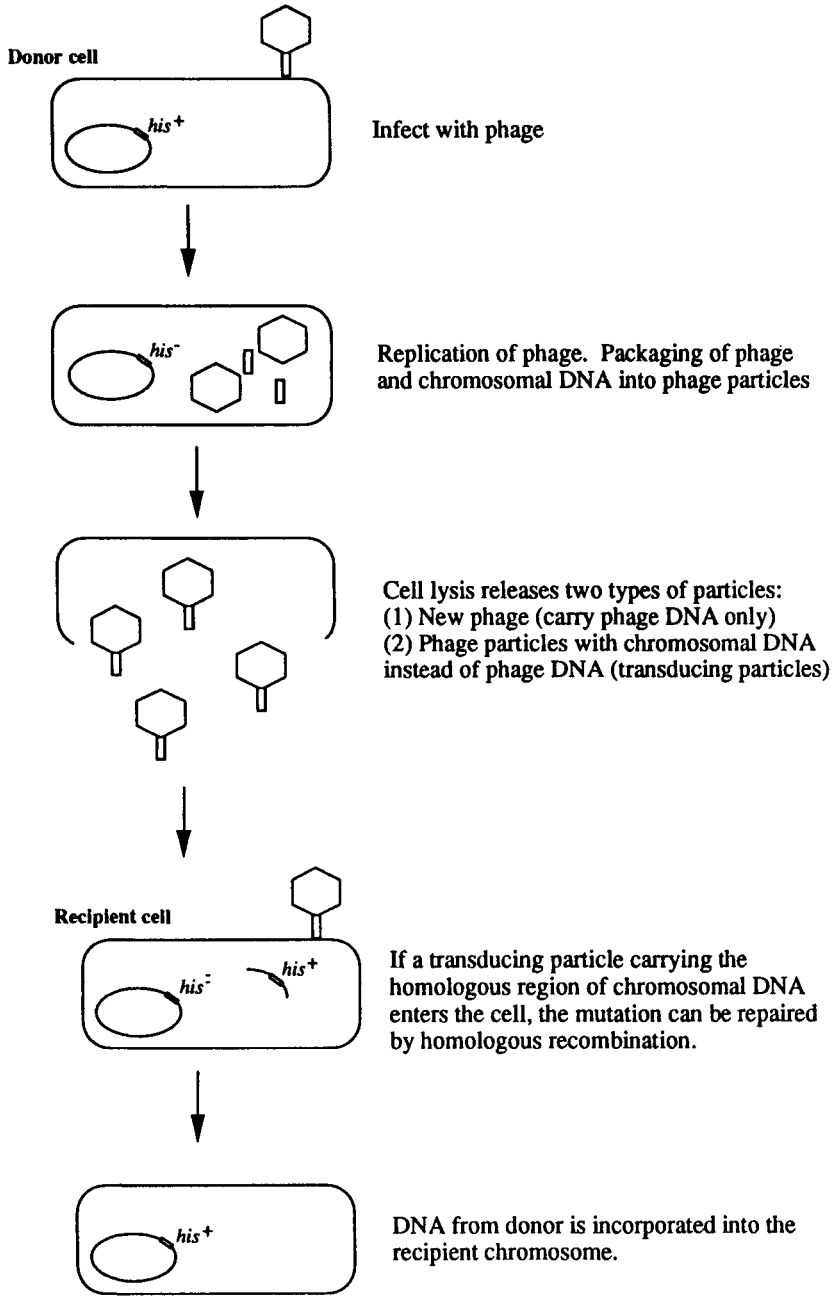

Figure 7. Gene transfer by generalized transduction.

example, when bacteria are growing very slowly because limiting nutrients are available in the surrounding medium (a common condition in nature), the infecting phage may not be able to grow lytically because phage only grow in a bacteria that are actively metabolizing. In contrast, if the phage lysogenizes the bacterium the phage genes can replicate as part of the chromosome until growth of the bacterium resumes.

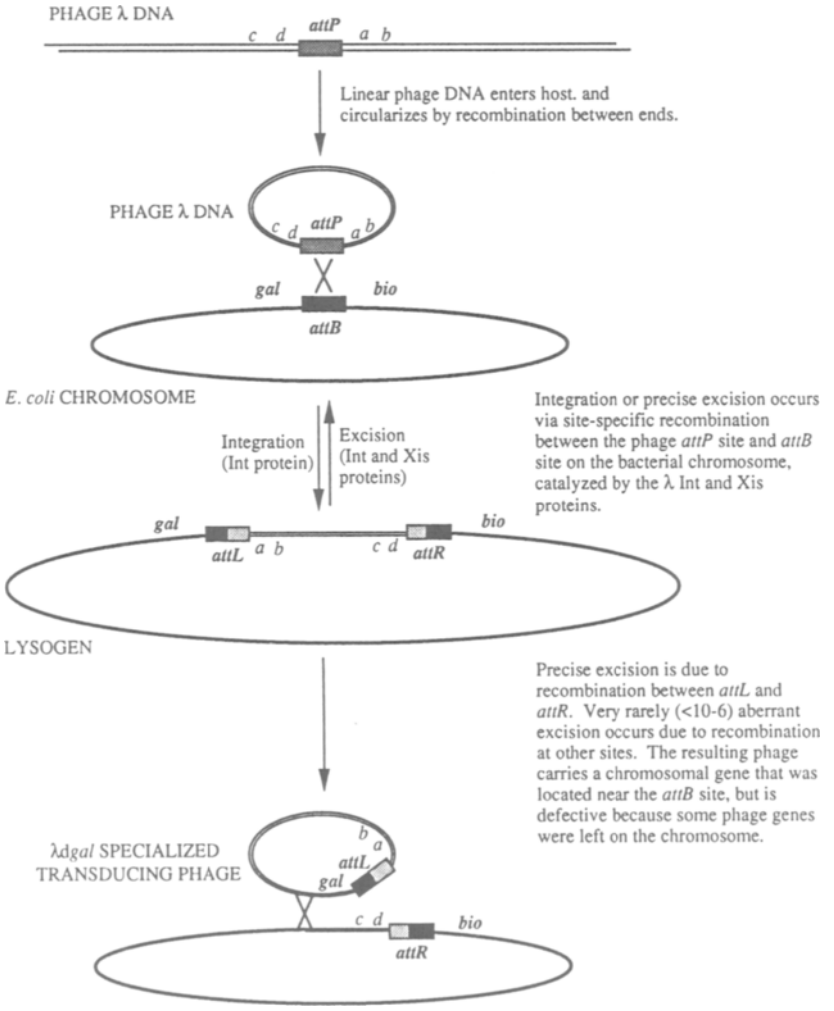

Figure 8. Formation of specialized transducing particles.

Although most phage genes are not expressed, lysogenic phage sometimes confer important phenotypes on the host cell. For example, the bacterium *Corynebacterium diphtheriae* only produces diphtheria toxin when it is lysogenized with certain phage. Several other examples of toxins produced by lysogenic phage are now known, suggesting that lysogenic phage may play important roles in bacterial pathogenesis (Salyers and Whitt, 1994).

Specialized Transduction

Specialized transduction occurs when an integrated prophage excises incorrectly and carries a piece of chromosomal DNA with the phage DNA (Figure 8). When the resulting phage particle infects a new cell the chromosomal DNA will be transferred together with the phage DNA.

SUMMARY

Although genetic processes are typically quite rare, the population size of bacterial cultures is sufficiently large that mutations and gene exchange allow bacteria to quickly evolve new functions. Many examples such as the rapid spread of antibiotic resistance in bacteria indicate that these processes have numerous practical applications in medicine.

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Chapter 5

Regulation of Cytoplasmic pH in Bacteria

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INTRODUCTION

Bacteria regulate their cytoplasmic pH (pHi) within relatively narrow limits despite their adoption of environments that vary widely in pH. Regulation of the cytoplasmic pH involves both passive and active components: the low permeability of the cytoplasmic membrane to protons and the cytoplasmic buffering capacity are both primarily passive factors that aid pH homeostasis. In contrast, regulated transport systems actively correct serious perturbations of the cytoplasmic pH (pHi) (Booth, 1985). Bacteria adapt to changes in external pH and this may increase their capacity for pH homeostasis (Goodson and Rowbury, 1989; Foster and Hall, 1990). Low pH is utilized as a defense mechanism by mammals. Invasion of the lower intestine requires survival of significant numbers of bacteria in the acidic environment of the stomach. Engulfment of bacterial cells in phagosomes, which exhibit pH values well below the threshold for the survival of most organisms, is also a major defense mechanism. In addition to this low pH, the bacteria are subjected to a variety of other stresses, such as exposure to superoxide ions, hydrogen peroxide and to the activity of small peptides that permeabilize the membrane. Survival in such hostile conditions requires major adaptive responses for which pH changes are only one of the triggers. Recent work suggests that there are strong links between pH homeostasis and apparently unrelated adaptive regimes, all of which potentiate the survival of pathogenic bacteria. This review considers our understanding of the mechanisms of pH homeostasis and how these relate to adaptive gene expression.

REGULATION OF CYTOPLASMIC pH

Passive Homeostasis

The major factor that militates against very rapid changes in pHi is the very low permeability of the membrane to protons and other ions. Large changes in the cytoplasmic pH of bacteria can only be readily effected if the cell is either treated with an ionophore or with a lipid-permeant weak acid. The cytoplasmic membrane is the major site of energy transduction and consequently, there are a large number of proton-translocating proteins inserted through the membrane. These systems should contribute little to the problem of pH homeostasis, since their activity is subject to the membrane potential ($\Delta\psi$). Changes in $\Delta\psi$ arise from small scale proton movements, but the proton movements needed to alter pHi are large (Booth, 1985). Even a small increase in the net entry rate for protons will reduce $\Delta\psi$, leading to changes in the activity of energy-transducing proteins and limiting further proton entry and consequently, preventing changes in pHi.

The other major factor preventing substantial perturbation of pHi is the high buffering capacity of the cell, which derives from protein content of the cytoplasm and from the synthesis of glutamate as a counterion for potassium accumulation. Glutamate accumulates to up to 400–500 mM in Gram negative bacteria and even

though its upper pK value is relatively acidic (pK 4.07) it could constitute significant buffering capacity at low pHi values. Basic and acidic amino acids are most frequently located at the surfaces of proteins and thus their side-groups provide the major reserve of buffering capacity. Consequently, peak values of buffering capacity are found at the extremities of the pH range (Figure 1). There is little evidence that microorganisms can specifically alter their cytoplasmic constitution with the objective of increasing the buffering capacity. However, mutants of *Salmonella typhimurium* that were selected for survival under conditions of acid stress were found to accumulate citric and isocitric acid (pK 6.4), due to a blockage in the enzyme isocitrate dehydrogenase. It has been proposed that the replacement of the glutamate (pK 4.07) with acids of higher pK might create extra buffering capacity which could enhance the survival of these organisms (Foster and Hall, 1991).

Active pH Homeostasis

For *Escherichia coli* a sudden change in the external pH of over one unit, induced by the addition of mineral acids, provokes a rapid fall in pHi of 0.1–0.15 pH units (Kihara and Macnab, 1981). Recovery of pHi takes several minutes and is dependent upon the presence of potassium and also upon the activity of the potassium uptake systems (Kroll and Booth, unpublished data) (Figure 2). The series of events that trigger the recovery of pHi has not been analyzed in detail but may involve

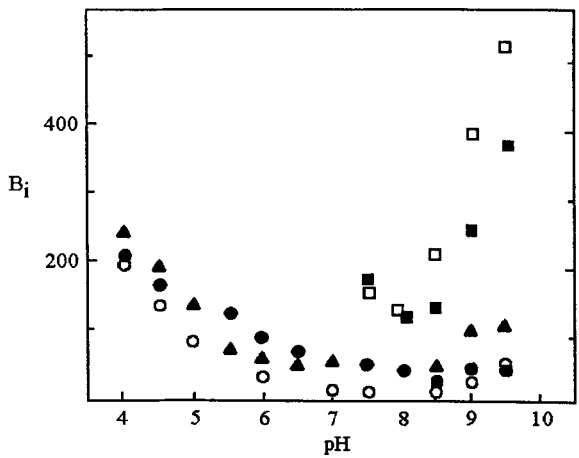

Figure 1. The buffering capacity of the cytoplasm of a range of microorganisms. The Figure is a modified form of Figure 3 in Krulwich et al. (1985) and is reproduced with the permission of the authors. Symbols: (○) *Bacillus stearothermophilus;* (▲) *Escherichia coli;* (●) *Bacillus acidocaldarius;* (■) *Bacillus alcalophilus;* (□) *Bacillus firmus* RAB.

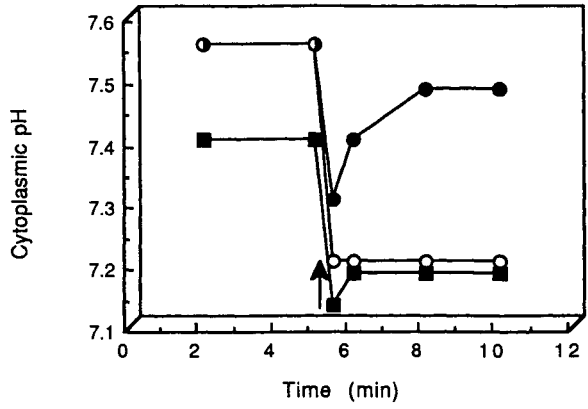

Figure 2. pH homeostasis during acid shifts in *Escherichia coli.* Cells of *E. coli* were incubated in the presence (●○) or absence (■) of 2 mM KCl at pH 7 and the time indicated (arrow) the pH of the medium was rapidly shifted to pH 6 by the addition of HCl. The cytoplasmic pH of the cells was monitored by incubations with the weak acid benzoate using ^3H_2O as a marker as described in Kroll and Booth (1981). Filled symbols: Wild type *E. coli;* open symbols; TK224O, a mutant lacking major potassium uptake systems.

several components in addition to the uptake systems. Potassium accumulation is recognized as the dominant mechanism by which the $\Delta\psi$ is depolarized leading to sufficiently large-scale proton movements to generate a pH gradient. Mutants of *E. coli* that lack potassium uptake systems display both a reduced cytoplasmic pH when incubated at acid pH (Booth and Kroll, 1981) and a reduced growth rate at pH 6 (White et al., 1992). There is no specific potassium uptake system that is required for pH regulation, merely a requirement for potassium entry to facilitate net proton extrusion by the major proton pumps. In the fermentative organism *Enterococcus fecalis* (formerly *Streptococcus*) mutants that lack either the sole proton pump, the F0–F1 H^+-ATPase or the potassium uptake system fail to flourish at acid pH (Kobayashi and Unemoto, 1980). These two systems are the major mechanisms for pH regulation and act in concert to raise the cytoplasmic pH in the fermentative bacteria. Indeed the expression of the ATPase genes is stimulated whenever the capacity for pH regulation is perturbed. Elegant studies showed that growth at acid pH, treatment with uncouplers or limitation for potassium elicited the enhanced synthesis of the membrane-located H^+-ATPase. Cells with elevated ATPase demonstrated an increased degree of pH homeostasis, an observation consistent with the known role of this enzyme in pHi control in fermentative bacteria (Kobayashi et al., 1984).

Alkaliphiles

Cation-proton exchange systems have been implicated in the controlled acidification of the cytoplasm after pHi has become too alkaline (Booth, 1985). For most alkaliphiles the dominant cation involved in pH homeostasis is Na^+. For example, *Exiguobacterium aurantiacum* fails to regulate pHi when incubated at alkaline pH in the absence of sodium ions (McLaggan et al., 1984) (Figure 3a). Addition of sodium ions brings about a rapid restoration of pHi to a value similar to that seen in growing cells. When Na^+ is in excess pHi overshoots the required pHi and is then corrected by mechanisms that have not been characterized (Figure 3a). For Na^+ to participate in pH control it must be present in the cytoplasm, but passive permeability of the membrane to Na^+ is very low and without other means of entry this would limit the participation of this ion in pH homeostasis. Two solutions to this problem have been proposed (Figure 3b): firstly, it has been proposed that Na^+-co-transport systems generate a constant supply of this cation in the cytoplasm and secondly, that a specific channel exists that allows pH-regulated sodium ion entry (Krulwich et al., 1985; Booth, 1985). Both solutions are plausible. A pH-regulated Na^+ channel could respond specifically to the need for pH regulation in the organism. Activation of the Na^+ channel would provoke a major influx of sodium ions and would transiently depolarize the membrane; respiration would rapidly restore the membrane potential ($\Delta\psi$) which could then drive the Na^+/H^+ antiport leading to proton entry and net acidification (Figure 3b). A pH-regulated Na^+/H^+ antiport has been characterized in a number of alkaliphiles and has been shown to be capable of acidifying the cytoplasm in a Na^+-dependent manner (Krulwich, 1985).

To summarize, the regulation of cytoplasmic pH in bacterial cells is facilitated by both passive and active mechanisms. The cytoplasmic membrane acts as a barrier to proton entry and the buffering capacity of the cytoplasm can limit the effects of any protons that do enter the cell. In addition, there is a significant over-capacity in many cytoplasmic enzyme pathways that may enable the cell to maintain adequate fluxes despite the apparent pH sensitivity of individual reactions. The active processes include the potassium uptake systems that generate an alkaline interior during turgor regulation, channels that may facilitate the entry of ions that are the substrates for cation-proton antiports that function to lower the cytoplasmic pH, and the antiport systems themselves. Despite the relative constancy of the cytoplasmic pH under many conditions it is likely that it is a dynamic parameter that is maintained by the combined activities of many systems. Inhibition of any one of these systems may lead to changes in cytoplasmic pH (e.g., Figure 2). The detailed analysis of the ion movements involved in pH homeostasis will require combined genetic and electrophysiological approaches analogous to those applied to animal cells. The recent development of patch clamping techniques to bacterial membranes (Delcour et al., 1989) will greatly facilitate the analysis of pH homeostasis.

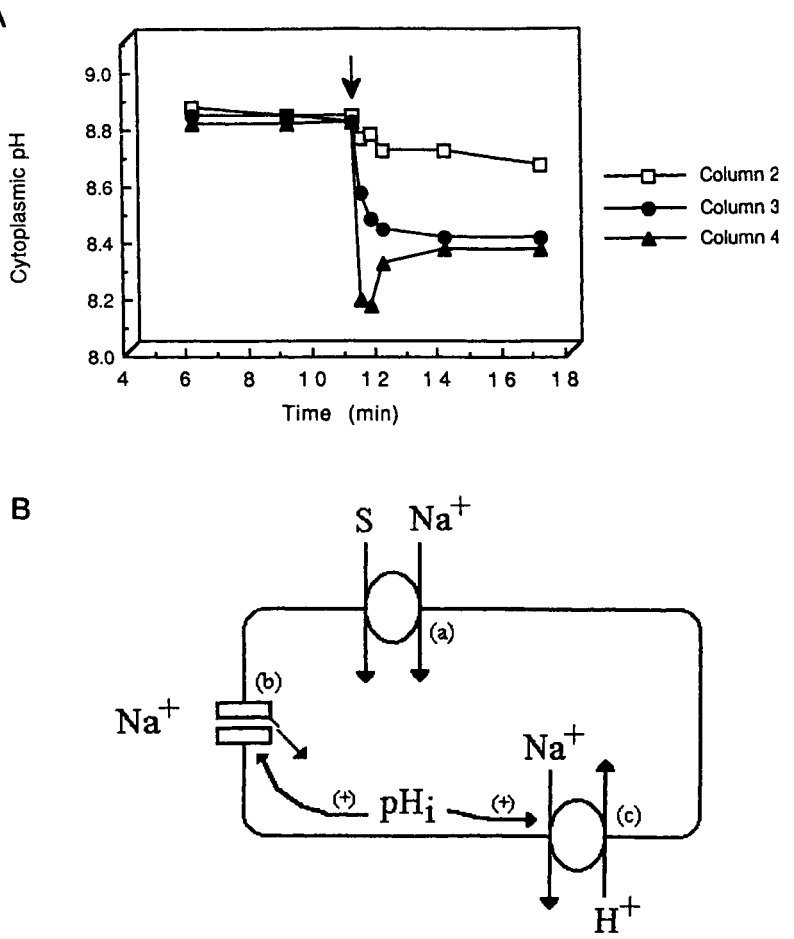

Figure 3. Regulation of cytoplasmic pH in the alkaliphile, *Exiguobacterium aurantiacum*. A. Regulation of cytoplasmic pH during pH shifts is dependent upon the sodium ion concentration; symbols: (□), 0.1 mM NaCl; (●), 2 mM NaCl; (▲), 10 mM NaCl. Data taken from McLaggan et al. (1984) with permission. Cells were incubated in 50 mM CHES-KOH buffer (pH 9) containing 1mM $(NH_4)_2SO_4$ in the presence of 10 mM glucose for 7 min. After a further 4 min NaCl was added. Throughout the incubation, cytoplasmic pH was monitored with methylamine. B. Na^+ circuits in alkaliphiles; The proposed elements of the circuit are: (a) the Na^+-symports that facilitate nutrient uptake, (b) a Na^+ channel that is predicted to be regulated by changes in cytoplasmic pH, (c) the Na^+/H^+ antiport which facilitates proton entry in exchange for cytoplasmic Na^+ ions. The antiport and the channel are the two elements of the circuit that are proposed to respond specifically to changes in cytoplasmic pH.

ADAPTATION TO CHANGES in pH

The earliest reports of acid-induced gene expression were made over 50 years ago, with the observation of the decarboxylases (see Gale, 1946; Booth, 1985; Olsen, 1993). Bennett has proposed that the induction of lysine decarboxylase at acid pH may make a significant contribution to cytoplasmic buffering capacity through the consumption of protons during synthesis of cadaverine from lysine (Meng and Bennett, 1992). Such mechanisms work well in amino acid-rich environments and are likely to be important for bacterial survival in foods; they are less favored mechanisms when the environment is amino acid-depleted, for example in the phagosome. Other decarboxylases are also induced at acid pH and the generality of this pattern has led to the suggestion that the primary role of these enzymes is the provision of carbon dioxide in acid environments (see Gale, 1946; Boeker and Snell, 1972). Carbonic acid (pK 6.2) will be present at only low concentrations at low pH but is essential for growth and consequently the amino acid decarboxylases may be induced to fulfill this requirement.

The Acid Tolerance Response

The discovery of the acid tolerance response for *E. coli* and *S. typhimurium* (Goodson and Rowbury, 1989; Foster and Hall, 1990) has led to further understanding of adaptation to acid pH. Bacteria that have been incubated at pH 4.3–6.0 for short periods show much greater survival at pH 3.0 than identical organisms grown at neutral or slightly alkaline pH (Figure 4). This adaptation is dependent upon protein synthesis but the functions of the induced proteins are unknown. Enteric bacteria that have been adapted to moderate pH have been found to possess greater ability to regulate their cytoplasmic pH when they are exposed to very acidic conditions (pH 3.3).

A number of genetic loci have been associated with altered acid tolerance responses, the ATPase operon (*unc*), the *phoP* gene and the iron regulatory protein (*fur*) (Foster and Hall, 1992). A common bond between iron regulation and acid survival is not unexpected. The *fur*-controlled genes are involved in iron acquisition and the activity of the genes under the control of *fur* is potentially a major pathogenicity index. When the human body mounts an antibacterial response, it uses several iron binding proteins to limit the availability of this ion to the invading bacterium (Hassett and Chen, 1989). One of these proteins, lactoferrin, is released into the phagosome after engulfment of a bacterium. Low pH is also a feature of the phagosome and the bacterium may use the limitation of iron to sense its location in this organelle. In addition, the ingestion of the bacterium into the phagosome elicits the respiratory burst, producing superoxide ions and hydrogen peroxide. The well characterized oxidation stress and redox cycling responses are induced by the presence of these toxic oxygen species (Demple, 1991). Detoxification enzymes, such as peroxidases, catalase, superoxide dismutase and their associated metabolic

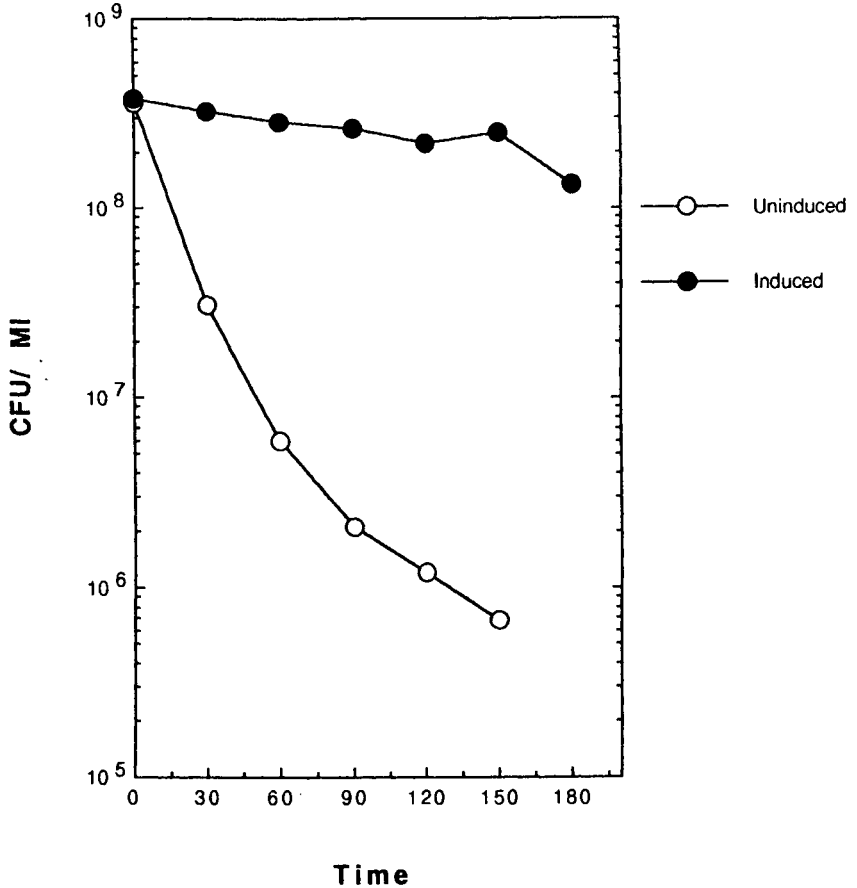

Figure 4. Effect of induction of the ATR on survival of *E. coli* MC4100 at pH 3.3. Exponentially growing *E. coli* MC4100 cells (Vogel Bonner medium E, pH 7.6, 0.2% glucose) were either acidified to pH 3.3 by the addition of HCl (○) or were first adjusted to pH 4.3 for 30 minutes prior to incubation at pH 3.3 (●). The survival of the cultures was estimated by viable plate counts.

systems, glucose-6-phosphate dehydrogenase and glutathione reductase, are part of this stress response. Limitation of the supply of iron would affect the ability to form active catalase and superoxide dismutase; consequently, the toxicity of active oxygen species would be enhanced.

Links Between pHi, Stationary Phase and Multiple Antibiotic Resistance

There are further connections to be built here between changes in cytoplasmic pH and adaptation to stress. DNA repair systems are induced by oxidation stress to

counteract the DNA damage caused by reactive oxygen species. Recently, it has become clear that acidification of the cytoplasm reduces the mutagenicity of powerful mutagens and reduces the lethality of electrophilic reagents (Booth, unpublished data), possibly by inducing DNA repair systems. Similarly, stationary phase cells induce elements of the oxidation stress response and are also more resistant to the effects of acidic pH (Matin, 1991). The starvation sigma factor *rpoS* (*katF*), which is required for transcription of some of the stationary phase survival genes, can be induced by acidification of the cytoplasm, although this is almost certainly not the only signal for induction of this gene (Schellhorn and Stones, 1992). The genes controlled by the PhoP protein (see below) are essential for pathogenicity of *S. typhimurium* and *phoP* mutants exhibit a diminished acid tolerance response (Groisman and Saier, 1990; Foster and Hall, 1991). While the mechanisms of protection by the induced proteins are not fully understood, the link to survival of acid pH in the phagosome cannot be ignored.

Recently, further exciting links between intracellular pH and adaptation to the human host have been suggested. This set of links is built around the *marA* locus, which controls the expression of a number of genes and operons, many of which encode outer membrane proteins and some of which affect the intrinsic resistance of *E. coli* cells to agents as diverse as chloramphenicol and menadione (Cohen et al., 1989). The sequence of the *marA* gene suggests that its product is a regulatory protein. Intriguingly, the protein displays considerable homology to the SoxS protein, which is the regulator of the genes controlling resistance to redox cycling agents, such as menadione (Gambino et al., 1993), and many of the effects caused by mutation at the *marA* locus can be mimicked by exposure of cells to redox cycling agents (Hächler et al., 1991). The MarA protein also regulates the synthesis of the OmpF porin through stimulation of synthesis of the *micF* RNA, which inhibits translation of the *ompF* mRNA. The *marA* gene has recently been recognized as the repressor of a gene of unknown function, *inaA*, which was recognized by its induction by weak acids at pH 6. Both *inaA* and the MarA phenotype are induced by salicylic acid. Redox cycling agents generate superoxide ions which may cause membrane damage causing changes in the membrane permeability similar to the effects of salicylate (Figure 5). This corresponds to the situation faced by the bacterium when it enters the phagosome: low pH coupled with high levels of superoxide and peroxides will generate membrane damage. The changes in cytoplasmic pH encountered by the bacterium under these conditions are mimicked by the addition of weak acids or mild uncouplers. Thus, the *marA* regulon may constitute an adaptive response to aid survival of the host's defense mechanisms. How resistance to chloramphenicol and tetracycline fit into this picture is not obvious, but it would not be surprising to find that the true substrates for these drug transport systems expressed under the control of the *marA* locus are cell toxic agents synthesized in phagosomes.

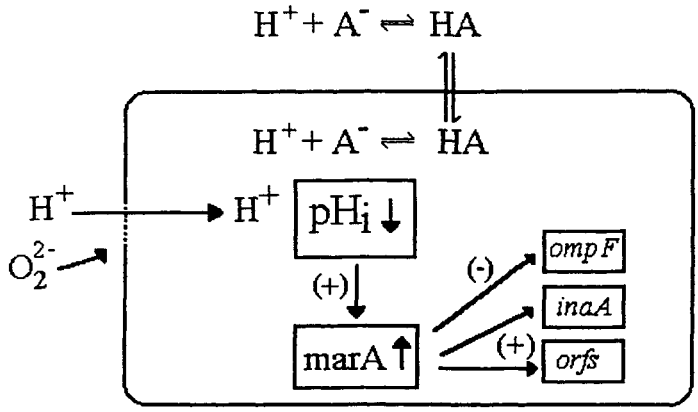

Figure 5. A model for the induction of the MarA response by oxidative stress and by weak acids. It is proposed that superoxide ions damage the membrane leading to acidification of the interior in a manner analogous to the addition of a weak acid to the medium. The change in cytoplasmic pH is sufficient to induce the expression of the MarA locus leading to changes in gene expression at other loci.

pH SENSING

Both adaptive gene expression and the ability to regulate pHi imply a capacity to sense changes in pH at the cell surface and in the cytoplasm. The mechanisms of pH sensing are far from clear but some insights are emerging. As was illustrated above (Figure 2), a rapid change in external pH will provoke a change in pHi that may be sustained for several minutes, sufficient to activate regulatory proteins and initiate changes in gene expression. Extreme acid-shock will also cause a major perturbation of cell metabolism. Not only is the cell likely to be cut off from a source of readily utilizable carbon leading to a change in the carbon and energy status of the cell, leading to imposition of the stringent response and changes in catabolite repression. Other changes may affect the balance of product and by-product, for example, the pattern of electron flow to oxygen and generation of superoxide may be affected, leading to signals for induction of stress genes. Proteins caught in the assembly process may unfold and provide signals for induction of heat shock proteins. These changes cannot be ignored when signals that effect adaptive changes in gene expression are being sought.

More specific control mechanisms must also be involved. Early work on chemotaxis demonstrated that a response is elicited both to acid external pH and to weak acids that alter the cytoplasmic pH (Kihara and Macnab, 1981). The chemosensors (MCPs) are generally transmembrane proteins that undergo changes in methylation in response to signal reception. The MCPI protein was shown to respond to reduced pH in the cytoplasm or in the environment. Thus, changes in the activity of

transmembrane sensors consequent upon changes in either external or cytoplasmic pH are likely to be very important mechanisms of provoking switches in gene expression.

Porin Gene Expression: A Paradigm?

Expression of the porin genes responds to a wide range of environmental stimuli including acidification of external and internal pH (Heyde and Portalier, 1987; Thomas and Booth, 1992). Regulation occurs at many levels including catabolite repression and DNA topology, but the crucial regulatory elements are the EnvZ/OmpR protein couple. The EnvZ protein is the transmembrane sensor that is essential for the expression of the porin genes. This protein is a member of the highly homologous, histidine protein kinase (HPK) family, members of which play a major role in regulating the bacterial response to environmental stresses (Stock et al., 1989). (PhoP, described above is also a member of this family of gene products). High osmolarity leads to suppression of expression of the OmpF porin and induction of the OmpC porin and this change arises from alteration of the level of the phosphorylated form of the OmpR protein (OmpR-P), which is the product of activation of EnvZ (Mizuno and Mizushima, 1990). The phosphatase activity of EnvZ is very sensitive to the potassium level in the cytoplasm and this is one of the primary mechanisms that modulates the level of OmpR-P. If the histidine protein kinase, transphosphorylase or phosphatase activities of other HPK enzymes is sensitive to pHi, this could be sufficient to effect significant changes in gene expression. Other changes in regulatory parameters, for example, DNA topology, are also likely to be important aspects of the regulation of gene expression by pH. Recent work suggests that a shift to alkaline pH is equivalent to an increase in osmolarity, i.e., increased negative DNA supercoiling in cells incubated at alkaline pH (Thomas and Booth, 1992; Karem and Foster, 1993; see also Higgins et al., 1990). Changes in DNA topology can be either inhibitory or stimulatory to gene expression and is likely only to be an important ancillary effector for most genes that are regulated by specific DNA protein interactions.

SUMMARY

Bacteria regulate the cytoplasmic pH principally through the controlled movement of cations across the membrane. The major cation fluxes are those of potassium and sodium ions, the latter achieving particular importance in the alkaliphiles. Regulation of the cytoplasmic pH is aided by the relatively low proton permeability of the membrane and by the cytoplasmic buffering capacity which prevent large pH changes in the cytoplasm. The bacterial cell has been demonstrated to possess adaptive regimes of two types that allow the cell to survive changes in the external pH. Some bacteria have the ability to sense acid pH and to swim away from acidic environments (pH taxis). In addition there is a general phenomenon of adaptation

to acidic conditions. Thus, bacteria that have been growing in mildly acidic conditions have been shown to survive more hostile pH values better than bacteria grown at neutral pH. The changes in gene expression needed to bring about this increased survival at very acid pH are not fully understood, but it is increasingly apparent that this adaptive phenomenon cannot be separated from other adaptive strategies, in particular those involved in survival of exposure to superoxide ions and redox cycling agents and those associated with adaptation to starvation conditions. The adaptation to changes in pH can thus be seen to significant aspects of the pathogenesis of the organism.

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Chapter 6

Vertebrate Hormones in Bacteria and Microbial Eukaryotes

JOHN LENARD

INTRODUCTION

We are accustomed to thinking of endocrinology in human, or at least in mammalian terms. A moment's reflection, however, might persuade us that the general problem that hormones address—intercellular communication so as to integrate responses from different cells—has existed for far longer than vertebrates themselves. Viewed in this way, it might not surprise us to find recognizable forerunners of our familiar

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endocrine systems in more ancient organisms. In this chapter we will review some of the evidence that this is indeed so, and speculate upon the possible medical implications.

INSULIN

Insulin is the most intensively studied hormone. Its universal presence in vertebrates, including the most primitive species, has been clearly established. Insulin is not limited to vertebrates, however. Insulin-like peptides have been found in several other phyla, including echinoderms, molluscs, crustaceans and insects. Removal of insulin-producing cells from certain insects results in elevated concentrations of trehalose and glucose in the hemolymph—a close parallel to the familiar hyperglycemic effects of insulin insufficiency in vertebrates (LeRoith et al., 1986). Genes for the bombyxins, a family of insulin-like proteins of still undiscovered function, have been cloned from moths (Iwami et al., 1989). In addition, a gene encoding a protein possessing high homology with mammalian insulin receptor has been cloned from *Drosophila*, based on its immunological cross-reactivity (LeRoith et al., 1986).

Even more surprising, perhaps, has been the finding of insulin-like molecules and insulin-induced responses in still more primitive organisms, including prokaryotes and others generally regarded as unicellular. So unexpected were these findings that they are still regarded as controversial, and will likely remain so until genes involved in the putative signaling pathways are identified. In the meantime, however, we may consider the numerous observations pointing to the existence of insulin-based signaling systems in both prokaryotic and eukaryotic microbial cells (Lenard, 1992).

The earliest findings were that *Escherichia coli, Tetrahymena pyriformis, Neurospora crassa* and *Aspergillus fumigatus* all secreted material that cross-reacted with antibodies specific for mammalian insulin. Amounts of cross-reacting material were low, but this could have arisen from the unavailability of authentic standards for the radioimmunoassays; partial cross-reactivity with the mammalian insulin standards would necessarily give incorrectly low figures. Further, this cross-reacting material chromatographed similarly to authentic insulin on gel filtration, and also possessed insulin-like lipogenic activity on mouse adipocytes. Activity was blocked by anti-insulin antibodies, and also by antibodies to the murine insulin receptor, demonstrating an insulin-like mode of action through the endogenous receptor (Lenard, 1992).

The effects of insulin on fungal cells were characterized using a wall-less strain of *N. crassa*. Numerous metabolic effects were noted following administration of modest (10–100 nM) levels of insulin. Production of CO_2, ethanol, glycogen and several other metabolites of glucose were all enhanced. Increased glycogen production was correlated with apparent exhaustion of intracellular levels of UDP-glucose, the substrate for glycogen synthase. Interestingly, *N. crassa* glycogen

synthase was activated by insulin, from a glucose-6-phosphate *d*ependent form (D) to a glucose-6-phosphate *i*ndependent form (I). An identical activation by insulin occurs in mammalian cells, where activation from the essentially inactive D form to the active I form results from dephosphorylation of the enzyme by a specific phosphatase, which is itself activated by insulin-dependent phosphorylation. Half-maximal activation of *N. crassa* glycogen synthase required 2 nM insulin, a level comparable to that required in mammalian cells. Insulin-induced phosphorylation of numerous other *N. crassa* proteins was detected by two-dimensional gel electrophoresis, and occurred at tyrosine, threonine and serine residues. These findings are all consistent with the existence in *N. crassa* of a protein kinase-mediated signal transduction pathway, specifically activated by insulin (Lenard, 1992).

Insulin appears to be present in cells of all life forms, as indicated by the finding of cross-reacting material in every organism in which it has been sought, extending even to species of archaebacteria. Insulin has recently been reported to accelerate the germination of fat-bearing seeds (e.g., cucumber, melon, squash), perhaps owing to its reported ability to enhance glyoxysomal enzyme activity (Goodman and Davis, 1993). In mammals, insulin is remarkable for the extraordinary diversity of its functions, including poorly characterized developmental effects. The importance of these developmental actions is evident in humans born without functional insulin receptors (a condition known as leprechaunism, Kahn et al., 1992), which results in severe defects and rapid death. Interestingly, insulin is present in the mammalian brain, suggesting that it is synthesized there, since the peptide hormone is unable to cross the blood-brain barrier (LeRoith et al., 1986).

All these considerations have led to the notion that insulin may be among the most ancient of hormones, having evolved early to coordinate certain essential intra- and intercellular events, perhaps those concerning nutrient acquisition and storage. If this is so, then insulin's prominent hypoglycemic action in mammals may be a relatively recent development, representing a specialized adaptation of this original function.

CATECHOLAMINES

The catecholamines have been less extensively studied than insulin, but their signaling pathways also appear to be widely distributed. Growth of *E. coli* in serum-containing medium was dramatically enhanced by addition of norepinephrine, under conditions chosen to simulate the blood during sepsis (Lyte and Ernst, 1991). Other catecholamines had more modest effects. Further studies showed that norepinephrine acted to reverse a bacteristatic effect of the serum itself (Lenard and Vanderoef, 1995). This may be of considerable medical significance, since sepsis is characterized by highly elevated catecholamine levels, suggesting that these agents may affect the course of the disease by directly stimulating growth of the pathogen (Lyte, 1993). Similar interactions have been proposed for other pathogenic microorganisms, notably *Trypanosoma*, in which adenylyl cyclase

activity could be modulated by adrenergic drugs (DeCastro and Luz, 1993). Functional effects of added catecholamines have been documented in *Tetrahymena*, and authentic catecholamines are synthesized by these cells (Lenard, 1992).

STEROIDS

There is good evidence for the existence of steroid hormone pathways in yeasts and fungi. Several pathogenic yeasts are affected by the steroid hormone status of their hosts. The disease caused by the yeast *Paracoccidiodes brasiliensis* is about 50 times more common in males than in females, despite a similar incidence of infection. The difference has been attributed to inhibition of the dimorphic transition, from the benign mycelial to the pathogenic yeast form, by the females' higher levels of estradiol. The organism possesses a cytoplasmic estradiol-binding protein, a putative receptor, which probably mediates this inhibition. An opposite effect has been suggested to explain the high rate of infection by *Coccidioides immitis* in pregnant women, where elevated levels of sex hormones promote pathogen growth, probably through interaction with a specific progesterone binding protein. In addition, corticosteroid binding proteins have been isolated from several yeasts, and endogenous steroidal ligands for some of these proteins have been found, suggesting the existence of complete steroid signaling pathways (Lenard, 1992).

Evidence for the existence or activity of other mammalian hormones in various microbial cells is much more fragmentary, and their significance correspondingly more speculative. The *Saccharomyces cerevisiae* sex hormone, alpha factor, is a small polypeptide whose signaling pathway has been extensively characterized in recent years using genetic approaches. The pathway is initiated by hormone binding to a membrane receptor, followed by the sequential actions of a trimeric G protein, several protein kinases and finally at least one transcriptional regulator—all familiar elements in mammalian pathways (Elion et al., 1993). A sequence homology was detected between alpha factor and the hypothalamic hormone GRH (gonadotrophin releasing hormone), and a GRH-like activity of alpha factor was demonstrated using mammalian pituitary cells. Glucagon has been reported to activate adenylyl cyclase in *N. crassa*, as it does in mammalian cells. The presence of several other polypeptide hormones was also detected by radioimmunoassay in both pro- and eukaryotic microbial cells, and corresponding activities were described for some of them (Lenard, 1992).

CONCLUDING REMARKS

Is there a coherent perspective from which to view these diverse reports and observations? Recent years have provided dramatic new evidence of the extent to which basic cellular processes, such as cell division and vesicular transport, have been conserved through vast stretches of evolutionary time. It is well known that all of the individual elements of mammalian signal transduction pathways—recep-

tors, G proteins, adenylyl cyclase, protein kinases, transcriptional activators—are present in even the most primitive eukaryotes, and many are present in some form in prokaryotes as well. We may reasonably suppose, then, that entire pathways that integrate essential physiological responses may have been conserved as well.

One might object, however, that hormonal effects are inherently multicellular, and therefore irrelevant to unicellular organisms. Against this objection two arguments can be made. First, there are many examples of mammalian hormones acting in an autocrine manner, i.e., the producing cell and the target cell are the same, the hormone acting directly upon the cell that produced it. This phenomenon may be expected to occur in other organisms as well. Second, it is becoming less clear just what a "unicellular organism" is, in light of findings that even bacterial colonies can show unexpected complexities, including differentiated regions exhibiting specialized functions (Shapiro, 1988). Organisms that we regard as unicellular might simply be those atypical few that survive under laboratory conditions, an experimental artifact extracted from a world of interdependent cells.

It seems clear from the few examples described above that molecular interactions between the endocrine signaling systems of hosts and invading pathogens may be widespread, and are likely to be of considerable medical significance. Host hormones may stimulate or inhibit the growth of invaders, or conversely, pathogens may send chemical messages out to subvert host endocrine networks to their own purposes. The possible adaptations and counter-adaptations, as yet only barely glimpsed, could be as richly diverse as any described by classical ecologists. Further elucidation of endocrine mechanisms in microbial species promises to yield new insights to basic scientist and clinician alike.

NOTE ADDED IN PROOF

Isolated reports related to this chapter continue to appear. The yeast *Saccharomyces cerevisiae* was found to exhibit enhanced growth upon addition of insulin to the growth medium (Berdicevsky & Mirsky, 1994). Morphological changes were also observed: the cells were almost round in the presence of insulin, whereas elongated forms appeared in its absence (Mirsky & Berdicevsky, 1994), reminiscent of published reports of the effects of insulin on growth of *Neurospora crassa* (Lenard, 1992). However, a search of the yeast genome revealed no open reading frames encoding protein sequences that were significantly homologous to human insulin or insulin receptor (unpublished observation).

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Chapter 7

Principles of Bacterial Pathogenesis

DANIEL C. STEIN

INTRODUCTION

The human fetus, at birth, is essentially devoid of microbial life. As the fetus passes through the birth canal, it encounters its first microbial flora. Within a few days, the infant is infected with a large number of microorganisms. In a short time, the number of microorganisms found in/on the body outnumbers the number of cells contained within the body (1×10^{14} bacteria versus 1×10^{13} human cells). However,

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in spite of the large number of microorganisms found on/in the body, this colonization does not imply that man lives in a disease state. In a healthy individual, these bacteria exist in and on the body without causing disease. In fact, some of these microorganisms provide the host with tangible benefits (e.g., the flora of the intestinal tract produce essential vitamins while the flora colonizing all body surfaces provide an antagonistic environment to transient microbes and protect the host from colonization by pathogens).

The terms infection and disease are often used interchangeably; however, it is important to note the distinction between the two. Disease is defined as an abnormal condition having characteristic symptoms. Infection is the multiplication or maintenance of a parasite on the surface or within a tissue. The presence of a microorganism in or on the body does not need to lead to disease. Likewise, disease may be present in the absence of microorganisms. This chapter will deal with microorganisms and their characteristics that allow them to cause disease.

NORMAL FLORA

The microorganisms found in/on a healthy individual can be considered normal flora. The composition of this flora remains relatively constant, with minor changes occurring: during or after disease; due to changes in diet, or due to hormonal changes. The relationship between the host and his/her normal flora can be mutualistic, where both organisms benefit, or commensal, where one organism benefits from the interaction, but the other is not harmed. Factors that determine whether a specific microorganism will grow in a given body site include: (1) whether oxygen is present, (2) the pH of the host site, (3) the types of other microorganisms found in these sites, and (4) the availability of nutrients.

The normal flora represent the best example of a successful host-parasite interaction, because these bacteria thrive without inflicting damage to the host, and the host defense mechanisms tolerate or ignore these microorganisms. The host response to the same bacteria can vary between individuals, and reflect a variety of parameters independent of the bacteria. Normal flora should not be confused with transient microflora (those microorganisms that we encounter in our daily activities).

Normal flora are found colonizing the skin, respiratory tract, oral and digestive tract and lower urogenital systems. Because each of these body sites provides a unique environment, the types of bacteria present in each will vary. For example, *Staphylococcus aureus* is part of the normal flora of the skin because conditions found there allow for its growth (acidic pH, high salt concentration, and dry environment). Conversely, *S. aureus* is not found in the large intestine because bile salts prevent it from growing in this niche.

Although all body surfaces open to the environment are colonized, organs not exposed to the environment are sterile. In a healthy individual, the circulatory system or cerebral spinal fluid is sterile, and the presence of any microorganisms

in these sites is abnormal. Normal flora growing in their particular niche are not considered disease causing. However, when they find the opportunity to establish a colonization in another niche, they may cause disease.

PORTALS OF ENTRY

Humans continually encounter a plethora of bacteria from the environment, yet the establishment of infection after each contact tends to be the exception rather than the rule. The ability of transient microflora to establish infection requires that they overcome the host defense mechanisms. The first barrier to infection is intact host surfaces. Bacteria, or their products, must breach this barrier, or the bacteria must be mechanically introduced into the host via a fomite or vector. Because of the unique characteristics of each host surface, bacteria possess varying abilities to enter at different sites.

When colonization is required for disease, the bacteria must gain access to the host, adhere to tissues, penetrate or evade defense mechanisms, and damage the host tissue. When microbial produced toxins are responsible for disease (intoxication), colonization is not required, but the toxin must enter the host to reach its site of action. The site at which a microbe enters the host is the portal of entry. The portals of entry of some pathogenic bacteria are shown in Table 1.

TRANSMISSION OF INFECTIOUS AGENTS

The portal of entry for a particular bacterium can be dependent on the transmission of the microbe from its natural habitat (source or reservoir of infection) to the human host. Transmission can occur through inhalation, ingestion, direct contact with contaminated objects (fomites) and infected individuals, or via vectors (e.g.,

Table 1. Portals of Entry for Some Bacterial Pathogens

Route	*Examples*
Intestinal tract	Salmonella sp., Shigella sp., *Clostridium botulinum, Bacillus cereus,* enterotoxigenic *Escherichia coli.*
Respiratory tract	*Mycobacterium tuberculosis, Mycoplasma pneumoniae, Bordetella pertussis.*
Skin	*Staphylococcus aureus, Clostridium tetani, Pseudomonas aeruginosa*
Oropharynx	*Neisseria meningitidis, Streptococcus pneumoniae*
Urogenital tract	*Neisseria gonorrhoeae, Treponema pallidum, Chlamydia trachomatous*
Transplacental	*Treponema pallidum*
Arthropod mediated	*Borrelia burgdorferi, Yersinia pestis*

Table 2. Routes of Transmission of Infectious Diseases

Mode	*Description*	*Examples*
Direct contact	Direct transmission of infectious agent; no intermediate object is involved	Gonorrhea, staphylococcal infections, anthrax
Indirect contact	Agent of disease is transmitted from its reservoir to susceptible host by a non-living object (fomite).	Pseudomonas infections
Droplet transmission	Microbes are spread by droplets (i.e., mucous droplets) that travel only a short distance; spread by coughing, sneezing, etc.	Pneumonia, whooping cough
Waterborne	Pathogens are spread through ingestion of contaminated water.	Cholera, waterborne shigellosis
Foodborne	Pathogens are transmitted in foods that are incompletely cooked, poorly refrigerated or prepared in an unsanitary manner.	Botulism, salmonellosis, food-borne shigellosis
Airborne	Spread by droplet nuclei in dust that travels more than one meter from reservoir to host	Tuberculosis, pneumonic plague
Arthropod borne	Insects carry pathogen from one individual to another.	Lyme disease, plague, rocky mountain spotted fever.
Transplacental	Infectious agents are transmitted from mother to fetus.	Syphilis

arthropods). If the agents that cause disease can be transmitted to others, they are considered infectious agents, and the diseases are infectious diseases. Any disease that spreads from one host to another, either directly (e.g., gonorrhea) or indirectly (e.g., shigellosis), is said to be a communicable disease. A noncommunicable disease is not spread from one host to another (e.g., Legionella pneumonia). These diseases are caused by normal flora (e.g., urinary tract infections in women), or by microorganisms that reside in the environment (e.g., cholera). Zoonoses are diseases that primarily occur in animals but can be transmitted to humans (e.g., bubonic plague). The different routes of transmission of bacterial infection are shown in Table 2.

PATHOGENIC MICROORGANISMS

The development of disease after exposure to an infectious agent depends on properties of both the bacterium and the host. Microorganisms vary in their ability

to cause disease. Those that are successful are pathogens and the manner in which a disease originates is referred to as its pathogenesis. Bacterial characteristics that contribute to pathogenicity are virulence factors. A pathogens' ability to cause disease in a given host varies due to individual differences in the host's defense mechanisms (immune response), and due to strain differences (these are sometimes referred to as differences in virulence or differences in pathogenicity). If one could infect the same individual multiple times with different strains of the same species of bacteria (and the host did not develop an immune response to the infection), differences in the outcome of each infection would reflect differences in the pathogenic potential of each strain. The severity of the disease reflects the total of the expression of the virulence determinants of the infecting strain(s). These differences in the severity of the disease are due to differential expression of virulence determinants. Some commensal microorganisms are unable to cause disease when present alone in certain biological niches. However, when other strains are present, the two "nonpathogens" can interact and cause disease. This synergistic interaction is commonly seen in infections caused by anaerobic microorganisms.

Opportunistic pathogens are organisms that proliferate and cause disease only when the hosts defenses are compromised or suppressed. They are found in our day to day surroundings and can be part of our normal flora. They fail to cause disease because other resident microflora keep their growth in check, or the hosts normal defense mechanisms prevent the organisms from causing disease. There are several organisms that are always associated with disease. These are obligate pathogens, and there presence in the host is always associated with a disease state (e.g., syphilis).

ESTABLISHMENT OF THE DISEASE STATE

Infectious diseases are established by colonization with a microorganism or intoxication with a microbial product, and a response from the host. Diseases resulting from colonization depend on the establishment of the pathogen within the host. In contrast, intoxication does not require infection, but depends upon the action of a bacterial product (usually a toxin).

Since humans live in a constant state of infection, disease can arise from the indigenous microflora (opportunistic pathogens). Opportunistic pathogens usually cause disease in response to changes in the host. For example, when an individual visits the dentist to have a tooth extracted, the removal of the tooth breaches the integrity of the oral mucosal membranes, allowing the normal microflora of the oral cavity access to the circulatory system. Some of these flora (e.g., α-hemolytic Streptococci) are capable of colonizing heart tissue and causing tissue damage. In the healthy individual, these flora cannot access heart tissue and therefore do not cause disease. The process of extracting a tooth allows them access to a new niche and they take advantage of this opportunity by colonizing it. While infection of the

oral cavity by α-hemolytic Streptococci is innocuous, infection of heart tissue results in disease.

Disease also arises from our interactions with the environment. Through a variety of transmission methods, potential pathogens gain access to body surfaces. These transient microflora can cause disease if they gain access to the appropriate niche, possess the needed virulence determinants (see below), and are able to overcome the host defense mechanisms.

There are many ways to measure the virulence of microorganisms. The infectious dose 50 (ID_{50}), is the number of microorganisms needed to cause infection in 50 % of the test animals. This measure of virulence relates to the number of organisms needed to establish the initial infection. This type of measure cannot be used to predict the outcome of an infection, but rather can be used to assess how readily a bacterium will be able to colonize a niche. The number of microorganisms required to establish infection varies from organism to organism and can even vary from host to host. Some microorganisms are so virulent that only a few organisms are needed to cause disease. The agents that cause plague, *Yersinia pestis*, and tularemia, *Francisella tularensis*, have infectious doses as low as 10 organisms. Disease caused by Shigella species have infectious doses of around 200 organisms, while diseases caused by Salmonella species require millions of bacteria in order to establish infection. These differences in infectious doses indicate that each bacterial species has or lacks certain components needed to establish infection.

The ability of an organism to kill its host is usually expressed as an LD_{50} (lethal dose 50). This is the number of microorganisms or dose of toxin that will kill 50% of the test organisms. When measuring LD_{50} for toxins, this is a quantitative number and indicates a dose that is responsible for death. When used as a measure of the number of organisms needed to kill a host, it is a measure of the number of organisms that must be administered to cause death in the host. Remember that after living microorganisms are administered to a host, they replicate. Death can be rapid, or death may occur only after the infecting organisms have undergone many rounds of replication. If the disease causing organisms must replicate many times before causing death, this provides a window where death can be prevented by some sort of interventive therapy (e.g. with antibiotics).

After the initial colonization by a bacterium, a certain period of time must pass before clinical illness appears. This time period is known as the incubation period. While this period is variable, each pathogen possesses a characteristic time between exposure and appearance of disease. Food poisoning caused by *S. aureus* is characterized by its rapid onset, usually within 4 hours of ingestion of contaminated food. This disease results from the ingestion of a preformed toxin, and no replication of the bacteria in the host is required. The rapid onset of symptoms (vomiting, abdominal cramps, diarrhea) reflects the fact that no replication of the organism is required to cause disease. Shigellosis, caused by a variety of Shigella species, is characterized by abdominal cramps, diarrhea, fever and bloody stools. Symptoms of disease appear within 1 to 3 days after ingestion of the microorganism. During

this incubation period, the organism multiplies within the intestinal mucosa. As the organism replicates, it secretes a variety of toxins and proteins that damage these cells. Symptoms of disease only appear after enough growth has occurred to produce enough toxic products to then damage the intestinal mucosa. The disease tuberculosis is caused by *Mycobacterium tuberculosis*. Infection is initiated by the inhalation of contaminated aerosol droplets. The organism replicates in alveolar macrophages. It is not unusual for the incubation period for this disease to exceed 2 years. This long incubation period reflects the long generation time of the causative agent.

Colonization of a tissue by a pathogen may or may not result in disease. The outcome of this initial colonization is dependent on properties of both the host and the parasite. Predisposing factors of the host can affect the outcome of the disease. A predisposing factor is one that may make the body more susceptible to disease or alter the course of disease. Gender can be an important predisposing factor. For example, women have more urinary tract infections than men, while men have a higher incidence of pneumonia. Genetic background may play a role in disease. Individuals with sickle cell anemia are more resistant to malaria than healthy individuals. Climate and weather can affect the incidence of infectious disease. It is often impossible to predict which predisposing factors may be important in the establishment of various diseases. However, what is clear is that a pathogen must overcome the body's lines of defense.

BACTERIAL DETERMINANTS OF PATHOGENESIS

The sum total of all traits found in an organism that are required for disease are referred to as the organism's virulence determinants. The expression of individual components or multiple traits will be reflected in the type of disease seen. Some microorganisms only cause local infections, while others are able to disseminate from the initial point of infection. Some microorganisms secrete components that cause local tissue damage, while others secrete compounds that cause systemic tissue damage. These pathogenic determinants can be loosely categorized into several classes: Those involved in adherence or attachment, those required for tissue damage, those required for invasion, and those required for evading the host immune response. With the exception of toxins, virulence determinants alone will not cause disease. They provide the bacterium with a series of products that when acting *in toto*, allow the organism to overcome the host's defense mechanisms.

A virulence determinant is a bacterial product that contributes to the disease state. Examples include toxins, capsules, extracellular enzymes, structural components of the bacteria or factors that facilitate colonization or invasion. Toxins may be the sole component responsible for disease (intoxication) and can occur in the absence of viable microorganisms. A disease caused by intoxication is botulism. This food poisoning, caused by the toxin produced by the anaerobe *Clostridium botulinum*, usually occurs as the result of ingestion of improperly prepared canned food. The

neurotoxin alone is responsible for the disease and disease occurs in the absence of the microorganism.

Some microorganisms are capable of causing a variety of diseases. The type of disease caused is a reflection of the virulence of the causative agent and the immunity and health of the host. *Escherichia coli* has been documented to cause many different types of diseases, including bladder and kidney infections, diarrhea, dysentery, hemolytic uremia, meningitis, pneumonia, and septicemia. In general, different strains are associated with the different diseases. These differences are due to differences in virulence of the various strains. *E. coli* is a normal flora of the intestinal tract. In 1993, an outbreak of diarrheal disease occurred in Seattle. The causative agent of this epidemic was *E. coli*. The strain responsible for the epidemic is quite similar to that found in our intestinal tract. However, the new strain had acquired the ability to produce a potent toxin.

VIRULENCE DETERMINANTS

Many microbial factors influence the outcome of a host-bacterium interaction. One factor that enables a microorganism to cause disease is bacterial adhesins or ligands. The presence of adhesins or ligands on a microorganism may not be enough to cause disease because nonpathogens possess these same components. However, before a microorganism can colonize an anatomical site, it must be able to adhere to the appropriate tissue. Bacteria have evolved a variety of structures that contribute to this adherence ability. These interactions are tissue specific and explain why certain pathogens are only found causing disease in certain anatomical sites. Some interactions require multiple factors, on either the host or the bacterium. Some species have a variety of adherence factors that allow them to adhere to multiple tissues. Adherence by other organisms shows tissue specificity. An organism like *Streptococcus mutans* adheres much better to surfaces found in the oral cavity, while an organism like *Neisseria gonorrhoeae* adheres better to urethral mucosal tissues. These tissue specific adherences are the result of the interaction of adherence factors with host tissues. Bacterial surface factors contributing to adherence are quite diverse, and reflect the diversity of tissue found in the human body. They include the F protein of *Streptococcus pyogenes*, the K-88 antigen of *E. coli*, pili from *N. gonorrhoeae*, and various polysaccharides of *S. mutans*.

After a microorganism adheres to a tissue it must replicate at that site in order to colonize it. In order to replicate, it must overcome colonization barriers provided by the normal flora, and be able to escape the defense mechanisms of the host. Many of our normal flora produce products (bacteriocins) that prevent the growth of closely related microorganisms. The production of bacteriocins is one way in which the normal flora prevent the establishment of a new strain of the same species. If an organism is able to overcome the barrier provided by the normal flora, it must then pass one of the hosts first lines of defense (phagocytosis). Phagocytic cells possess the ability to engulf (phagocytose) and destroy microorganisms. Many

bacterial species are virulent because they are able to evade phagocytosis, or if phagocytized, avoid being killed by the phagocyte. Bacteria produce a variety of products that can prevent or block phagocytosis. The role of capsules in bacterial virulence is to protect the bacterium from the host's inflammatory response. Capsules minimize complement activation and prevent ingestion of bacteria by phagocytes. Bacterial capsules produced by organisms like *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Bacillus anthracis* and *Y. pestis* block phagocytosis in a nonimmune individual. An effective host response against encapsulated bacteria is to produce antibodies that bind the capsule. Capsules, although protective, do not prevent phagocytic killing if the bacteria are ingested. Resistance to phagocytosis is not limited to bacterial capsules. *S. aureus* produces a surface antigen and Protein A which interfere with bacterial uptake by the phagocyte. Many nonpathogenic bacteria produce capsules, so the presence of a capsule on a bacterium does not mean that it must cause disease.

Some bacteria have evolved the ability to survive within phagocytic cells. One strategy is to escape from the phagosome before it merges with the lysosome. *Legionella pneumophilla* produces phospholipase C that allows it to escape killing by the phagolysosomal enzymes. *M. tuberculosis* seems to produce some factor that prevents phagolysosome-lysosome fusion from occurring. Still another strategy is to produce compounds that detoxify the compounds produced by the phagolysosome. *Salmonella typhimurium* produces the enzymes superoxide dismutase and catalase to detoxify various forms of reactive oxygen produced by the phagolysosome. Other pathogens, like *Brucella abortus*, produce proteins that block the release of killing factors by the phagocyte.

Bacteria have evolved mechanisms for entering host cells that are not normally phagocytic. This provides them with a mechanism of invading host tissues. Bacterial proteins that cause this response are called Invasins. *Listeria monocytogenes* produces several proteins required for induced phagocytosis. This induced phagocytosis is a bacterial initiated event because mutants of invasive organisms have been isolated that lack this ability. This does not mean that specific host functions are not required for invasion.

The role of extracellular proteins in certain disease processes is quite clear. Some of these proteins and their role in disease are summarized in Table 3. Exotoxins secreted by many Gram positive and Gram negative bacteria have been shown to play a role in a variety of bacterial diseases. Exotoxins vary in their activities and in the types of host cells that they act upon (see Table 4). The nomenclature for exotoxins is confusing. Some are named after the type of host cell that they attack (neurotoxin, leukotoxin, hepatotoxin, etc), others are named after the species of bacteria that produces them (Shiga toxin, cholera toxin, etc), while others are named on the basis of their activity (adenylylcyclase, lecithinase, etc). One toxin produced by *E. coli* has been called Shiga-like toxin because it acts via the same mechanism as the Shiga toxin. Another toxin has been called a verotoxin because it is toxic for a cell line called Vero. Enterotoxins have been so named because they cause

Table 3. Bacterial Products Involved in Virulence

Product	*Role in Disease*	*Function*	*Producing Organism*
Coagulase	Inhibition of phagocytosis	Causes production of fibrin clot	*Staphylococcus aureus*
Streptokinase	Unknown	Converts plasminogen to plasmin	*Streptococcus pyogenes*
Hyaluronidase	Aids spread of organism throughout tissue and organs	Destroys connective tissue	Streptococci Staphylococci Clostridia
Hemolysins	Many	Lyse red blood cells; lyse variety of other cells	Many different species
Lecithinase Collagenase	Promote colonization, spread	Digest tissue components	Clostridia species
Capsules and slime layers	Adherence; antiphagocytic	Allows bacteria to evade immune response	Many bacteria
Siderophores	Growth of microorganism	Acquisition of iron	Most bacteria
DNases	Aid in spread through body cavities	Destroy DNA; reduce viscosity from debris.	Most bacteria

diarrhea, while enterohemorragic toxin gets its name from the result of the tissue damage that it causes, producing blood in the stools.

Some diseases are the direct result of the action of the bacterial toxin, and disease will occur whenever the toxin is administered to the host. The best examples of this are botulism and staphylococcal food poisoning. In other instances, toxins are not the primary determinant of pathogenicity, but are required along with other factors to cause disease. *Clostridium perfringens* causes gas gangrene. This organism produces a toxin that kills tissue in the immediate vicinity of the bacteria. This allows the bacteria to grow and spread from the site of infection in a slow progressive manner.

Because exotoxins are foreign proteins, they are highly immunogenic. If one could detoxify the protein (making it a toxoid) without destroying its immunogenicity, these toxoids would be good vaccine candidates. A variety of toxoids are currently used as vaccines to protect against tetanus, diphtheria, and so on.

Pathogenic organisms can secrete proteins with hydrolytic activities. These enzymes (hyaluronidases, hemolysins, collagenases, etc) damage host tissues by disrupting their structure. It is often difficult to distinguish between bacterial mediated tissue damage and that which results from a normal immune response. Select pathogens directly attack the immune response by producing a protease

Table 4. Bacterial Toxins and their Role in Disease

Toxin	*Source*	*Specificity*	*Mode of Action*	*Role in Disease*
Diphtheria toxin	*Corynebacterium diphtheria*	Broad	Stops protein synthesis by ADP-ribosylation of host elongation factor 2.	Tissue damage
Tetanus toxin	Clostridium tetani neurons		Causes decrease in neurotransmitter release	Spastic paralysis
Botulism toxin	*Clostridium botulinum*	GD1b gangliosides	Blocks release of acetylcholine	Flaccid paralysis
Cholera toxin	*Vibrio cholera*	GM1gangliosides	activates adenylyl cyclase	Causes secretion of electrolytes into the bowel lumen
Pertussis toxin	*Bordetella pertussis*	Unknown	ADP-ribosylation of G1	Lymphocytosis, islet activation
Toxic shock toxin	*Staphylococcus aureus*	T cells, macrophages	Elicits cytokine production	Fever and other general symptoms associated with shock
à-Toxin	*Clostridium perfringens*	Many cell types	Phospholipase removes polar head groups from phospholipids	General tissue damage; also can kill phagocytes
Streptolysins	*Streptococcus pyogenes*	Many cell types	Cytolytic activity	Cardiac damage, inhibition of chemotaxis
Shiga toxin	Shigella species	Intestinal cells	Inhibits protein synthesis by inactivating 60S ribosomal subunit.	Diarrhea?

specific for IgA. IgA protease attacks the host's immune response by proteolytically cleaving secretory IgA.

Not all toxic substances produced by bacteria are proteins. Structural components of bacteria are toxic to humans. The best example is a glycolipid, lipopolysaccharide (LPS), found in the outer membrane of all Gram negative bacteria. LPS has also been called endotoxin. This molecule is made up of two different components. The toxic portion of the molecule, lipid A, anchors this molecule in the outer membrane of Gram negative organisms. Lipid A is attached to a variety of sugar

Table 5. Comparison of Properties of Endotoxins and Exotoxins

Endotoxins	*Exotoxins*
Integral component of the cell wall of Gram-negative bacteria.	Secreted or released by bacterial cells into the surrounding environment
Complex molecule made up of lipid and carbohydrate	Protein
Heat stable	Generally heat labile
Antigenic, although antibody directed against them may not prevent underlying infection or disease.	Stimulate the production of protective antibodies.
Toxic effects are non-specific; produce similar symptoms, irrespective of the producing organism.	Toxic effects specific for each exotoxin; clinical symptoms are distinctive.
Antitoxins are not available.	Antitoxins available that can prevent disease.

molecules, whose structure and composition can vary, depending on the species from which they were isolated. This molecule can have a repeating sugar component (polysaccharide) which contains only a few sugars (oligosaccharide). This molecule provides many Gram negative organisms with a variety of biological functions. It can protect them from the killing action of various antibiotics and host-mediated processes, and they can be directly involved as adherence factors.

Lipid A only exerts its toxic effect on the host when it is released from the cell membrane. This release occurs when host defense mechanisms interact with the Gram negative cell (the membrane attack complex of complement, digestion and killing by phagocytes, killing by various antibiotics, etc). Lipid A toxicity is due to its ability to activate complement and stimulate the release of bioactive host proteins such as cytokines. The properties of endotoxin, as compared to exotoxins, are shown in Table 5. Other molecules found as structural components of bacterial cells can also be toxic (e.g., cell wall {peptidoglycan} of *S. pyogenes* and cord factor {glycolipid}from *M. tuberculosis*).

Some pathogens are able to evade the immune response by coating themselves with host product. *T. pallidum* evades the immune response by coating itself with fibronectin. When growing in vivo, *N. gonorrhoeae* adds host supplies sialic acid to its LOS. This modified LOS is structurally identical to human glycosphingolipids. These additions effectively mask the pathogen from the host immune response.

SUMMARY

Microorganisms are able to cause disease, if given the right opportunity. The outcome of infection depends on a combination of host and bacterial properties. Disease can result from the introduction of a single factor into the host, or require

complex interactions between the host and the colonizing microorganism. The diversity of microbial products accounts for the spectrum of diseases seen in man.

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Chapter 8

Selected Bacteria of Medical Importance

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INTRODUCTION

It is neither the intent nor the charge of the discussion which follows to catalogue and describe the pathophysiology of each and every infectious disease, nor compile a listing of virulence factors and tactics, currently known, employed by bacterial pathogens to incite the seemingly diverse infectious processes.

During the first five to six decades following universal acceptance that certain bacteria cause infectious disease (circa 1880) countless volumes were filled detailing morphological information and grouping of the 200 or so recognized human bacterial pathogens. These monographs and texts offer a plethora of identification schemes, based upon taxonomical considerations, specimen collection and handling and rather extensive listings of the physiological activities of each pathogen on a spectrum of bacteriological media. As long as one knows where to look for such information there is no need to replay it here.

Early studies, by Frederick Loeffler in Berlin and Alexander Yersin and Emile Roux, revealed that certain pathogens, such as the diphtheria bacillus, produce potent toxins which when released into the tissues mediate events leading to death or very serious illness. Simultaneously (circa 1890) it was discovered, by Emil von Behring, Paul Erhlich and Shibasaburo Kitasaoto in Berlin and Yersin and Roux in Paris, that blood serum collected from sheep and horses previously immunized with crude diphtheria toxin preparations reduced mortality rates when administered to infected children. These were the first efforts at immunotherapy. These early successes gave incentive to others to seek therapeutic solutions to other infectious diseases as the relevant etiological agents were identified.

By the 1930s school children were being skin tested to determine their immune status for diphtheria, scarlet fever, and tuberculosis. Vaccines became available for diphtheria, tetanus, whooping cough, anthrax, cholera, and tuberculosis. Bacteriological protocols were in place to facilitate isolation and identification of most bacterial pathogens, which enabled public health personnel to institute household quarantine measures widely employed in the 1930s and 1940s which were designed to prevent or stall epidemics of whooping cough, scarlet fever, and the various childhood viral maladies. This period also witnessed the discovery, by Alexander Fleming, of an antimicrobial agent secreted by the common bread mold *Penicillium notatum*. Diagnosis of infectious disease, laboratory identification, immunotherapy, vaccination and antibiotic therapy were each realized during the first half of life of this new discipline, first called *Bacteriology*.

During the last half of its century-old existence the discipline *Bacteriology* adopted a more comprehensive name, *Microbiology*, and invited chemists, biochemists, immunologists, clinicians, molecular biologists, and geneticists to join the field, adopt a pathogen and ply their respective trades—the collective goal to elucidate the fundamentals of pathogenesis. In concert with these efforts others began to employ the emerging molecular technologies to examine the host-side of the host-parasite relationship with an eye toward understanding and then manipu-

lating host-sponsored innate resistance and provide it with a defensive advantage over the pathogen. A rather clear blueprint of the defensive strategy, which has evolved in the vertebrate in response to infectious disease, is beginning to emerge. While individual bacterial pathogens have each adopted diverse and often unrelated offensive tactics to breach the host defense, the host has settled upon a unified defensive position. The vertebrate has developed: (i) phagocytosis; (ii) humoral immunity which is antibody based and designed to promote phagocytosis, neutralization of toxic items, and initiate complement-mediated killing, and (iii) cell mediated immunity during which specialized killer cells are encouraged to kill cells infected with intracellular parasites. Each of these three tasks are carried out by highly specialized white blood cells (WBC). The WBC, which include neutrophils, macrophages and lymphocytes are often prompted to perform their assignments following reception or recognition of a soluble chemical signal or message emitted by activated WBCs or families of inflammatory proteins. As you will see the chemical-WBC-communication network programmed to set off events, often called inflammation, designed to eradicate or confine infection is rather extensive. It is obvious that this defensive strategy can be overwhelmed. One-hundred-fifty years ago, 1 in 2 humans died of infectious disease, if they avoided cancer, organ failure or death by accident. Translated these figures suggest that in the absence of modern-day measures such as vaccination, antibiotic therapy and public health measures (milk Pasteurization, water purification and waste water treatment), the pathogen can be very successful.

Four diverse infection processes, bacterial meningitis, toxic shock, septic shock and enteric infections were chosen for the discussion which follows with the express purpose of (i) familiarizing the reader with microbiological nouns, verbs and adjectives which pervade the vocabulary and grammar of this discipline, (ii) give sufficient understanding of infectious processes so that the reader will be adequately prepared to pursue related topics, and (iii) provide the reader with an appreciation of the sophistication pathogens have attained to exploit a seemingly sound defensive posture.

Bacterial Meningitis

Inflammation of the meninges, the membranous structure composed of the dura-mater, arachnoid and the pia-mater layers which cover the brain and spinal cord, will be the first infectious disease state examined. The inflammatory events, which characterize meningitis, are usually the consequence of overt infection within the subarachnoid space which exists between the arachnoid and pia mater membranes. Spinal fluid, a modified component of blood plasma, is secreted into the ventricles by highly vascularized villi which protect from the pia mater. The spinal fluid, approximately 400 ml, circulates freely throughout the continuous subarachnoid space between the arachnoid and pia mater layers and is eventually

absorbed into the blood by subarachnoid villi located on the inner roof of the skull (Gray and Fedorko, 1992; Tuomanen, 1993).

The sterile subarachnoid tissue and spinal fluid present little opposition to those pathogens which conspire to invade this tissue space. While small numbers of phagocytic cells called microglial cells and astroglia populate brain tissue, the spinal fluid is essentially devoid of protective immunoglobulins and complement proteins which are normal protective constituents in blood plasma. As a consequence, once the blood-brain barrier (BBB) has been breached by bacterial pathogens, brain tissue is most susceptible to infection. The primary deterrent to infection, the blood-brain barrier is a tightly tiled, seemingly impenetrable vascular endothelium so constructed to exclude most substances from entering the brain.

A select number of bacterial pathogens, once blood borne, manage to penetrate the blood-brain barrier and gain access to the subarachnoid spaces in which they induce meningitis, an infectious state which afflicts 50,000 Americans annually (mostly children aged 2 years and less). At 3 to 6 months of age when the umbrella of passive maternal immunity has waned, the infant becomes most susceptible to colonization and invasive infection by a select group of encapsulated bacterial pathogens, some of which can produce bacterial meningitis.

Hemophilus influenzae type b (Hib) (an erroneous name since this bacterium is not the agent of influenza), is a Gram-negative bacillus, and has until recently been the leading cause of invasive bacterial disease in children less than 5 years of age in United States. Before the advent and licensure of conjugate vaccines in the 1980s (discussed below) one-in-200 children developed invasive Hib disease before the age of 5 years, and fully two-thirds of these infections occurred in children less than 15 months. Mortality rates in children less than 5 years of age ranged from 3% to 6%. The clinical disease in greater than 60% of Hib cases was meningitis, and 30% of survivors suffered permanent neurological sequelae.

Every effort to rank etiological agents of community-acquired bacterial pneumonia in adults lists *Streptococcus pneumoniae* (pneumococcus), a Gram-positive coccus as number one. *Streptococcus pneumoniae* is also the most common cause of bacterial meningitis in adults and places second behind *H. influenzae* as the cause of bacterial meningitis in children. Before the era of antimicrobial therapy, the mortality rate for pneumococcal meningitis approached 100% and for pneumococcal bacteremia (irrespective of infection focus) was approximately 80%. Following introduction of the beta lactam family of antibiotics, effective against *S. pneumoniae*, mortality rates for these two infection states were reduced to 30% and 20%, respectively. Though these reductions in mortality are commendable, they were not acceptable, and this discussion will eventually reveal a plausible explanation for the high failure rates. *Neisseria meningitidis*, a Gram-negative diplococcus, often called meningococcus or mgc, represents the third most common cause of meningitis which occurs across all age groups, often in epidemic form. While, *H. influenzae, S. pneumoniae* and *N. meningitidis* constitute the most-frequently encountered etiological agents of meningitis, Lancefield Group B *Streptococcus*

agalactiae, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Escherichia coli* and the yeast *Cryptococcus neoformans* are encountered frequently (Quagliarello et al., 1992).

How do these bacterial pathogens gain access, colonize the pharyngeal surface and from this site cause meningeal infection? More specifically, how, following colonization of the naso-pharyngeal mucosa, do these pathogens deflect and evade the non-specific defense mobilized and deployed by the host, such as the complement cascade and phagocytosis, to eradicate the trespassers? What processes and mechanisms (virulence factors) do these pathogens employ to invade and then survive the hostile anti-microbial environment of the vascular compartment? Finally, how do these organisms cross the seemingly impenetrable blood-brain barrier and incite life-threatening inflammation in the subarachnoid tissues and fluids? Variations on these queries are continually posed as infectious disease states emerge or are reassessed, with the same overall objective—to develop and implement effective preventative or treatment measures. In each of the last decades new infectious agents have surfaced (Legionnaires disease in the 1970s, toxic shock syndrome, Lyme disease, Brazilian Puerperal Fever in the 1980s and Streptococcal Toxic Shock syndrome and *Helicobacter pylori* and peptic ulcers in the 1990s), as well as the reemergence of "old" infectious agents (tuberculosis, syphilis, Group A streptococci) each demanding assessment or reassessment.

Though the pathogens Hib, meningococcus (mgc) and pneumococcus are each taxonomically disparate, they exhibit one common structural feature, a well defined extracellular polysaccharide capsule. In addition, they elaborate virulence factors which enables each to first colonize and depart the pharynx and ultimately enter the spinal fluid where they foment and orchestrate the inflammatory events defined as meningitis. Invasive *Hemophilus influenzae* strains express a single capsule type (polyribosyl ribitol phosphate [PRP], serological type b), while invasive *Neisseria meningitis* strains may exhibit any one of nine different antigenic capsular groups (serogroups A, B, C, D, X, Y, Z, W135, and 29E). *Streptococcus pneumoniae* strains can express one of 84 antigenically-unique polysaccharide capsular types. Invasive pneumococcal strains, however, are usually associated with one of approximately 20 select carbohydrate capsular groups (Johnson, 1991). The extracellular, often acidic, carbohydrate capsule which enshrouds each of these three organisms functions to deflect and repel the phagocytic efforts of tissue-based and blood-borne phagocytic cells. The capsule represents the single-most important virulence factor expressed by these three pathogens, while non-encapsulated Hib, mgc and pneumococcal strains may be transient inhabitants of the nasopharyngeal surface, they are typically non-invasive.

Colonization

Both mgc and Hib bind selectively to non-ciliated cells in the nasopharyngeal epithelium. It is possible that *N. meningitidis* cells utilize adherence pili which

project from the cell wall (termed adhesins) to recognize and adhere to nonciliated cells, while Hib probably do not use pili for this task. *Streptococcus pneumoniae* routinely colonize the upper respiratory tree, and from that site may translocate to produce an assortment of invasive infections. Pneumococcal strains which express capsular types 6, 14, 19, and 23 account for greater than 50% of all middle ear infections (acute otitis media), meningitis, lobar pneumonia, and bacteremia. As a result pneumococcus may enter the vascular compartment from various primary infection foci, as a prelude to inciting meningitis. During colonization virtually all clinical Hib, mgc and pneumococcal strains secrete a protease, which cleaves the proline-rich hinge region of secretory IgA present in the mucous blanket covering the pharyngeal surface. Enzymatic digestion of secretory antibody may clear the way for successful colonization. One clever evasion tactic attributed to the meningococci when faced with an impending humoral IgM and IgG antibody response, is to induce production of capsule-specific secretory IgA. Capsule-specific IgA delivered to the mucosal surface reacts with the capsule and blocks the complement-mediated bacteriolytic events promoted by blood-based capsule-specific IgM and IgG antibody (discussed below). Resistance to or susceptibility to a potential clinical meningococcal infection is determined ultimately by the immune status of the host, which translates into the presence or absence of serum IgM and IgG acquired as a consequence of previous exposure or immunization. While meningococcal capsular groups A and C will provoke production of protective levels of antibody, the group B capsule is relatively nonimmunogenic. Group B capsule is composed of neuraminic acid which is structurally similar to human host tissue constituents, a circumstance which may permit mgc cloaked in a neuraminic acid capsule to employ stealth technology and sneak through the tissues undetected. Surprisingly type B mgc does not represent the most frequently encountered clinical mpc isolate.

Epithelial cell colonization may lead to overt invasive infection in persons who lack humoral immunity (capsule-specific antibody), or to a transitory pharyngeal carrier state in those individuals possessing humoral immunity. A correlate exists between the mgc carrier rate (usually an immune, asymptomatic nasopharyngeal carrier) and the potential for onset and decline of an epidemic. For example, when mgc nasopharyngeal carriage rates approach 20% in the community, an epidemic may be imminent since it is the immune carrier who usually starts and perpetuates meningitis outbreaks.

Direct injury to the ciliated epithelial cells, paralyzing the synchronous beating of the cilia, may enhance colonization of Hib and mgc, by impeding the cleansing action of the mucous flow. *Neisseria meningitidis* enter epithelial cells by an endocytic process (pathogen directed phagocytosis), and are transported within a membrane-enclosed vesicle to the basement membrane side into the submucosal tissue. Alternatively, *H. influenzae*, by some undefined process, create a separation of the tight apical junctures of the columnar epithelial cells and Hib slip through this opening into the subepithelial tissues by intercellular passage. While prolifer-

ating in the subepithelial tissues mgc and Hib slough lipopolysaccharide (LPS, endotoxin), a toxic constituent of the Gram-negative cell wall, into the infection foci. As will be documented later LPS can incite the fulminant phase of meningococcal sepsis and the consequent high mortality (see septic shock).

Invasion

When the epithelium barrier has been breached, the pathogen is confronted by the second tier of innate defense, circulating antibody (humoral immunity) acquired passively at birth and present only during the first months. Humoral immunity is also acquired actively through previous exposure, or following immunization with first generation capsular vaccines or second generation polysaccharide-protein conjugate immunogens (Hib, licensed in late 1990). In immune individuals, blood-borne capsule-specific IgM and IgG when delivered to the developing infection foci reacts with the capsule surface and marks the pathogen for phagocytosis by neutrophils or macrophages infiltrating the infection site. Neutrophils exhibit an antibody receptor on their cytoplasmic membranes (termed an F_c receptor) which permits the phagocyte to recognize the antibody-labeled pathogen for ingestion. In addition to promoting phagocytosis, capsule-specific IgM and IgG antibody can also kill the pathogen by complement-mediated bactericidal action (classical complement pathway, Figure 1). Following reaction with the capsular antigen, receptor sites for complement protein 1q (C1q) are exposed on the IgM or IgG antibody molecule. Next, C1r and C1s complex with C1q forming an active enzyme complex which sets off the complement cascade depicted in Figure 1. Taken to its completion a membrane attack complex (labeled MAC) composed of the terminal complement proteins C5b, C6, C7, C8, and C9 is formed in the bacterial cell membrane, in a manner which simulates staves in a barrel. Pores created in the cytoplasmic membrane by the MAC permit rapid egress of vital cellular constituents, thus leading to death of the target cell. Antibody mediated phagocytosis (a process often called opsonization) and complement-mediated bacteriolysis can protect and spare the victim from any further inconvenience (solid immunity to invasive infection).

In the absence of humoral immunity encapsulated pathogens often invade the blood compartment with impunity. Once blood-borne, encapsulated organisms must continue to evade the unrelenting efforts of phagocytic cells. Even in the absence of protective levels of anti-capsular antibody, the pathogen must side-step the lethal action of the alternative complement cascade (Figure 1). You may wish to consult Table 1, which lists the complement split products generated from various complement proteins following activation. The alternative pathway (so named because it bypasses the requirement for antibody) is activated when complement protein 3b (a product of C3, a major plasma protein) binds to surface OH or NH_2 groups present on constitutive monosaccharides and amino acids in the capsule and cell wall of the invading pathogen. Activated C3b, bound to the capsule or cell wall surface, is stabilized when complexed with factor B (a second plasma protein), a

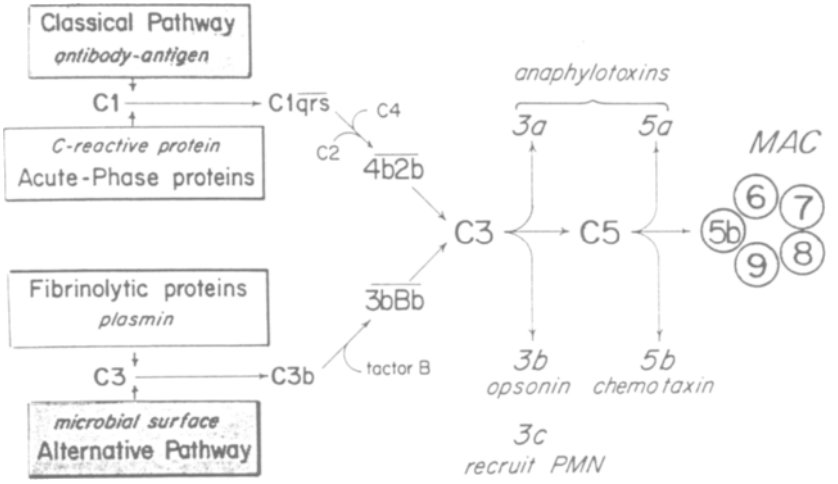

Figure 1. The complement proteins, a family of 20 plasma proteins, can be activated in a sequential manner to generate products (defined further in Table 1) which will initiate inflammatory events designed to confine or eliminate infection. Each of the four diverse activation events (picture to the left) ultimately converge through activation of complement protein 3 (C3) which lead to the formation of the bacteriolytic membrane attack complex (MAC). The classical complement pathway is activated by serum antibodies IgM or IgG, following their reaction with the relevant target bacterium (antigen). The alternative complement pathway is activated when C3b becomes stabilized on a microbial surface (bypassing the requirement for antibody). In addition the classical and alternate complement cascade can be turned on by acute phase proteins (such as C-reactive protein) and as a consequence of the enzymatic action of the fibrinolytic proteins.

union which creates an active enzyme complex called C3bBb convertase. Through its enzymatic action C3bBb convertase cleaves its substrate C3 to generate a galaxy of C3b molecules which shower the tissue landscape and blanket proliferating bacterial cells. A second product of convertase activity is 3a, defined as an anaphylatoxin, elicits histamine release from basophilic cells (Table 1). The resulting C3b shower labels each bacterial pathogen within reach for phagocytosis by neutrophils which in addition to F_c-receptors also possess C3b-receptors. Secondly, 3b can amplify the inflammatory reaction by the generation of additional C3bBb convertase activity in the affected tissue. Thirdly, C3bBb convertase will mediate enzymatically the formation of membrane attack complexes (MAC), formed by association of the terminal complement protein components 5b, 6, 7, 8, and 9. When the MAC is inserted into the bacterial membranes, the target bacterium is killed (complement-mediated bacteriolysis).

Table 1. The Biological Activities of Selected Complement (C) Split-Products Generated Following Activation of the Classical or Alternative Complement Pathway During the Infection Process.

Complement Split-products	*Inflammatory Event Produced*
C3a	Anaphylatoxin. Causes degranulation of tissue Mast cells and blood basophils, release of histamine causing: smooth muscle contraction increased vessel permeability enhanced mucous production by goblet cells neutrophil chemotaxis
	Aggregation of blood platelets
	Degranulation of eosinophiles
C3b	Opsonin. Reacts with and labels bacteria for phagocytosis
C3c	Promotes release of neutrophils from bone marrow
C5a	Anaphylatoxin. see C3a
	Aggregation of platelets
	Chemotactic signal for neutrophils, basophils, eosinophils and blood monocytes
	Provoke degranulation of neutrophils spilling bactericidal superoxides, hydrogen peroxide and digestive enzymes into tissue environment causing tissue cell injury
C5b6789	Membrane Attack Complex. Creates pores in cytoplasmic membrane of target pathogen, lethal to the cell

One defensive tactic employed by bacterial pathogens to evade complement-mediated death resides with the monosaccharide makeup of the polysaccharide capsule. Capsules of several *N. meningitidis* serogroups contain sialic acid as a constitutive sugar, and this monosaccharide favors binding of factor H (a plasma protein inhibitor of C3 activation). When factor H complexes to C3b it supplants factor B and aborts formation of the 3bBb convertase. On the other hand C3b readily binds to the capsular polysaccharides of most pneumococci, but in a manner and in a molecular orientation that prevents efficient binding of factor B, and in the absence of factor B convertase activity is not created. Conversely, the polyribosyl ribitol phosphate (PRP) makeup of the Hib capsule will not permit direct binding of C3b. In the absence of humoral immunity these three pathogens can successfully deflect complement-mediated death by the alternative complement pathway and thus unopposed survive and flourish in the vascular space.

Breaching the BBB

By mechanisms yet to be elucidated blood-borne Hib, mgc and pneumococci cross the blood-brain barrier into the spinal fluid, where they multiply unimpeded

to critical cell densities. Obviously, entry into the subarachnoid space is not happenstance, or most organisms, after gaining access to the blood, should accomplish this task. Since mgc express pili, it is possible that adhesion pili play a role in recognition of the cerebral vascular endothelium. The most likely portals of entry into the subarachnoid space are areas of minimal resistance such as the chorion plexus, aural venous sinuses, cerebral capillaries and sites of surgical, traumatic or congenital system defects. Once invasive mgc, Hib or pneumococcal cells enter the cerebral spinal fluid they are almost certain to survive since neither antibody nor complement proteins are present in spinal fluid. When the proliferating pathogens attain critical numbers, a chain of inflammatory events are triggered which are commensurate with the signs and symptoms of acute bacterial meningitis.

INFLAMMATION PROCESSES IN THE SUBARACHNOID SPACE

In the supporting tissues of the brain reside macrophage-like cells, called microglia and astrocytes. They respond to invading bacteria and cell wall structural components, such as lipopolysaccharide, teichoic acid and peptidoglycan elements that dividing and dying bacterial cells slough into the spinal fluid (Figure 2). Confronted by these foreign elements the resident astroglial cells are stimulated to release soluble mediators (cytokines) designed to initiate protective inflammatory events. The inflammatory cytokines tumor necrosis factor (TNF, Table 2) and interleukin 1 and 6 (IL-1, Table 3); and IL-6 (Table 4) act directly on the capillary endothelium, leading to a change in permeability which will eventually lead to the accumulation of blood plasma in the spinal fluid (*vasogenic edema*) (Saez-Llorens and McCracken, 1991; Quagliarello and Scheld, 1992).

The synergistic action of TNF and IL-1 on the endothelium, can facilitate movement of bloodborne neutrophils across the capillary endothelium into the

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Figure 2. Diagrammatic representation of neutrophil exudation into the spinal fluid and consequent breakdown of the blood-brain barrier. Panel A: bacteria have [1] entered the spinal fluid where they [2] release cell wall constituents such as LPS which induce activated microglial cells to release [3] the inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor (TNF). These cytokines induce transient expression of adressin molecules, such as endothelial leukocyte adherence molecule-1 (ELAM-1), on the capillary endothelium. Panel B: blood-borne neutrophils adhere to ELAM-1 through membrane receptors, pave the endothelial cell surface and degranulate releasing superoxides and peroxides which damage the vascular endothelium. Interleukins 6 and 8 secreted by the glial cells and C5b released following complement activation on the pathogen surface act as chemotactants, attracting neutrophils into the subarachnoid spaces.

(continues)

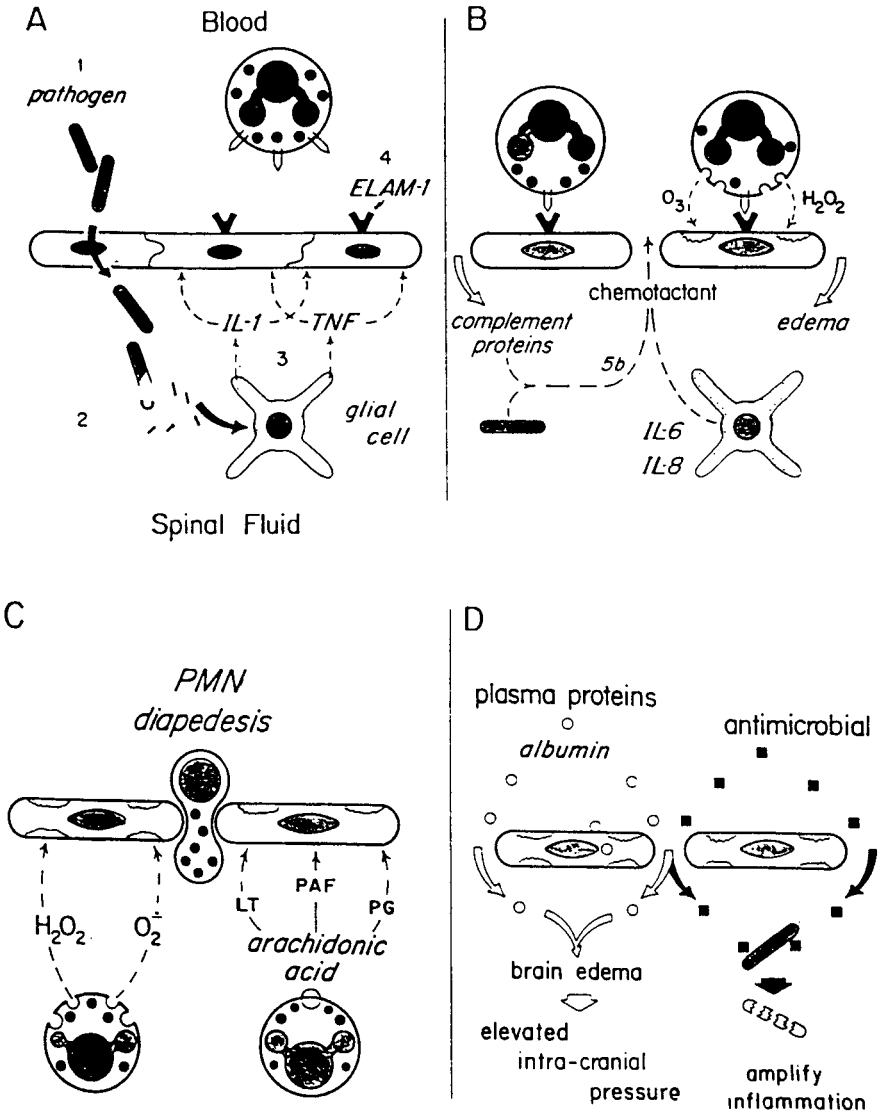

Figure 2. (continued) Panel C: adherent neutrophils follow a gradient of chemotactants and enter (by an event called diapedesis) the spinal fluid where they continue to damage the vasculature and can also generate platelet activation factor (PAF), leukotrienes (LT family) and prostaglandins (PG) which cause continued vasoconstriction giving rise to edema. Panel D: increased cranial pressure results from edema as a consequence of prolonged inflammatory activity. Administration of antimicrobials can exacerbate the inflammatory events by detonating and killing the pathogens, and scattering cell wall fragments in the subarachnoid space.

Table 2. Biological Effects of Tumor Necrosis Factor

Site of Action	*Biological Effect*
Endothelial Cells	Promotes adhesion of endothelial cells, neutrophils, eosinophils, basophils, and monocytes, by inducing the expression of adhesion molecules
	Is directly toxic to vascular endothelium, and increases microvascular permeability
Monocytes	Stimulates release of IL-1, IL-6, IL-8, PAF, LT, and PG, and may function as an autocrine to stimulate its own production
Neutrophils	Enhances phagocytic activity of neutrophils
Lymphocytes	Only weak effect on lymphocytes
Fibroblasts	Stimulates collagenase release in synovial cavity
Bone Marrow	Stimulates production of neutrophils by the bone marrow
Blood Plasma	Activates coagulation pathways and the complement system
Heart	Reduces transmembrane potential of muscle cells and depresses cardiac myocyte shortening
Brain	Acts directly on the hypothalamus to elevate body temperature
All Tissue Cells	Induces expression of MHC class I molecules
	Hemorrhagic necrosis of tumor cells

Key: IL = interleukin, PAF = platelet activation factor, LT = leukotrienes, PG = prostaglandins, and MHC = major histocompatibility complex

spinal fluid (Figure 2). Neutrophils, circulating in the blood, must be furnished an exit sign (an address to read) which directs these phagocytes to leave the blood compartment. They enter the infected tissues which are signaling (through cytokine release) for assistance in the removal and eradication of invading bacterium. Once released, by stimulated macrophages, both TNF and IL-1 act on the capillary endothelium and as a result, endothelial cells mobilize several families of neutrophil adherence molecules and display these molecules (called adressins) on their membrane. One adressin called the endothelial leukocyte adhesion molecule-1 (ELAM-1) is delivered to the endothelial cell membrane surface, where it promotes adherence of neutrophils to the vessel walls (pavementing). Neutrophils in turn express an assortment of membrane receptors which they can employ to recognize adhesion molecules, newly placed on the vessel endothelium. Neutrophils utilize a glycoprotein receptor which contains both sialic acid and fucose to recognize and bind to ELAM-1. Following immobilization onto the endothelium surface, the adherent neutrophils respond to chemotactic signals emitted from inflammatory cells in the infected spinal fluid, squeeze between endothelial cell junctions (by a process termed diapedesis) and then following a chemotactic gradient, migrate into the subarachnoid spaces (Figure 2). Adherent neutrophils are prompted to degranulate and empty the contents of their lysosomes, which includes hydrogen peroxide and superoxide ions, onto the endothelial cell surface inflicting damage to these

Table 3. Biological Effects of Interleukin-2

Site of Action	*Biological Effect*
Endothelial cells	Promotes adhesion of neutrophils, basophils eosinophiles and monocytes, by inducing formation of adhesion receptors
	Acts synergistically with TNF, enhancing tissue cell sensitivity to TNF
Macrophages	Stimulates release of TNF, IL-6, IL-8, PAF, LT, and PG, and may stimulate its own production
Neutrophils	Promotes neutrophil (other polymorphonuclear cell) activation and accumulation
Lymphocytes	Activates resting T-cell to proliferate, and release cytokines
Bone marrow	Increases production of granulocytes, and neutrophil mobilization
	Osteoclast activation
Liver	Synthesis of acute-phase proteins
	Decreases synthesis of albumin, transferrin
Blood plasma	Increases copper, decreases levels of iron and zinc
Brain	Endogenous pyrogen, acts directly on the hypothalamus to elevate body temperature
Adrenals	Promotes adrenocorticotropic hormone

Key: TNF = tumor necrosis factor, IL = interleukin, PAF = platelet activation factor, LT = leukotrienes, PG = prostaglandins

cells. The chemotactic signals that neutrophils follow emanate from the activated complement split-protein C5b, in addition to IL-6 and -8 produced by stimulated macrophages. At the height of neutrophil influx, as many as one-million white cells may occupy each milliliter of spinal fluid. Both TNF and IL-1 function as endogenous pyrogens and they can act on the hypothalamus and readjust the thermal-set-point leading to elevated body temperature. Also, both cytokines can function in an autocrine manner, which means they stimulate themselves (autostimulation)

Table 4. Interleukin Molecules which Participate in Inflammation

Interleukin-2	Promotes TNF and Interferon (INF) release
	Promotes helper function to B-cell and cytotoxic T-cell
	Increases cardiac output, decreases arteriole pressure, decreases vascular resistance, hypotension
Interleukin-6	Promotes neutrophil activation and accumulation
	Acts as additional helper signal for T- and B-cell activation
Interleukin-8	Acts as chemotactic signal for neutrophils and lymphocytes and induces tissue infiltration
	Inhibits endothelial-neutrophil adhesion

Key: TNF = tumor necrosis factor, INF = interferon

as well as bystander glial and endothelial cells to release additional TNF, IL-1, -6, and -8.

Subsequent to TNF and IL-1 release, both macrophages and infiltrating neutrophils may be stimulated to release a family of inflammatory mediators, which at one time were known collectively as the slow-reactive substance (Table 5). They include the following chemically defined substances: platelet activation factor (PAF), the leukotriene family (LT) and the prostaglandins (PG). Each of these mediators are generated metabolically from arachidonic acid derived from cytoplasmic membrane lipids. Both PAF and LT act directly on the vascular endothelium and alter permeability permitting egress of adherent neutrophils into the spinal fluid. In concert with PGE_2, PAF, and LT promote increased capillary blood flow (hyperemia). Altered vascular permeability, through the collective action of TNF, IL-1, PAF, and LT, permits movement of blood plasma into the CSF, which carries complement proteins to the brain-side of the blood-brain barrier. The magnitude of endothelial permeability (impairment of the blood-brain barrier) correlates directly with the numbers of bacteria present in the subarachnoid space (Saez-Llorens and McCracken, 1991; Quagliarello and Scheld, 1992; Tuomanen, 1993).

As neutrophils arrive in the infected subarachnoid space they come under the influence of TNF and IL-1 and are simulated to degranulate and release toxic

Table 5. Mediators of Inflammation Generated Through Metabolism of Membrane Associated Arachidonic Acid Secreted by Basophilic Cells, Neutrophils and Macrophages

Mediator	*Biological Effect*
Platelet Activation Factor (PAF)	Stimulates release of TNF, leukotrienes and prostaglandins
	Activates neutrophils to degranulate, release free radicals
	Promotes platelet aggregation, leading to thrombosis
	Induces vasoconstriction
Leukotrienes (LT)	
LTB_4	Promotes neutrophil adhesion to endothelium, neutrophil chemotaxis
	Increases vascular permeability
LTD_4	Increases vascular permeability
	Contraction at adjacent endothelial cells
	Decreases coronary blood flow
Prostaglandins (PG)	
PGE_2	Inhibits IL-1 production
	Suppresses TNF production
	Inhibits T-cell, B-cell mitosis
	Inhibits ability of endotoxin to elicit hypotension
	Increases blood flow

oxygen metabolites and also release leukotrienes, prostaglandins and platelet activating factor. These factors continue to modify and induce the capillary endothelium to take up plasma albumin (vesicular imbibement) which in concert with pericellular leakage of albumin into the spinal fluid leads to vasogenic brain edema. Given the rigid confines of the skull and spine, an increase of intracranial pressure can lead to dire consequences. Vasogenic edema results in increased spinal fluid volume, which gives rise to increased intracranial pressure.

To summarize, inflammation of the meninges is triggered by invasion of the subarachnoid spaces and subsequent cytokine overproduction. Leukocytes adhere to cerebral capillary walls, release toxic items that cause endothelium injury resulting in increased permeability. An influx of serum proteins into the spinal fluid causes vasogenic edema, as well as blockage of spinal fluid outflow at the arachnoid villi. These inflammatory events promote increased spinal fluid volume and viscosity (due to neutrophil exudate), which augment intracranial pressure, decreased blood flow to the brain and depressed oxygenation of brain tissue (hypoxemia). Hypoxemia triggers a switch to anaerobic metabolism marked by increased lactate and decreased glucose levels in the spinal fluid. Ultimately, as blood flow to the brain is curtailed, ischemia results (tissue necrosis) that may lead to neuronal injury and irreversible brain damage.

Antimicrobial Therapy: A Trojan Horse

The mere suspicion that a case of bacterial meningitis is at hand, demands a quick response. In an attempt to identify the microbial pathogen the victim's spinal fluid is examined bacteriologically and serologically. Because of the speed by which meningitis progresses, time does not permit waiting for laboratory identification and antibiotic sensitivity testing on the isolate. The victim must be given immediate antimicrobial therapy. When administered in high dose, antibiotics may penetrate the blood-brain barrier in sufficient levels to kill the bacterial pathogen. Antibiotics in the penicillin family generally kill pneumococcus, meningococcus and Hib, and yet until very recently fully one-third of meningitis victims died while undergoing antibiotic therapy. Why, if the inciting bacterial agent is killed (active infection eradicated) do such high numbers of victims die? Recently, several groups of investigators may have unraveled this enigma, by examining in an animal model each stage of bacterial-induced meningitis (Saez-Llorens and McCracken, 1992; Toumanen, 1993).

Brain edema, determined by a measurable increase in fluid content, was induced in rabbits by introducing live pneumococcal or *H. influenzae* cells into the subarachnoid space. The first discernible signs of infection, fever and presence of white cells in the spinal fluid, usually did not occur until bacterial numbers reached 100,000 cells per milliliter of spinal fluid. Fever and inflammatory events commenced, in concert with the appearance in the spinal fluid of detectable levels of TNF and IL-1.

Surprisingly, instillation of dead pneumococcal cells, equivalent to 100,000 cells per ml of spinal fluid, into the subarachnoid spaces of the rabbit provoked the same cycle of inflammatory events (meningitis) incited by live cells. How does one reconcile these findings? There exists an intriguing explanation—one-third of meningitis victims die during the course of antibiotic therapy)—findings which suggest bacteria killed by antimicrobial therapy in the infection site may continue to play a role in maintaining the inflammatory consequences initiated by the primary infection. First, when dead pneumococcal cells were introduced into the rabbit, meningeal inflammation resulted, which was indistinguishable from that induced by active infection. Second, these investigators were able to provoke an identical inflammatory reaction when they injected pneumococcal cell wall fragments or lipopolysaccharide extracted from *H. influenzae* cells. Third, instillation of the inflammatory cytokines TNF or IL-1 into the subarachnoid space, in the absence of bacterial cells, provoked neutrophil infiltration, fever and brain edema. And, one final piece of evidence revealed that when 1,000 living pneumococcal cells, 100-fold below that of an inflammatory inciting dose, were introduced into the subarachnoid space and the rabbits were treated simultaneously with penicillin, an intense inflammatory reaction commenced within 30 minutes. The short time interval would not permit the pathogen to attain the critical cell density required to incite the inflammatory events, suggesting that the antimicrobial therapy may augment and amplify the inflammatory events.

Penicillin treatment does not simply kill the bacterial agent and leave in its wake a bacterial cadaver, but rather this antimicrobial shatters the bacterial cell scattering cell wall fragments, which includes lipopolysaccharide, teichoic acid and peptidoglycan, across the tissue terrain. Each fractured cell disseminated as 1000's of cell wall fragments may imitate the consequence of a rapidly proliferating bacterial population. Resident macrophages unable to discriminate cell wall fragments from intact living cells respond accordingly. Thus, the lytic action of antibiotic which should mitigate the consequences of an overt infection, mimics a sudden escalation in bacterial numbers, setting off the release of a cascade of inflammatory cytokines. The increased permeability of the capillary endothelium invites an influx of neutrophils and plasma proteins to the brain-side of the vascular endothelium, which continue to promulgate intense inflammation. As a consequence, for the first hours following antibiotic therapy brain swelling, fever and intracranial pressure may increase to critical levels, during which time the intensive inflammatory response can deface the cellular architecture of the brain and supporting tissues.

The rabbit meningitis model revealed that antibiotic treatment, intended to kill the inciting pathogen, can worsen an already desperate situation, a scenario often recorded in human meningitis victims during antibiotic treatment. To rescue the victim from possible lethal inflammation initiated by antimicrobial therapy, demanded a new approach to therapy: kill the pathogen and treat simultaneously with antiinflammatory drugs to dampen the inflammatory response.

Can the lessons learned from the meningitis model in which inflammation incited in the sterile confines of the subarachnoid spaces by an infectious agent which is both prolonged and intensified by antimicrobial therapy, be applied to seemingly unrelated infectious states? One possible infection correlate is pneumococcal-induced inner ear infections (otitis media). These tissue spaces are normally sterile and confining. Painful inflammation in children during antibiotic treatment seems to continue even in the face of negative bacterial cultures (Kawana et al., 1992) Could arthritis and pleurisy represent similar infection inflammation sequelae?

Prevention Through Vaccination

It has been known for decades that the capsular polysaccharides of *Streptococcus pneumoniae* exhibit diverse antigenic specificity, and account for at least 84 different capsular serotypes or groups. Capsule-specific antibody when administered to laboratory animals will provide protection to invasive infection. The currently licensed pneumococcal vaccine consists of a combination of purified capsular polysaccharides prepared from 23 serotypes. The 23 capsule serotypes incorporated into the polyvalent vaccine were selected because it was estimated they were responsible collectively for greater than 87% of bacterial pneumonias in the United States (Burman et al., 1985; Musher, 1992).

The pneumococcal polyclonal subunit vaccine in its current construct is not an effective preventive measure for meningitis. The problem is that the immune system in children before their second birthday (the primary risk group for meningitis) fails to respond immunologically to carbohydrate antigens with an elevated, persistent and boostable antibody response. Unfortunately, if the subject does respond immunologically, it is with a T-cell-independent, mostly IgM, response of short duration without benefit of immunological memory. Since there appears to be a correlation between the number of pneumococcal induced attacks of acute otitis media in pre-school age children, with the risk of developing hearing impairment, there is additional incentive to attempt vaccination in this risk group (Baltimore, 1992).

The picture for *H. influenzae* type b, brightened dramatically in the 1980s. The first Hib subunit vaccine introduced in the 1970s, consisted of polyribosylribitol phosphate (PRP), the capsular carbohydrate extracted from serotype b. This vaccine demonstrated 90% efficacy in children ages 18 to 70 months, but as was the case with the pneumococcal polysaccharide vaccine, it was ineffective in infants 3 to 17 months. A new generation of conjugate vaccines exhibited substantially improved immunogenicity. They were developed in the 1980s by conjugating, through a covalent linkage, the PRP-capsule to immunogenic protein carriers, such as diphtheria toxoid or an outer-membrane protein (OMP) extracted from *Neisseria meningitidis*. The strategy behind constructing PRP-protein carrier conjugate vaccines, was to recruit and stimulate T-helper cells ($CD4^+$ cell) into the immune response loop. T-helper cells are stimulated by protein antigen exclusively, and then only when foreign protein is processed and then presented on the macrophage

membrane surface in association with the MHC-II antigen presenting molecules. The B-lymphocyte can, on the other hand, through membrane bound antibody receptors recognize and interact with native non-processed antigen, protein or carbohydrate, when introduced into the body. The B-cell must, however, receive cytokine help signals from antigen-stimulated T-helper cells in order to switch from IgM to IgG synthesis. Without benefit of T-cell derived help signals, B-cells will synthesize IgM antibody exclusively. Polysaccharide subunit vaccines stimulate B-cells only, especially in the very young, while the PRP-protein conjugate vaccines stimulate both T-helper and B-cells, leading to a long-lasting, protective and boostable IgG anti-capsule response. Over a three year period (1988 through 1992) as a consequence of an active immunization program with protein conjugate vaccines, the age-specific incidence of Hib disease among children less than five years of age decreased 71% (Adams et al., 1993; Murphey et al., 1993).

TOXIC SHOCK AND MICROBIAL SUPERANTIGENS

In 1978 Todd and colleagues described and defined an infectious disease state they called toxic shock syndrome (TSS), that eventually gained widespread recognition and fame. Toxic shock was the expression first applied to a serious systemic condition which occurred primarily in young women who during menses used tampons produced by a specific manufacturer. It was ultimately concluded that a structural component in the tampon fabric supported colonization and growth of the notorious dermal pathogen *Staphylococcus aureus*, a Gram-positive coccus, and also the number-one cause of bacterial-induced food poisoning. Blood from TSS victims when examined bacteriologically is seldom positive for *S. aureus*. It was proposed that staphylococci confined to an isolated infection foci secrete exotoxins which incite the pathophysiology characterized as a multisystem disease of fulminant onset. And now, a decade later it has became apparent that infection foci produced by Group A *Streptococcus pyogenes* may lead to a condition similar to TSS, called streptococcal-induced toxic shock syndrome (STSS) (Stevens, 1993).

Modern medicine has had a major impact on many fronts and most significantly on the diagnosis, treatment and prevention of infectious diseases and their sequelae, infectious diseases which in earlier times exacted high morbidity and mortality rates. Penicillin, while problematic when used to eradicate bacterial pathogens at select sites, such as the meninges, has been employed successfully to prevent rheumatic fever and puerperal sepsis, both serious consequences of recent or current Group A *Streptococcus pyogenes* infections. As an aside and also somewhat difficult to explain, however, is that mortality rates for certain complications which follow *S. pyogenes* infections, such as scarlet fever, were on the decline well before the introduction of antibiotics.

During the last decades several new infectious disease states have emerged and some "old" infectious disease patterns have changed, in part through the creation of a growing population of immunocompromised individuals. Cancer therapy,

administration of immunosuppressive agents to transplant recipients and acquisition of immunodeficiency disease have in part created this risk group. Recently, aggressive *S. pyogenes* strains have surfaced, characterized in their infectious form by septic shock and multiorgan system failure, an example of which is acute respiratory distress syndrome (ARDS). Streptococcal-induced toxic shock syndrome, however, occurs in apparently healthy immunocompetent adolescents and adults to whom first world medical care is available. The speed by which *S. pyogenes* produces local infection, multiorgan failure and death, in 30% of cases, is alarming and not matched currently by any other infectious disease (Stevens, 1992). The infection process which accounts for the serious and often fatal consequences of STSS, may be similar to the events which lead to staphylococcal-induced TSS.

A number of unrelated infectious states caused by *S. aureus* and *S. pyogenes* are listed on Table 6. The primary infections originate in diverse tissue settings, such as the gastrointestinal tract, the genitourinary tract and the dermis. The common feature is that each infectious state is perpetrated through elaboration of toxic proteins by each pathogen, collectively called exotoxins. Each infectious disease state identified in Table 6 occurs as a consequence of the nonimmunological stimulation of T-helper cells. Recall, CD4 T-helper cells stimulated by processed protein antigen, presented by macrophages, proliferate and then release helper cytokines. Help factors when secreted in quantities greater than normal may launch the temporary, non-lethal events which accompany staphylococcal-food poisoning and cause the potentially fatal consequences of toxic shock.

Before the unique stimulatory properties that certain staphylococcal and streptococcal exotoxins exhibit when deployed during infection—a few moments must be taken at appropriate points to review the activities of thymus-derived lymphocytes, germane to their immunological assignments. When T-helper (CD4) cells are placed in culture and stimulated by conventional antigen less than 1 in 10,000 T-helper cells (0.01%) will respond immunologically, first through antigen recognition and then proliferation, events termed clonal selection and expansion respec-

Table 6. Microbial Superantigens

Pathogen	*Infectious State*	*Superantigen*
Staphylococcus aureus	Gastroenteritis (food poisoning)	Enterotoxin A, B, C, D and E
	Toxic Shock Syndrome	TSS-toxin 1 (TSST-1)
	Scaled Skin Syndrome	Exfoliative toxins (ExF) EXF-A, EXF-B
Streptococcus pyogenes	Shock, pyrexia	Streptococcal exotoxins (SPE) SPE-A, B, C
Pseudomonas aeroginosa	Septicemia	*Pseudomonas aeroginosa* exotoxin (PAE) PAE-A

tively. Each T-helper cell clone is by definition antigen-specific and can recognize a single antigen through a membrane-bound receptor, termed the T-cell receptor (TCR). A finite number of T-helper cells comprise each antigen-specific clone, and express the appropriate antigen-specific TCR. The immune facilities, the thymus specifically, have been charged with the task to educate millions of T-helper clones, to recognized one of the potentially millions of antigens on nature's menu.

When T-helper cells are exposed *in vitro* to *S. aureus* enterotoxin, however, greater than 20% of all helper cells (across many clones) may be stimulated to proliferate, resulting in explosive polyclonal T-cell expansion, instead of the 0.01% one would predict. Those microbial products, which incite global T-cell proliferation were appropriately labeled *superantigens* (Johnson et al., 1992). How can massive polyclonal superantigen induced T-cell expansion be explained in light of the conservative response which occurs when T-cells are confronted by "legitimate antigen?" First, it would be highly irregular and counter to prevailing dogma, if one in five T-helper cells possessed a TCR specific for each *S. aureus* enterotoxin. Second, very low concentrations of enterotoxin (picomoles) will trigger pan-stimulation of T-helper cells, a response level that could occur only with a billion times that concentration of conventional "legitimate antigen." One explanation suggests that T-cells stimulated to proliferate by superantigens do so through a nonimmunological event, which bypasses or ignores antigen recognition by the TCR.

T-helper cells stimulated immunologically by conventional "legitimate antigen" proliferate and secrete the "helper" cytokines interferon-gamma (INFγ) and interleukin-2 (IL-2). The biological activities of each helper cytokine are reviewed in Tables 4 and 7, respectively. Helper cytokines secreted into the immediate tissue environment provide the help signals that both T-cytotoxic (CD8) cells and B-lymphocytes solicit and require to urge each into immunological activity, target cell killing (tumor cells) and antibody synthesis, respectively. Enterotoxin-superanti-

Table 7. Biological Effects of Interferon-gamma (INFγ)

Site of Action	*Biological Effect*
Endothelial cells	Produces marked changes in endothelium
Neutrophils	Enhances neutrophil accumulation and activation, and promotes phagocytosis
Macrophages	Promotes release of TNF, IL-1, and IL-6
	Synergistically increases IL-2 promotion of TNFα release
	Promotes activation of macrophages, microbial cidal activity
Lymphocytes	Helps activate B-cells to produce antibody
	Enhances adhesion of lymphocytes to endothelial cells
Hypothalamus	Endogenous pyrogen, acting on brain, to elevate body temperature
All tissues	Induces expression of MHC I and II

gens, in addition to stimulating polyclonal T-helper cell proliferation, provoke the expanding clones to secrete excessive levels of INFγ and IL-2.

Does it follow that hypersecretion of helper cytokine accounts for the signs and symptoms which accompany food poisoning (fever, nausea, vomiting and diarrhea), TSS or STSS (fever, nausea, vomiting, diarrhea, hypotension, and multiorgan dysfunction) or dermal cell desquamation associated with a condition termed the scaled skin syndrome (SSS)? The pivotal question then is, What feature, property, or molecular attribute does each exotoxin exhibit, that permits these microbial products to incite massive T-cell stimulation?

One piece of evidence which implicated cytokines as possible mediators of toxic shock was gleaned from findings obtained from experimental cancer therapy studies done at the National Institutes of Health. Administration of high levels of IL-2 into the circulation of cancer victims caused disturbing side-effects; fever, nausea, vomiting, and diarrhea—the hallmarks of overt staphylococcal food poisoning (and most certainly other infection states). Do enterotoxin superantigens make people ill, by promoting excessive cytokine production? Is surplus cytokine introduced into the circulation from a localized infection site, or does bloodborne exotoxin incite these events in the blood compartment and in those tissues visited by blood? How does each exotoxin-superantigen, independent of microbial source, foster pan-stimulation of T-helper cells, a feat which "conventional antigen" cannot accomplish?

Figure 3 (panel A and B) depicts the manner in which T-helper cells recognize and engage processed protein antigen. Foreign items, which include extracellular infectious agents, and their products (defined as exogenous antigen), are phagocytosed by tissue-based phagocytic cells called macrophages. Macrophages are charged with the task of metabolically dismantling ingested items, and during this process, the foreign item is enclosed within a membranebound vesicle called a phagosome. Digestive enzymes are shuttled into the phagosome, from lysosomes gathering at the periphery of phagosome. Small fragments of processed microbial protein, no larger than 9 to 18 amino acid residues, are preserved during enzymatic digestion. Each processed peptide fragment (processed antigen) is complexed with an antigen presentation molecule, termed the major histocompatibility complex class II molecule (MHC II). Those macrophages which present and display the MHC II-processed antigen complex on their membrane surface are called antigen presenting cells (APC). Usually only APCs and B-lymphocytes express MHC II molecules. To visualize, in your minds eye, as to how the T-helper cell "sees" the MHC II-antigen complex displayed on the APC surface—picture a hot dog bun representing the MHC II and tucked into the bun crevice, the hot dog representing the processed protein antigen fragment. Prior to acquiring their immunological helper assignments, T-helper cells during maturation in the thymus were instructed to recognize *self* MHC-II and engage only those MHC II molecules holding a foreign *non-self* antigen fragment.

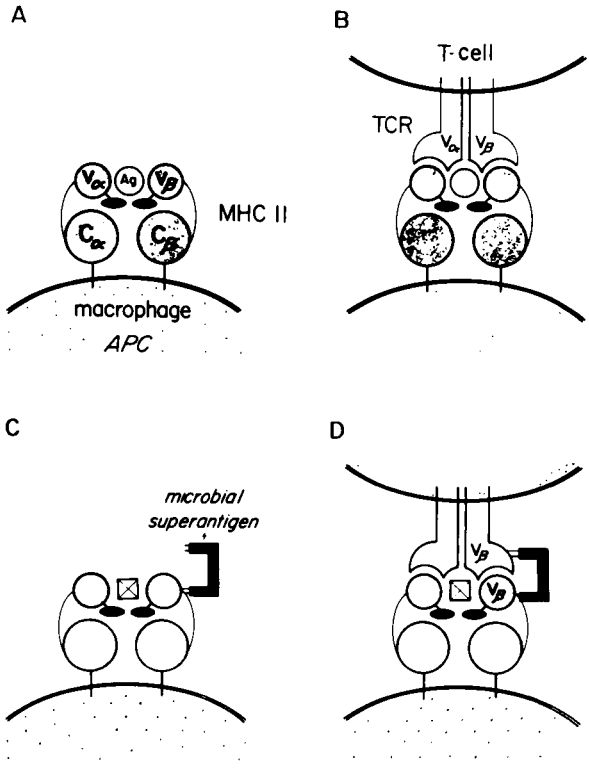

Figure 3. Superantigen panstimulation of T-helper cells. Panel A: The antigen presenting cells (macrophage) process protein antigen (Ag) and preserve peptides of 8 to 19 amino acids which they exhibit on their membrane surface in association with an antigen presentation molecule called the major histocompatibility complex class II (MHC II). The MHC II is composed of two chains (alpha and beta) anchored in the APC cytoplasmic membrane. Each chain is composed of an external (V) variable domain and an internal constant (C) domain. The processed peptide becomes associated with the V domains when presented by the APC. Panel B: T-helper cells which possess the appropriate receptor (T-cell receptor, TCR) must recognize both the processed peptide and the V-domain of the MHC II to be immunologically stimulated to proliferate and secrete helper cytokines. Panel C: Microbial exotoxin superantigens can interact with an external site on the V-domain of the beta chain on the MHC II which now opens a site on the toxin permitting it to react next with a site on the beta chain on the T-cell TCR (Panel D), which in effect "staples" the MHC II to the TCR. This event occurs independent of the antigenic specificity of the peptide in the MHC cleft, or the specificity of the TCR. T-helper cells can be stimulated to proliferate and secrete helper cytokines bypassing the immunological requirement to recognize specific antigen.

T-cells display and utilize the TCR to recognize processed foreign antigen associated with MHC II molecules on the APC surface Each antigen-specific TCR is composed of two peptide chains, which together comprise the antigen reactive site. One TCR family is composed of an alpha and beta chain, while a second TCR family is composed of a gamma and a delta chain. Each protein chain, which makes up the TCR, possesses an internal constant region (C), closest to the T-cell membrane surface, and an external variable (V) domain in which the antigen recognition site is located (Figure 3, Panel B). The great majority of circulating T-cells express α/β TCR, and a small percentage carry γ/δ TCR. The receptor-diversity that permits each TCR to recognize one of the millions of different antigens is generated by random rearrangement of germline genes in a manner similar to that which occurs in B-cells as they assemble antibody molecules which also express a vast repertoire of antigen specificity. Unlike antibody, however, the TCR reactive site must also recognize the MHC II presentation molecule (self) in concert with the processed peptide (non self). Each T-helper cell (or members of that clone) can potentially interact with any MHC-II, but this interaction is not consummated (does not result in immunological stimulation) unless the TCR also recognizes the foreign processed peptide (Figure 3, Panel B). As a consequence of dual recognition, only a finite number of T-helper cells possess the correct antigen-specific TCR to form a perfect MHC II-antigen fit. As stated previously only 1 in 10,000 T-cells can be stimulated by one defined antigen.

Superantigens, unlike conventional antigen, are not phagocytosed, processed and presented in association with MHC II as a prerequisite to T-cell stimulation. What in fact happens, prior to T-cell stimulation, is that superantigens bind to the MHC II at an external site outside the antigen presenting cleft (Figure 3, panel C). The superantigen MHC II binding event occurs independent of the antigen peptide currently present in the MHC cleft. Following binding to the MHC II, the molecular conformation of the superantigen may change to create or expose a site which the superantigen now utilizes to bind to the V-region of the beta-chain or gamma chain of the TCR. To paraphrase one investigator, the superantigen "staples" the MHC (on the APC) to the TCR (on the T-helper cell). The superantigen staple, ignores the requirement for "specific antigen" recognition, and can stimulate T-cells in a nonimmunological manner to proliferate and release helper cytokines (Figure 3, Panel D). The stapled macrophage may be activated and as a result will secrete TNF and IL-1. Because approximately 30 beta-chain isotypes exist, and each superantigen reacts with a limited number of isotypes, not all T-cells can be stimulated by a superantigen staple.

Exotoxin superantigens examined and compared to date are polypeptides of 20 to 30 kD, and despite a conserved retention of function, there exist only a few amino acid sequences that have been conserved in each exotoxin. It is now assumed that superantigenicity cannot be accounted for by a conserved amino acid sequence (if they evolved from a common ancestral gene) but rather to a common function

created by a complex three-dimensional structural orientation encoded by nonhomologous genes.

Cytokine-mediated Streptococcal Toxic Shock Syndrome

It had been debated as to whether toxic shock induced by *S. aureus* or *S. pyogenes* occurs as a result of the direct toxic effect of TSST-1, SPE-A, or SPE-B on host target tissue or occurs as a consequence of the collective biological effects produced by helper cytokine mediators. Little evidence has been advanced to support the direct toxicity position, rather it appears that excessive levels of endogenous mediators orchestrate toxic shock.

Recently, cases of severe invasive Group A *S. pyogenes* infections have been reported with increasing frequency, principally in North America and Europe (Stevens, 1992). The usual victim is between the ages of 10 and 50, who does not suffer predisposing underlying disease, which is in sharp contrast to earlier times when the majority of streptococcal-induced infections occurred in persons 10 years of age and under, or 60 years and older. In former times patients were often burdened with cancer, kidney disease, tertiary burns or the recipients of corticosteroid or immunosuppressive therapy. The current invasive streptococcal infections are launched following colonization of the mucosal membrane, yet rarely do infected individuals exhibit the classical symptomatic pharyngitis ("strep throat") associated historically with Group A *S. pyogenes* infections. Many victims experience an influenza-like syndrome before onset of the first signs of STSS, and extreme pain in an extremity.

Streptococcus pyogenes strains which express M-protein type 1 or 3, from among 60 possible serologically diverse M-serotypes, constitute the majority of invasive strains isolated and identified from STSS victims (Stevens, 1992). M-protein, which is anchored in the cytoplasm and projects through the cytoplasmic membrane and cell wall layers to the cell surface, in concert with the extracellular hyaluronic acid polysaccharide capsule constitute the two major virulence factors expressed by Group A streptococci. Each of these cell-associated structures defend the invading streptococcal cells by deflecting and repulsing the phagocytic efforts of neutrophils which infiltrate the infection site. If the host subject possesses circulating or mucosal antibody specific for M-protein, the infection process will be terminated by antibody-mediated phagocytosis (opsonization). In the absence of anti-M antibody, local infection may progress as the streptococci replicate unimpeded. The invading streptococci also synthesize and secrete a plethora of virulence factors (called spreading factors) which include streptokinase, hyaluronidase, streptolysin O and S, and Dnase which enhance tissue invasion. However, non-encapsulated, M-protein-minus Group A *S. pyogenes*, in spite of spreading factor activity, are generally avirulent.

Once Group A streptococcal cells penetrate the mucosal or dermal barrier, deep tissue invasion and bacteremia may result. If invading cocci secrete the pyrogenic

exotoxins SPE-A or -B, then these exotoxin-superantigens through T-cell:macrophage stimulation can induce shock, and multiorgan failure. Toxic shock develops only in persons lacking anti-SPE-A or anti-SPE-B specific antibody, which suggests exotoxin neutralization and immune clearance will preempt toxic shock. The susceptible subject can be defined as those individuals who lack either anti-M protein or anti-SPE-A or -B antibody.

Helper cytokines when produced and secreted at "normal" levels, following conventional antigen T-cell stimulation, remain localized in the tissue of origin, and while an intense but regional inflammatory reaction may result, it is largely beneficial. Cytokines mobilized and secreted as a consequence of infection are programmed to promote the following: to attract and then immobilize phagocytic cells onto the vessel endothelium serving the infected tissue, to create gaps in the vascular endothelium and invite the immobilized adherent phagocytic cells to vacate the blood and enter the infection site, to encourage purposeful movement of the phagocyte toward the inciting pathogen, and finally to promote efficient phagocytosis to clear the tissue of infection. Tables 1, 2, 3, 4, 5, and 7 attest to the fact that there exists considerable overlap and redundancy in inflammatory mediator function. However, as long as these activities are confined to the infection site, the consequences are beneficial.

The pyrogenic exotoxins SPE-A and SPE-B, produced by *S. pyogenes*, fit the description and functional profile of superantigens. These microbial products modulate the host defense inciting an exaggerated inflammatory cascade, the aftermath of excessive cytokine production, following unrestricted T-helper cell stimulation. Superantigen stimulated T-cells secrete INFγ which activate macrophages, prompting these cells to secrete TNF and IL-1, and now the events take on a familiar theme. TNF and IL-1 (Tables 2 and 3) incite the inflammatory events leading to shock. TNF blood plasma levels are often elevated in septic or toxic shock states. The administration of large doses of TNF into experimental animals duplicates many of the signs of toxic shock. The biological effects of TNF (also known as cachectin), when TNF is released in concert with IL-1, exert a collective multiplicity of effects. TNF, when injected into laboratory animals to achieve levels attainable during natural infection, induces hypotension, acidosis and death within several hours (Parsonnet, 1989). Pathological examination of tissues removed from these animals at necropsy revealed inflammation, ischemia and hemorrhage in various organs. Since shock and multiorgan failure are the terminal events in both toxic and septic shock, these events will be examined in the next section.

It is unlikely that a single endogenous mediator is responsible for the multisystem dysfunctions which characterize TSS and STSS. In all likelihood toxic shock is the clinical consequence of the cumulative effects of several primary and secondary mediators, possibly compounded by yet to be defined direct toxic effects of the exotoxins. It must be emphasized that the cytokine mediators under consideration, TNF, IL-1, IL-2, INFγ and others, have each been implicated in septic shock (Gram-negative induced endotoxin shock) and were cited as perpetrators of men-

ingeal inflammation. While activated macrophages and stimulated T-cells are the usual source, it must not be ignored that these cytokines can be secreted by the vessel endothelium.

IL-1 secretion explains the high fever that presages the onset of TSS and STSS. In addition IL-1 promotes far-reaching non-immunological activities which affect various organs (Table 3). The liver is prompted to release acute phase proteins, such as C-reactive protein, which can activate the complement cascade to generate protein split-products which augment inflammatory cytokine activity (Figure 1 and Table 1). Conversely the liver is asked to sequester Fe and Zn and halt serum albumin and transferrin synthesis. Skeletal muscle proteolysis occurs under the influence of IL-1, which may explain the myalgias which accompany STSS and TSS.

In summary, TSS and STSS have been divided for therapeutic purposes into four infection stages: stage I, localized infection foci; stage II circulating exotoxins; stage III detectable cytokines in the circulation; and stage IV shock and multiorgan failure. The suggested treatment approaches are as follows: to eradicate infection during stage I with antimicrobials, preferably agents that suppress protein synthesis; neutralize circulating exotoxins at stage II with toxin-specific monoclonal antibody (immunotherapy); and preemptive action of circulating cytokines at stage III with TNF or IL-1 receptor antagonists.

Secondary Consequences of Superantigen Stimulation: Autoimmunity

It has been suggested that superantigens secreted by infecting pathogens could potentially activate "auto-reactive" T-helper and T-cytotoxic cells, and give rise to an autoimmune response. Auto-reactive T-cell clones, which recognize "self antigen" displayed by MHC-II or MHC I molecules, are deleted during T-cell maturation in the thymus. Unfortunately *auto-reactive* clones "sneak-through" and escape elimination during T-cell maturation in the thymus, evidenced by the 20 to 30 documented autoimmune disorders which afflict man.

Usually only macrophages (APC) and B-lymphocytes can express MHC II in which they present processed foreign peptides to helper T-cells. Conversely, all nucleated cells, which include APCs and B-cells, express MHC I molecules, a correlate to the MHC II molecule. The MHC I molecules are also utilized to exhibit processed protein, which in normal times is derived from self cellular components which are usually metabolically recycled. Because these processed peptide fragments are derived from within, they are referred to as endogenous or self antigen, which should not provoke an immune reaction. On those occasions, when tissue cells become infected by intracellular pathogens, such as viruses, the virus may commandeer the protein synthesizing capacity of the host cell for their selfish interests—production of new viral particles. As a result, the infected cell may now process and present virus peptide fragments in the MHC I molecules on their surface. T-cytotoxic cells (CD8) were educated in the thymus to recognize foreign

processed peptides presented in self MHC I molecules, will commence killing (we hope) the infected target cell when they receive the appropriate help signal (IL-2) from immunologically-stimulated T-helper cells. These events are part of the immune response defined as cell-mediated immunity.

Superantigens bridge the MHC on the APC to the TCR of the T-cell, an event which we now know is not influenced by either the presence processed peptides in the MHC or the antigen specificity of the TCR. It is possible that an *autoreactive* T-helper or T-cytotoxic clone, which carries TCRs which recognize autologous or self tissue antigen could be stimulated by a superantigen. And, during polyclonal expansion an *autoreactive* clone undergoes nonimmunological expansion to reach numbers which may promote autoimmunity disease. Large numbers of autoreactive T-cytotoxic cells produced by clonal expansion can now engage MHC I molecules on normal cells displaying the self *autologous* peptides. Following superantigen stimulation sufficient helper IL-2 is available to prompt these autoreactive cytotoxic cells to initiate destruction of normal tissue, an event which may be difficult to shut down.

Similarly, an autoimmune response could be set off through the antibody producing B-lymphocyte. B-cells also express MHC-II molecules in addition to surface immunoglobulins D or M, which in a given B-cell clone carries specificity for a single antigen. If autoreactive B-cell clones, bearing surface Ig receptors specific for autologous antigen, escape elimination in the bone marrow during B-cell maturation events, the potential exists for autoreactive antibody to be produced. Superantigen activity could drive the B-cell to begin synthesizing and secreting autoantibody. B-cells capture native antigen (protein or carbohydrate) through membrane Ig, internalize the captured antigen, and process it in a manner similar to the APC. The processed self peptide is displayed in the cleft of an MHC-II molecule and presented on the B-cell membrane surface. In this manner the B-cell solicits help from the appropriate expanding T-helper clone through its antigen-specific TCR. A randomly placed superantigen staple could stimulate B-cell expansion, again bypassing specific antigen recognition.

GRAM-NEGATIVE INDUCED SEPTIC SHOCK

Multisystem organ failure (MSOF), exemplifies the oft-stated dictum "that patients usually expire as a consequence of the complications brought on by a disease state, rather than of the disease itself." MSOF is the final complication of critical illness, and Gram-negative induced sepsis is often the triggering event. The fundamental process at work in the septic victim is a progressive failure of host-defense to maintain homeostasis. The ultimate event may be impairment of gut wall integrity, which permits the translocation of Gram-negative organisms from the intestinal surface into the circulation. The systemic inflammatory response to a bacterial spill into the circulation is termed *sepsis* (Bone, 1991 and 1992).

The presence of bacteria in the blood compartment, termed bacteremia, may be transient, intermittent, or continuous. In its transient form the organisms, introduced as a consequence of instrument probing on a colonized mucosal surface, are cleared in a matter of minutes. Dental procedures, urinary tract examinations and surgery at an infected tissue site constitute the most frequent causes. Transient bacteremia is usually inconsequential, except in persons with preexisting heart valve disease, in whom blood-borne oral, pharyngeal, and intestinal microbial flora, can colonize the diseased valve which may lead to endocarditis. Intermittent bacteremia results when innate defenses fail to contain local infection, most notable examples are: pneumonia, meningitis, pyelonephritis, pyogenic arthritis, cutaneous soft tissue infection and undrained abscesses. Continuous bacteremia is usually the consequence of an intravascular foci of infection, such as infective endocarditis or contaminated intravenous catheters, which contributes to the incessant presence of bacteria in the blood.

A fulminant variant of Gram-negative bacteremia may be the outcome following infection by a select group of classical infectious agents, which include: meningococcemia (*N. meningitidis*), bubonic plague (*Yersina pestis*), Rocky Mountain spotted fever (*Rickettsia*), typhoid fever (*Salmonella typhi*) and Brazilian puerperal fever (*H. influenzae*, biogroup *aegypticus*). Each of these pathogens evade elimination by innate defense at the primary infection site and enter the blood stream directly or via the lymph channels (*Y. pestis* and *S. typhi*) which funnel through the thoracic duct into the blood stream. Hematogenous spread of each of these Gram-negative pathogens follows colonization of the nasopharynx, dermal inoculation through the bite of an arthropod vector, transportation to the intestinal surface following ingestion of contaminated food or colonization of the conjunctivae, respectively. Meningococcal-induced sepsis leads to hypotension, disseminated intravascular coagulation (DIC), petechial dermal hemorrhage and adrenal gland failure (Waterhouse-Friderichsen syndrome), each a sign of septic shock.

Most septic episodes begin with a foci of tissue infection, a depot of replicating bacteria, at either an extravascular or intravascular site, which gives rise to secondary bacteremia. Most frequently these infection foci are found in the urinary and respiratory tracts, but can include intraabdominal infections (abscesses, peritonitis, biliary tract), wound infections, meningitis, bone, and the dermis, and those conditions identified previously which include indwelling catheters and prostheses. Septic episodes, however, may not be associated with a definable infection foci, a circumstance defined as primary bacteremia. The normal Gram-negative bacterial flora of the intestinal tract are well documented as major agents of primary bacteremia.

Persons most at risk for severe sepsis and septic shock are the elderly, the immunocompromised, individuals with cancer and persons with endocrine, kidney, liver and heart disorders. Sepsis-prone individuals often possess pre-existing elevated levels of one or more of the inflammatory mediators as well as high numbers of circulating neutrophils, macrophages and lymphocytes. Advances in medical

practice and technology have increased the risk for sepsis and septic shock. The widespread use of catheters and implanted prosthetic devices coupled with improvement in care have given longer life to those whose immune facilities have waned.

Mortality rates from Gram-negative sepsis remain alarmingly high, particularly following the onset of septic shock, despite recent advances in understanding the pathophysiological mechanisms of sepsis, and in spite of improved antimicrobial treatment regimens.

The first signs and symptoms of sepsis include: fever, chills, hyperventilation, a confused mental state, and occasional hypothermia. A serious consequence of sepsis is the development of hypotension, which may progress to severe loss of blood flow to vital tissue (hypoperfusion), and organ failure. Additional complications which accompany sepsis are disseminated intravascular coagulation (DIC) and a significant decrease in the number of circulating white cells (neutropenia). When sepsis evolves into hypotension and multiorgan dysfunction the condition is termed septic shock, the most common cause of death in intense care facilities, and the 13th most common cause of death in the United States (Parrillo, 1990). Infectious disorder produces 400,000 cases of sepsis, 200,000 episodes of septic shock and 100,000 deaths annually. A subset of victims with Gram-negative sepsis develop adult respiratory distress syndrome (ARDS), a complication which occurs in 18 to 25% of patients resulting in mortality rates which approach 90% (Martin and Silverman, 1992).

The systemic inflammatory events which occur following wholesale excitation of T-helper cell by exotoxin-superantigens may lead to toxic shock, but this has to be distinguished from endotoxin-induced septic shock. In the former case, blood-borne exotoxins (toxemia) mediate the inflammatory consequences, while bacteria in the blood (bacteremia) trigger the inflammatory events leading to septic shock. While both Gram-positive bacteria and fungi can also incite the cascade of inflammatory events which lead to sepsis and septic shock—the discussion that follows will center on Gram-negative sepsis, which accounts for 80% of septic shock episodes.

Lipopolysaccharide-endotoxin

In 1892 Richard Pfeiffer while toiling in Robert Koch's laboratories, first reported detecting a heat-stable toxic component in Gram-negative bacteria. During his investigations of *Vibrio cholerae*, Pfeiffer noted that cell lysates prepared from heat-killed cholera vibrios produced shock and death when injected into guinea pigs. The expression endotoxin was coined by Pfeiffer to differentiate the toxic substance released following bacteriolysis, from the poisonous toxins (exotoxins) secreted by the viable bacterial pathogens *Corynebacterium diphtheriae* and *Clostridium tetani*, discovered and described only a few years earlier in the same institute

by Frederich Loeffler, Emil Behring and Shibasaburo Kitatsato and, in Paris, by Emile Roux and Alexander Yersin.

During this period William B. Coley, a New York City surgeon, reported some success in treating cancer patients by infecting them with live bacteria, which in some cases resulted in tumor regression. For obvious safety concerns, in this preantibiotic era, he substituted killed bacterial suspensions (called Coley's toxins), though non-viable Coley's toxins produced many of the signs of bacterial infection, which included fever and chills. The tumoricidal effects of Coley's toxins when administered to cancer victims were inconsistent and this treatment approach was abandoned, giving way to radiation therapy. In follow-up studies, the observations reported by Coley were confirmed. Gram-negative organisms, in particular, when injected into tumor-bearing mice caused hemorrhagic necrosis of the tumors, during which the tumors seemingly bled into themselves, turned black and crusted over. The tumor killing factor released from Gram-negative bacteria, which Pfeiffer first called endotoxin, was subsequently isolated in 1942, purified and determined to be a large lipid-sugar complex.

Figure 4 depicts the generic structure of Pfeiffer's endotoxin, a structural component which is located in the outer-leaflet of the outer-membrane in Gram-negative cell walls. Endotoxin is a long chain lipopolysaccharide (LPS) molecule composed of three distinct regions: a lipid moiety, a core oligosaccharide and a large polysaccharide, consisting of repeat units of two to six monosaccharides (called the

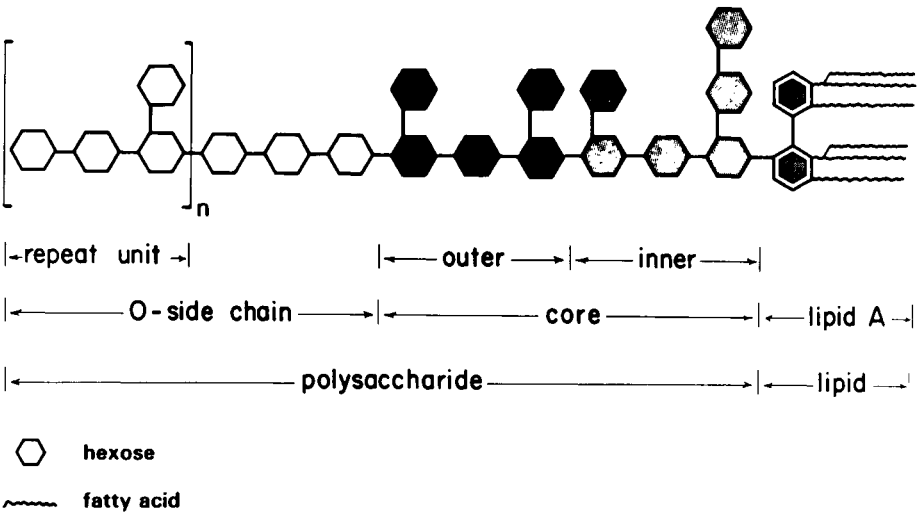

Figure 4. Schematic diagram of lipopolysaccharide (LPS). LPS is composed of three distinct regions: a lipid moiety located proximal to the peptidoglycan layer, which contains the toxic substance associated with LPS (endotoxin), the core oligosaccharide is the region which shows little or no diversity across Gram- negative genera, and the O-side chain which is responsible for the cell wall associated antigenic properties.

O-antigen side chain). Lipid A, located proximal to the peptidoglycan layer, is composed of a diglucosamine moiety to which long chain fatty acids are linked through -OH and -NH_2 groups. There may be one, two or three fatty acid chains linked to each glucosamine residue and these fatty acids contribute the toxic property to the LPS molecule. The core oligosaccharide is a region in the LPS molecule which shows little diversity among the bacterial genera which comprise the *Enterobacteriaceae*. The eight carbon sugar 2-keto-3-deoxyoctonate (KDO) is a monosaccharide common to the inner core of most organisms. The large polysaccharide portion, designated the O-side chain, when separated from Lipid A is not toxic, and bestows the dominant cell wall-associated antigenic properties to Gram-negative cells. The monosaccharide composition of the O-side chain is quite diverse even within a single genus. For example, *Escherichia coli* exhibits 160 different O-antigens (O-serogroups), each expressing a unique repeat unit made up of different monosaccharides. In *E. coli* 0157, the designation 0157 implies this strain (O-serogroup) expresses O-antigen 157, exclusively. The enterohemorrhagic *E. coli* (EHEC), secrete an extremely toxic exotoxin (called a Veroloxin) which acts on the kidney, and has been responsible for sporadic outbreaks of lethal food poisoning. This toxigenic strain can be identified in contaminated meat products through detection of the 0157 polysaccharide antigen, with the appropriate antiserum. In a similar manner, the human enteric pathogens, *Salmonella, Shigella* and *Vibrio* can be identified when isolated from clinical specimens. If the appropriate O-antigen-specific serum is used *Salmonella typhi*, O-serogroup D, *Shigella dysenteriae* O-serogroup A, and *Vibrio cholerae* O group 1 isolates can be identified within a few minutes, following isolation. The O-antigen polysaccharide serves as a very useful diagnostic marker, by which to detect specific Gram-negative pathogens in clinical specimens and to trace O-specific pathogens epidemiologically during infectious disease outbreaks. Currently, *V. cholerae* 0139 has displaced the classical 01 serogroup, in India, as the primary epidemic toxigenic agent of Asiatic cholera. The 0139 antigen marker will be quite useful in tracking and monitoring the spread of cholera over the next decade.

Gram-negative–induced Sepsis

The following statement describes and defines, eloquently, the septic inflammatory holocaust that can occur in the high-risk group, when the innate defense is marshalled to respond to a Gram-negative pathogen. "It is the information carried by the bacteria that we cannot abide. The Gram-negative bacteria are the best examples of this. They display lipopolysaccharide-endotoxin in their cell walls, and these macromolecules are read by our tissues as the very worst of bad news. When we sense lipopolysaccharide, we are likely to turn on every defense at our disposal; we will bomb, defoliate, blockade, seal off and destroy all the tissues in the area" (L. Thomas, in "The Lives of a Cell," 1974).

It should become clear, that as the events which surround *sepsis* and *septic shock* are revealed, it is the Gram-negative pathogen, the macrophage, the cytokine TNF and the vascular endothelium which are the central players in the pathophysiology of these inflammatory events.

Shock is the expression assigned to a variety of syndromes characterized by overall inadequacy of tissue perfusion (diminished blood supply), during which the blood flow is diverted or there occurs severe loss of plasma volume. Shock exists in three forms: hypovolemic, vascular (endotoxin-induced) and cardiac shock. Hypovolemic shock occurs as the result of sudden and massive loss of blood plasma volume following hemorrhaging, tertiary burns, or bouts of continuous diarrhea and vomiting (Asiatic cholera and bacillary dysenteriae). Tissues deprived of blood become oxygen starved, forcing the deprived tissue cells to resort to anaerobic glycolysis to purchase energy, which in turn leads to lactic acid accumulation (acidosis). To compensate for acidosis the lungs release increased levels of carbon dioxide.

It is vascular shock, or endotoxic-shock, which Gram-negative bacteria incite. During progressive Gram-negative infection, blood flow is diverted to peripheral vessels and the extravascular spaces. The first clinical signs of septic shock are: a sudden precipitous drop in blood pressure (hypotension), peripheral vasodilation (warm shock) followed by peripheral vasoconstriction (cold shock, as the physiology attempts to maintain blood flow to the heart, brain and kidneys), fever, severe decrease in numbers of circulating neutrophils (neutropenia) and platelets (thrombocytopenia). As the inflammatory events worsen disseminated intravascular coagulation may occur, evidenced by the appearance of hemorrhagic petechiae on the dermis and multiorgan failure in vital organs such as the adrenals and the lungs.

Laboratory animals challenged with nanogram quantities of LPS (a central player) have been reported to produce the following: fever, hypotension, leukopenia, thrombocytopenia, disseminated intravascular coagulation, abortion, hemorrhagic destruction of tumors and activation of both the complement cascade and blood coagulation—mimics infection-induced septic shock. Early in the infection process each Gram-negative pathogen may promote distinct and recognizable infection states (i.e., typhoid fever, bubonic plague). However, there is considerable overlap in the terminal signs and symptoms as septic shock occurs, which may reflect endotoxin-induced inflammation.

On the path to sepsis, the initiating event is entry into the circulation by the Gram-negative pathogen, endotoxin or a comparable inciting substance (teichoic acid, lipoteichoic acid [Gram-positive cell wall constituents] or exotoxins in the superantigen family). *Escherichia coli* is the most commonly-encountered pathogen, followed by *Klebsiella* and *Enterobacter* species. *Pseudomonas* species though encountered less frequently are associated with the highest mortality rates among all agents of sepsis. Sepsis may commence through the activation of one or more of the inflammatory pathways outlined in Figure 5. These events are orches-

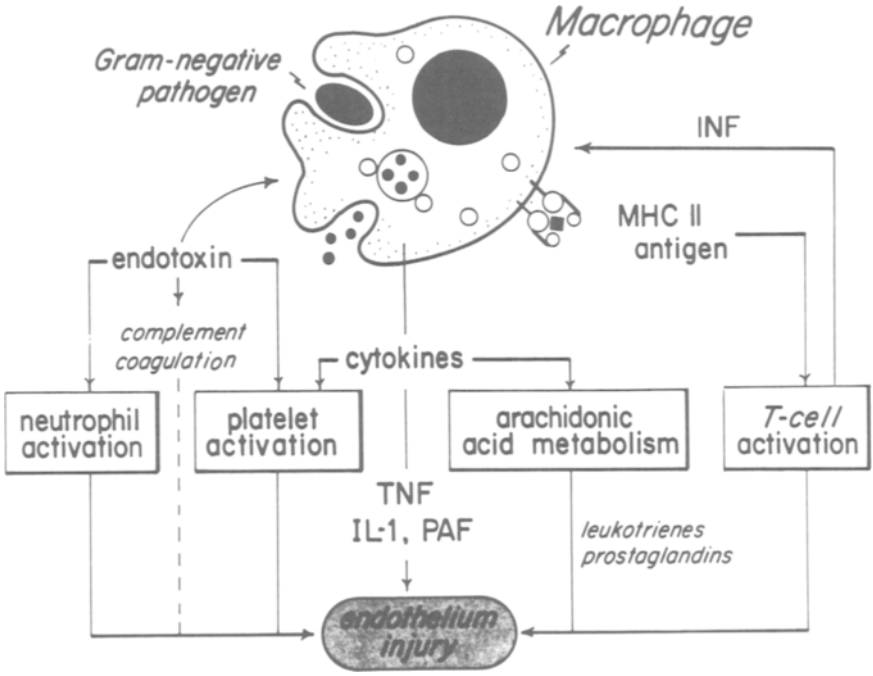

Figure 5. The phagocytic macrophage, which may also serve as an antigen processing cell, is a pivotal player during the initiation and maintenance of inflammatory events which are designed to promote eradication of infection and promote healing. These events when sustained, prolonged and amplified can lead to damage of the vascular endothelium. Macrophages will become activated to initiate these activities following stimulation by Gram-negative bacilli, activation by T-cell cytokines (INFγ) or following presentation of processed antigen in the MHC II to T-helper cells. Activated macrophages secrete TNF and IL-1 which act directly on the endothelium. Simultaneous activation of neutrophils, the complement cascade and mobilization of arachidonic acid can generate additional inflammatory mediators which can amplify inflammation and inflict permanent damage to the endothelium as a prelude to sepsis and septic shock.

trated by blood monocytes or tissue macrophages following stimulation by free endotoxin or through phagocytosis.

Recall, tumors on laboratory animals became hemorrhagic and necrotic when these animals were challenged with LPS. Conversely, tumor cells propagated in the test tube were neither inhibited nor killed when exposed to LPS. It was hypothesized and subsequently determined that the LPS-induced tumoricidal action *in vitro* was an indirect activity, in that tumor cells were killed by a factor produced by the tumor-bearing host in response to the presence of endotoxin. Following up on this idea, Old and his colleagues (1988) discovered and characterized a host derived

tumoricidal substance, which they designated tumor necrosis factor (TNF). They went on to prove that both Gram-negative bacteria and endotoxin stimulated macrophages to secrete TNF, which in addition to it's tumoricidal activity can also mediate a plethora of seemingly unrelated biological effects reviewed in Table 2.

TNF is synthesized and secreted by macrophages in response to nonspecific challenge by a variety of invasive stimuli, among which endotoxins are probably the most potent. The role TNF plays in the induction of septic shock was suspected, when TNF was detected in the serum of laboratory animals following challenge with a lethal dose of endotoxin. Also, animals injected with TNF exhibited many of the signs recorded previously for endotoxin-induced shock: fever, hypotension, shock lung syndrome and acute tubular necrosis of the kidney. In addition, mice pretreated with antibody specific for TNF, exhibited reduced mortality rates following subsequent endotoxin exposure. TNF plays a central role in fomenting inflammation and shock, by promoting the release of secondary or terminal mediators. The primary tissue targeted during septic shock is the vascular endothelium (recall meningeal inflammation). Unless macrophages are stimulated to secrete TNF and IL-1, circulating endotoxin exerts little direct toxic effect, despite making contact with the vessel endothelium throughout the body.

When released in proximity to the endothelium, TNF exerts a change on the normally anticoagulant surface of the vascular endothelium converting it to a procoagulation state, during which time the vessel walls are primed to promote blood coagulation. The effect seems to be a down-regulation of thrombomodulin, an endothelium membrane component that redirects thrombin from its normal function, that of cleaving fibrinogen. Following contact with TNF, the endothelial cells express a tissue factor, which favors blood coagulation, and may promote coagulation which can progress to disseminated intravascular coagulation, a prologue to vessel wall necrosis.

In concert with TNF induced blood coagulation, the Hagemann factor, a plasma protein (Factor XII) becomes activated on the negatively charged endotoxin surface, an event which can trigger the intrinsic coagulation pathway and lead to the formation of acellular thrombin or fibrin clots within the vessel lumen. The growing fibrin polymers may occlude the vessel, thus blocking blood flow to the affected tissues. Platelets (thrombocytes) also promote blood clotting through the extrinsic coagulation pathway, by binding Factor XII to a membrane receptor. Activation of the extrinsic pathway leads to the formation of cellular-thrombin clots, and as platelets are activated through aggregation and become entrapped in developing clots, they too become scarce in the general circulation (thrombocytopenia). Prolonged vessel occlusion, as a consequence of clot formation, deprives the affected tissues of oxygen, leading to ischemic necrosis and hemorrhaging, exemplified by the appearance of dermal petechial or purpuric rash.

Recall, endothelial cells express adhesion molecules, such as ELAM-1, when prompted by TNF and IL-1. Blood-borne neutrophils marginate as blood flow slows to the affected tissue and adhere to vessel walls in the same tissue. Widespread

neutrophil pavementing contributes to the disappearance of these phagocytic cells from the circulation. Gaps created at endothelial cell junctions, permit blood plasma to leak into the supporting tissues (transudation) and provide exit ports for adherent neutrophils to move through and enter the tissue (Beutler, 1990; Parrillo, 1990; Bone, 1991).

TNF may incite adherent neutrophils to degranulate and discharge their contents, superoxide ions and hydrogen peroxide, onto the endothelium surface, thereby damaging the vessel walls. During phagocytosis, phagocytic cells exhibit a burst of respiratory oxidative activity, increased oxygen consumption and exhibit a shift in glucose metabolism to the hexose monophosphate shunt. In this metabolic pathway oxygen is converted to bactericidal hydrogen peroxide and to extremely toxic superoxides, which are packaged in intracellular lysosomes. Following phagocytosis the lysosomal contents are delivered to the phagosome to promote killing and digestion of the ingested pathogen. TNF and C5b, however, direct the neutrophil to discharge their lysosome contents onto the endothelium, causing injury to vessel walls, which when coupled with blood coagulation events leads to tissue necrosis.

Sepsis may in part be mediated by intravascular activation of the complement cascade by endotoxin through the alternative pathway (Figure 1). The complement system, a family of approximately twenty chemically-distinct plasma proteins, exerts collective actions that are instrumental in the development of an effective (protective) inflammatory response. The complement proteins are inactive while circulating in the blood, but once activated they interact in a highly regulated enzymatic cascade and generate reaction products that direct activities leading to clearance of infectious agents (Table 7). There are two pathways by which complement is activated—the classical pathway and the alternative pathway—which converge to form a terminal reaction sequence, the creation of a macromolecular membrane attack complex, which when inserted into the bacterial membrane will kill the target cell. As the complement cascade precedes a variety of reaction products, so-called split-proteins, are generated which induce vasodilation (C3a, C5a), attract neutrophils chemotactically (C5b) and promote phagocytosis by labeling the target pathogen with C3b (Table 7). It is through the alternative pathway that Gram-negative cells, endotoxin or cell wall constituents of Gram-positive pathogens may activate complement (Figure 1, bottom). When C3bBb convertase is activated and stabilized upon complexing with Factor B, complement split-products are generated which can amplify the inflammatory events initiated by TNF, IL-1, and blood coagulation.

Additional secondary inflammatory mediators may also be mobilized and when released augment the activities of the primary mediators. TNF and IL-1 stimulate phospholipase A_2 activity in macrophage, neutrophil and endothelial cell membranes, leading to the generation of three families of late acting lipid mediators (Figure 5). Following methylation of membrane phospholipids, a membrane-associated enzyme, phospholipid A_2, generates the inflammatory mediator platelet

activation factor (PAF) and a 20-carbon fatty acid, arachidonic acid. Arachidonic acid can be metabolized through the lipoxygenase pathway to yield the leukotriene (LT) family of mediators which includes LTC_4, LTD_4 and LTE_4, which were once called, collectively, slow-reactive-substance. When metabolized through the cyclo-oxygenase pathway arachidonic acid gives rise to the prostaglandin (PG) family of mediators. The biological effects these secondary mediators contribute to the inflammatory events are listed in Table 8 (Bone, 1990 and 1993; Parrillo, 1993).

Finally macrophages collecting at the infection foci, phagocytose, process and present bacterial-derived antigen fragments in the MHC II. Antigen-specific T-helper cells engage the MHC-antigen complex and are stimulated to secrete helper cytokines, which include INFγ. INFγ can activate bystander macrophages to express MHC molecules and secrete additional TNF and IL-2, which amplify the inflammatory events developing on the vascular endothelium.

In the tissue microenvironment the beneficial effects each inflammatory mediator exerts probably outweigh the negative effects, for they enhance host defense against infection and contribute to healing. If infection is not resolved successfully and one or more of these mediators or endotoxin leak into the circulation, the consequences may not be beneficial. Once in the blood compartment, endotoxin or TNF (and other mediators) can trigger the sepsis cascade. Under the best of circumstances the entrance of TNF or endotoxin into the circulation may not cause sepsis, for superimposed on the inflammatory cascade are points at which a runaway inflammatory cascade can be downregulated. For example, prostaglandin E_2 may suppress the capacity of macrophages to release cytokines and other mediators (Table 5) while macrophages may exert a "calming effect" on T-cells, restoring homeostasis.

In risk groups, identified at the outset, downregulation may not be operative, or excessive endotoxin or cytokine activity is being continuously sustained, or too many mediators were released simultaneously, and as a result the victim exhibits the first signs of systemic response to infection. Disseminated intravascular coagulation and ischemic hemorrhaging occur, and the victim shows the early signs of septic shock and organ failure. Extensive damage to the endothelium of any single organ, such as the pulmonary vasculature (ARDS), can prove fatal. If the endothelium cannot repair itself, and additional mediators are released into the blood, additional damage sites will result, and ultimately blood pressure drops. The precise mechanism by which hypotension occurs has not been fully elucidated. It may result from direct TNF action on the heart, or release of myocardial depressant factor, or an endothelium derived relaxing factor or a combination of these factors.

Summary

Sepsis begins with an infection foci from which the microorganisms invade the bloodstream resulting in positive blood cultures, or the microorganisms may proliferate at an infected site and release large quantities of various mediators into the bloodstream. These mediators may consist of elaborated endotoxin, exotoxin

and other cell wall constituents or host-derived cytokines such as TNF, the interleukins or complement activation. While some mediators may be more important than others, there could be 20 to 30 molecular mediators that can produce long lasting effects on the peripheral and organ vasculature, and give rise to septic shock and organ failure.

INFECTIOUS DIARRHEA

In 1881, Carl Joseph Eberth a German anatomist-pathologist reported observing bacilli in the lymph nodes and spleen tissue of typhoid fever victims (an infectious disease called "abdominal typhus" in those times, since many thought this syndrome was a form of typhus fever). The organism was consequently called *Eberthella typhosa* for a period of time. Two years later, George Gaffky, a pupil of Robert Koch, and his successor as director of the Institute for Infectious Diseases isolated and described the typhoid fever agent. Daniel E. Salmon and Theobold T. Smith isolated bacilli from diseased swine and called it *Bacillus cholera-suis.* Eventually the cholera-suis and typhoid fever organisms were recognized to be closely related bacteriologically—and are now classified in the genus *Salmonella.*

Theodor Escherich was an early bacteriologist and docent for children diseases in Munich and later Vienna, where he published his classical papers from 1879 to 1885 on intestinal bacteria. During this period he isolated *Bacterium coli commune*, which now bears the name *Escherichia coli.* He recognized this bacterium to be a normal inhabitant of the intestinal tract and a prominent member of those organisms, which also includes *Enterobacter* and *Klebsiella species* referred to as the "coliforms."

In August of 1882 Robert Koch headed the German Cholera Commission to Alexandria, Egypt and then to Calcutta later that year. During this period Koch isolated a curved bacillus (vibrio) from the diarrheic feces and the intestinal surfaces of cholera victims, which could not be recovered from "normal non-infected persons." Considerable controversy surrounded Koch's claims that the vibrio was the etiological agent of Asiatic cholera—primarily because the disease could not be reproduced in the laboratory animal—meaning he could not fulfil his "postulates." The organism was first called *Vibrio comma*, defining its cellular morphology. Ultimately, most accepted this organism as the etiological agent of Asiatic Cholera, and has become known as *V. cholerae.*

There were those who advocated the position that all intestinal infectious diseases had a common etiology and they argued that while each disease state may exhibit different clinical signs, these were merely manifestations of the same infectious disease. These advocates stated that typhoid fever, cholera and bacterial dysentery were one and the same disease, caused by a single bacterial agent. This position was silenced in 1896 in a paper published by the Austrian bacteriologist Max von Gruber and Herbert E. Durham an Englishman working with Gruber in Vienna. Their paper entitled "A New Method for the Rapid Identification of the *Cholera*

vibrio and the Typhus (Typhoid) Bacillus," was published in the Munich Medical Weekly. They reported: "When cells from agar cultures of the cholera vibrio or the typhoid bacillus were mixed with immune serum (obtained from victims of these infections), the bacterial cells clump together in masses and motility ceases." "We have named the specific substance in the immune serum producing the effect Klumper (or Agglutinin) and highly immune sera produce the agglomerative effect in astonishingly high dilution." The Table below summarizes their agglutination findings:

	Immune Serum Obtained From:	
	20 cholera patients	*20 typhoid patients*
cholera vibrio	20/20	0/20
typhoid bacillus	0/20	20/20
colon bacillus	0/20	0/20

The agglutination reactions show that serum collected from persons afflicted with Asiatic cholera possess in their blood serum a Klumper (antibody) which will clump or agglutinate the cholera vibrio only, and not the typhoid bacillus nor the colon bacillus (*Escherichia coli*). If cholera and typhoid fever were caused by the same bacterial agent, then the blood serum collected from both disease states should clump either the cholera vibrio or the typhoid bacillus. Gruber and Durham suggested serum possessing cholera-specific antibody could be employed to identify unknown bacterial isolates on streak plates prepared from diarrheal specimens—in a like manner serum possessing typhoid antibody could be used to identify the typhoid agent. The slide agglutination test is used today for just that purpose. Conversely, a disease state can be diagnosed by collecting blood serum from the victim and mixing it with the typhoid or cholera agent and observing an agglutination reaction. Modifications of this approach are used frequently today to diagnose both bacterial and viral infectious disease states.

In 1897 dysentery was raging in Japan, and other areas in the Far East, and from June through December 89,400 dysentery cases were recorded in Japan with 24% mortality. Kyoshi Shiga, an assistant to Shibasaburo Kitasato (Institute for Infectious Diseases, Tokyo), examined 36 dysentery victims at post-mortem and isolated a bacillus, in almost pure culture. He reported the isolate was non-motile (both the cholera vibrio and the typhoid bacillus are motile) and gelatinase-negative (the cholera vibrio possesses gelatinase activity), and as a consequence he argued a new agent of disease (acute dysentery) had been isolated which Shiga named *Bacillus dysenteriae*. In quick succession Simon Flexner, John Boyd, and Claude Sonne isolated similar (but distinct) organisms which caused dysentery-like epidemics in the Philippine Islands and India. Today these isolates are classified as *Shigella dysenteriae* (Shiga's bacillus), *Shigella flexneri, Shigella boydii*, and *Shigella*

sonnei. Shiga also demonstrated that serum harvested from the blood of dysentery victims agglutinated the dysentery bacillus *exclusively* and not the cholera vibrio or the typhoid bacillus, providing further proof that the dysentery bacillus was unique and different from other recognized enteric pathogens.

As a group those agents producing infectious diarrhea are the leading killers of small children in developing nations where the typical child suffers 10 to 20 bouts of diarrhea before age three. Malnutrition exacerbates the severity, duration and frequency of these episodes, thus creating a debilitating cycle of malnutrition and infection. Some estimates suggest 750 million cases of infectious diarrhea occur worldwide on an annual basis, resulting in 5 million deaths. The annual death rate in New York City from diarrheal disease in 1890 was 5500 per 100,000 infants, and in 1990 it was 3.5 per 100,000 infants. Levine and colleagues (1983) categorized bacterial-induced enteric infections into five groups, based upon their degree of ultimate invasiveness following ingestion by a susceptible host. The five enteric infection groups (summarized in Table 8) are as follows:

I. Mucosal adherence and enterotoxin production. *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) represent enteric bacterial pathogens which manifest this infectious process. These agents adhere to the intestinal mucosa, do not destroy the brushborder, invade the mucosa or produce histological lesions in this tissue. From an extracellular site each secretes an enterotoxin, which promotes an outpouring of fluids from the mucosa.

II. Mucosal adherence and brush border dissolution. The enteropathogenic *E. coli* (EPEC) fit this infection profile. The EPEC adhere tightly to the mucosa of

Table 8. Features of Common Enteric Infectious Diseases in Humans

Enteric Pathogen	*Diarrhea*[a]	*Dysentery*[b]	*Exotoxin*	*Tissue Site*
I *Vibrio cholerae*	+	0	+	small intestine
enterotoxigenic *E. coli*	+	0	+	small and large intestines
II enteropathogenic *E. coli*	+	+/0	0	small and large intestines
III *Shigella*				
dysenteriae	+	+	+	small intestine
flexneri	+	+	+	colon
sonnei	+	+	+	colon
IV *Salmonella spp*	+	0	0	small and large intestines
V *Salmonella typhi*	0	+/0	0	small intestine

Notes: [a]diarrhea = profuse watery stool with no inflammatory cells present
[b]dysentery = abdominal cramping, tenesmus, pus cells and blood in stools

both the small and large intestine, deface the brush border, yet do not invade the mucosa.

III. Mucosal invasion and intraepithelial cell proliferation. This infection pattern is characteristic of those agents which cause bacillary dysentery. Shigella species invade the mucosa, proliferate within the infected cell, disrupt cell metabolism, kill the host cell and invade laterally into neighboring mucosal cells. While the invading bacilli may penetrate to the lamina propria, they rarely spread beyond.

IV. Mucosal translocation followed by bacterial proliferation in the lamina propria and mesenteric lymph nodes. This infection process characterizes enteric infection caused by *Salmonella* species (other than *S. typhi*), *Campylobacter* spp and *Yersina enterocolitica*. These agents invade enterocytes, are translocated inside pinocytic vesicles to the lamina propria, and from this site infection may progress to the regional lymph nodes. Infrequently the infection leads to bacteremia and systemic infection.

V. Mucosal translocation followed by generalized infection. The agents of the enteric fevers *Salmonella typhi* and *S. paratyphi* A and B are delivered to the lamina propria by the translocation process employed by group IV pathogens. They possess the potential to penetrate the lymph node barrier and enter the blood stream by way of lymphatic drainage and are cleared rapidly by phagocytic cells in tissues such as the liver and spleen. They remain viable while sequestered in these phagocytic cells and following a 10 to 14 day incubation period they emerge from these cells to give rise to the first clinical signs of typhoid fever—a bacteremic state which can lead to septic shock.

Bacillary Dysentery

Invasive diarrhea can be defined as the response of the gastrointestinal tract to the enteric pathogens *Shigella, Salmonella, Campylobacter*, enteroinvasive *Escherichia coli* (EIEC), and to various viruses and parasites. These agents possess the capacity to invade, infect and injure the mucosal cells which line the small and large intestine. Among those bacterial agents which invade and infect the mucosa and give rise to bloody, frequent stools (dysentery), the *Shigella* organisms are probably the most frequently encountered. In the discussion that follows the terms diarrhea and dysentery are used to distinguish two distinct infectious syndromes. Dysentery refers specifically to the clinical signs: abdominal cramping, tenesmus, and the presence of inflammatory cells and blood in the stool. These signs and symptoms are the result of bacterial invasion of the intestinal wall, giving rise to epithelial necrosis, focal ulceration and inflammation, which accounts for pus cells and red cells in the feces. Bacillary dysentery is caused by the Gram-negative bacilli in the genus *Shigella* and the expression *shigellosis* is almost synonymous with the term dysentery (Mathan and Mathan, 1991). As for the expression diarrhea, it refers to the profuse watery discharge, usually produced in the small intestine. Diarrhea is the consequence of the rapid and profuse secretion of fluid across the mucosal

surface often in response to the action of specific enterotoxins such as those secreted by ETEC and *Vibrio cholerae*. In specific instances preformed toxin may be present in food items which is the case for staphylococcal-food poisoning and botulism. This latter mechanism is termed intoxication and is not dependent upon ingestion and infection by the bacteria which secrete the toxins.

Shigellosis: The Pathogenesis

Only humans and apes seem able to serve as the natural reservoir for *Shigella* organisms and surprisingly as few as 10 viable bacilli can produce clinical disease in these subjects. While the shigellae are quite susceptible to acidic conditions *in vitro*, they readily survive the hostile low pH of the stomach as they traffic through to the surface of the small intestine. In order to produce disease in the intestine the invasive shigellae must: (a) exhibit smooth LPS, which includes an intact O-side chain, (b) possess genetic elements which encode for invasive capabilities, and (c) secrete an exotoxin which exacerbates the infection process.

Lipopolysaccharide as a Virulence Factor

Endotoxin (LPS) can trigger serious inflammatory consequences as outlined in the previous section, and now we must determine if this cell wall component functions as a virulence factor necessary to promote dysentery. In addition to producing the noxious toxic effects that lead to septic shock, LPS appears to play a role in the pathogenesis of the invading *shigellae* and innate host defense. To remain virulent the *shigellae* must maintain an intact LPS structure, termed smooth LPS which actually describes and gives rise to the smooth or creamy colonial morphology the *shigellae* exhibit on an agar surface. The lipid A and inner core region of the LPS (Figure 3) in shigellae are almost identical to that of those regions in *Escherichia coli*. The O-antigen polysaccharide chain in the LPS of the *shigellae* is constructed of polymerized repeat units composed of di- to hexasaccharides (Brown et al., 1991).

The O-side chain polysaccharide functions as a virulence factor by providing resistance to phagocytosis and the bacteriolytic action mounted through activation of the alternative complement pathway (Figure 1). Rough mutants of shigellae (again this expression defines colony morphology) which have lost the capacity to produce the O-polysaccharide side-chain that comes from smooth strains are found to have lost their virulence. Each rough mutant examined to date has retained its invasive property, but lost its capacity to multiply within the target epithelial cell.

Shiga Toxin

Before proceeding it may be relevant to examine the exotoxin now known to be synthesized and secreted by the *shigellae*. Ever since Kyoshi Shiga first isolated and described the etiological agent of dysentery, it had been speculated that this

enteric pathogen produced a toxin. Crude cell-free extracts, prepared from *S. dysenteriae* type 1, when injected into rabbits produced paralysis in the extremities and hence it was defined as a neurotoxin. *In vitro* studies revealed that these same extracts were lethal for tissue cells propagated in culture and it was defined as a cytotoxin. Finally, crude toxin preparations were reported to elicit the secretion and accumulation of fluid in the rabbit ileum when injected into ligated intestinal loops. Until the time the toxin was purified, it was uncertain whether these multiple manifestations, neuro-, cyto- and enterotoxicity, were the consequence of three different toxins or that of a single entity. It is now known that a single protein toxin molecule promotes multiple activities, and this exotoxin is called the Shiga toxin. As is often the case when a virulence factor has been isolated and characterized, secretion of Shiga toxin was not confined to the shigellae.

A toxic activity produced by *Escherichia coli*, distinct from the previously characterized heat-labile (LT) and heat-stable (ST) toxins, was described and found to be cytotoxic to Vero cells cultured *in vitro*. This activity is now known as Verotoxin which is secreted by the notorious *E. coli* 0157. It has been implicated in the pathogenesis of both the hemolytic-uremic syndrome and hemorrhagic colitis. The finding that the toxic activity of Verotoxin produced by *E. coli* 0157 could be neutralized by antiserum produced against purified Shiga-toxin suggested a structural relationship between Shiga and Verotoxins. It was therefore proposed that Verotoxin be called Shiga-like toxin (Keusch et al., 1991).

Further investigations revealed that *E. coli* 0157 cells harbored two toxin converting phages (bacterial viruses), which carried the structural genes for two distinct cytotoxins. The toxic activity of one of these toxins was neutralized by anti-Shiga toxin antibody while the other toxin was not. These two toxins have been designated Shiga-like-toxin I (SLT-I) and SLT-II, respectively. The structural genes for each toxin have been sequenced and SLT-I was found to be nearly identical to Shiga toxin, while SLT-II showed 56% homology to the genomic sequence of Shiga toxin. Each SLT exhibits neuro-, cyto- and enterotoxicity. Finally, there are many who would argue that what are now known as *Shigella* and *Escherichia* are from a genetic point of view one and the same organism.

Structure and Function of Shiga Toxin

Shiga toxin, depicted on Figure 6, is the prototype molecule in the Shiga-like toxin family. The Shiga toxin molecule consists of one A subunit, a polypeptide of 32 kD, and five B subunits each of which is a peptide of 7.7 kD (Keusch et al., 1991). Most bacterial exotoxins described and characterized to date conform to the two domain or the A-B model, in which the A subunit expresses the biological or toxic activity (A), while the B subunit facilitates binding (B) of the toxin to receptors on the target cell. The diphtheria toxin molecule for example carries the A (active toxic property) and the B (binding) functions in different regions of the same

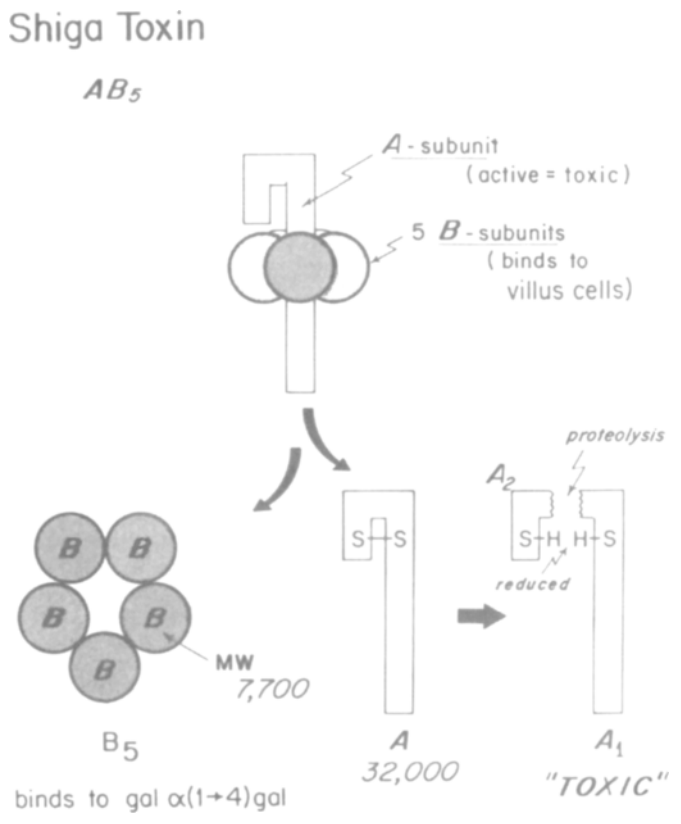

Figure 6. Schematic diagram of Shiga toxin. Shiga toxin is a bipartite toxin composed of an A-subunit which carries the toxic activity attributed to this molecule, and a B-subunit composed of five identical peptides which permit this molecule to bind to galactose-galactose receptor sites on colon villus cells. The A-subunit expresses functional enzymatic activity following reduction of an intrachain disulfide bridge and proteolytic digestion of the A-chain to a toxic A_1 fragment.

polypeptide chain. Conversely, in the cholera toxin the A and B functional domains, like the Shiga toxin, are located on different polypeptide chains.

The toxic A subunit which mediates the toxic biochemical effect, namely inhibition of protein synthesis, contains a stretch of amino acids that is susceptible to the action of proteolytic enzymes. Following enzymatic digestion of this sensitive region and reduction of an internal disulfide bridge, the A subunit is separated into two fragments. The larger A_1 fragment which retains the toxic properties now possesses enzymatic function which can inactivate eukaryotic ribosomal elements, which results in the inhibition of polypeptide chain synthesis. The specific site for

A_1 enzymatic action is the N-glycosidic bond of adenine at nucleotide position 4324 in the 28s rRNA of the 60s ribosomal unit (Donahue-Rolf et al., 1991).

The Shiga toxin when secreted onto the intestinal surface, binds through its B subunit to galactose (α1-4) galactose residues located in a glycolipid expressed exclusively by the absorptive villus epithelial cells and not by crypt or goblet cells. The gal(α1-4)gal residue, the receptor for both Shiga and Shiga-like toxins has been detected in these glycolipids only. The galactose–galactose disaccharide also comprises the human PI blood group antigen which raises some interesting considerations.

Uropathogenic strains of *E. coli* colonize the urinary tract by first binding to the galactose–galactose disaccharide through surface adhesins. Expression of PI by urinary tract tissue cells may contribute to recurrent ascending pyelonephritis. Individuals who do not synthesize the enzyme *N-acetylgalactosyltransferase* will display blood group PI on their tissue cells. It will be interesting to determine if these persons are more susceptible to hemorrhagic colitis, hemolytic-uremic syndrome or dysentery, because both *Shigella* and *Escherichia* secrete Shiga and Shiga-like toxins which interact with the PI epitope.

Shiga toxin, when taken up by an endocytic process into mucosal cells, inhibits protein synthesis in villus epithelial cells and may secondarily alter ion transport (Brown et al., 1991). Since Na^+ absorption and Cl^- secretion are functions segregated into the villus and crypt cells, respectively, the Shiga toxin blocks Na^+ absorption but spares Cl^- secretion by the crypt cells. The net effect, in the presence of unchanged Cl^- secretion, leads to fluid accumulation on the mucosal side. The much greater secretory activity following intoxication with cholera toxin is attributable to the double effect cholera toxin exerts by reacting with both villus and crypt cells, leading to simultaneous reduction in Na^+ absorption and an increase in Cl^- secretion (Keusch and Jacewicz, 1991). When the Shiga exotoxin gene is removed or disabled in *Shigella dysenteriae* 1 cells, there is a measurable reduction in the course of disease in laboratory animals.

Pathogenic Mechanisms: Shigellae

The crucial first step in the pathogenesis of bacillary dysentery is establishment of intracellular infection of the colon mucosa. Mucosal invasion can be conveniently divided into four stages: ***stage I***, entry of the bacillus into the surface mucosal cell, ***stage II***, escape from the phagosome and intracellular multiplication, ***stage III***, lateral spread of bacilli into neighboring mucosal cells, and ***stage IV***, death to the host enterocyte. Progression through these infection stages leads to amplification of bacillary numbers in the colon epithelium. The infection process stops at the lamina propria where the bacilli incite a severe inflammatory process which leads to ulceration and abscess formation.

Stage I: The entry process. The bacilli enter the colon mucosal cells by either receptor-mediated endocytosis or by phagocytosis, an event which heretofore was thought to be the exclusive domain of the professional phagocytes, neutrophils, monocytes, and macrophages. Adherence of the shigellae to colon cells induces polymerization of actin into microfilaments in the host cell cytosol and the subsequent accumulation of actin binding proteins, such as myosin, which are the key events preceding phagocytosis. Pretreatment of these tissue cells with cytochalasins, which are known to block polymerization of actin, will preempt phagocytosis and inhibit entry of *shigellae* into cells. Current reports suggest that the dysentery bacilli enter mucosal cells by a process similar to phagocytosis. The *shigellae*, it seems, have perfected a plan to induce nonphagocytic mucosal cells to perform this energy-requiring task and internalize these pathogens, when properly prompted. Before the phagocytic event is triggered, however, the microvilli or brush border is destroyed to expose the mucosal cell surface which possesses the flexibility required to initiate phagocytosis. As of the moment the chemical signals emitted by the shigellae to encourage phagocytosis by the mucosal cell are under investigation (Sansonetti, 1991).

Almost 40 years ago it was discovered that only those *Corynebacterium diphtheriae* strains infected with bacteriophage (bacterial virus) produced the potent exotoxin that made this pathogen infamous. In turn, it was reported, phages carried and contributed the genetic information that permits *S. pyogenes* and *Clostridium spp* to synthesize exotoxins. During this same period small circular pieces of extrachromosomal DNA, now called plasmids, were discovered in the cytoplasm of those shigellae which permitted them to express antibiotic resistance. When *Shigella spp* were examined for virulence plasmids, a large 220 kD plasmid was consistently found which seemed to associate with virulent strains. These plasmids, isolated from various *Shigella* species, are homologous and contain specific genomic regions which are essential for entry into epithelial cells. At least five independent genetic loci have been identified on these plasmids. This suggests that a rather complex strategy has evolved which permits the shigellae to entice mucosal cells to phagocytose and internalize these bacilli. Four plasmid-encoded invasin polypeptides (Ipa) have been detected, now termed IpaA, IpaB, IpaC, and IpaD. The Ipa polypeptides are thought to be "invasins," or virulence factors that orchestrate the entry of the *shigellae* into colonic mucosal cells. Current thought speculates that the dysentery bacilli respond to a microenvironmental colon cell stimulus (yet to be identified) emitted or present on the colon following which the *shigellae* express large quantities of plasmid-encoded Ipa invasins.

Stage II: Intracellular multiplication. Plasmid genes may also be required to permit multiplication of the *shigellae* within colon cells. These bacilli exhibit an intracellular generation time of approximately 40 minutes which is not influenced by the production and elaboration of Shiga toxin or Shiga-like toxins. Sequential electron microscopic studies have revealed that the phagosome membrane, which

encases the bacilli, lyses following ingestion and within 30 minutes following entry most bacilli are seen lying free within the cytoplasm of the infected cell. A correlation seems to exist between plasmid-driven intracellular multiplication and the capacity to lyse the membrane-bound phagocytic vesicle. Some investigators suggest that the molecular construction of the defoliated mucosal cell surface which now accommodates bacterial entry also promotes lysis of the phagosome releasing bacilli into the cytoplasm. The *shigellae* infect the epithelial cytoplasm and multiply therein, whereas the *salmonellae* employ epithelial cells as a transport escalator (remaining confined to the phagosome) to gain access to the lamina propria mucosa.

Stage III: Intracellular spread to adjacent mucosal cells. The capacity of the *shigellae* to spread laterally, from cell to cell in the mucosal lining appears to be crucial to the progress of the infection. Lateral invasion into neighboring mucosal cells, in a domino fashion, gives rise to a sizable ulcerative infection plaque. The invading bacilli may interact directly with microfilaments of the host cell to insure intracellular spread and infection of neighboring cells. A plasmid gene *icsA* (intra and intercellular spread), encodes for a 120 kD outer membrane protein, which has been implicated as integral to promoting intercellular infection. Interruption of *icsA* expression or function will abort intracellular multiplication and intercellular spread.

Stage IV: Killing of host epithelial cell. The presence of virulence plasmids also appears to be associated with the rapid killing of the infected host cells. Host cell injury and death seem to be correlated with the ability of the invading *shigellae* to rapidly dismantle the phagosome membrane which frees them into the cytoplasm. The metabolic events which accompany host cell death are: a rapid drop in intracellular concentration of ATP, increase in pyruvate levels and a halt in lactate production. It is important to point out that intracellular production of Shiga toxin does not seem to play a significant role in the killing process. Toxin-negative *Shigella dysenteriae* mutants can kill target cells with the same efficiency as the parental toxin producing strain.

It is recommended that the reader peruse an article entitled "Progress in Oral Rehydration Therapy (ORT)" (Hirschhorn and Greenough, 1991) which reviews progress made with this treatment approach to restore health to children suffering the consequences of severe diarrheal disease. The authors suggest that almost no adult or child would die of diarrheal disease if everyone knew how to prepare and deliver these recipes.

Summary

A multistage invasion and intracellular proliferation strategy has evolved in the *shigellae*, directed by virulence proteins encoded for by a large virulence plasmid. The bacilli upon making contact with the intestinal mucosa orchestrate their entry into epithelial cells by delivering transmembrane signals (Ipa polypeptides) that

solicit a phagocytic effort by these normally nonphagocytic cells. Once internalized in a membrane-encased vesicle the bacilli induce lysis of the membrane. Once free in the cytosol the *shigellae* proliferate, invade and infect neighboring cells following interaction with microfilaments. Host cells are killed, not by intracellular elaboration of Shiga toxin, but rather by a process that blocks mitochondrial respiration. The colonic enterocyte layer is killed and these cells slough along with white cells and red cells into the developing diarrheic stool.

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Chapter 9

Tuberculosis

PETER ZWADYK

INTRODUCTION

The genus *Mycobacterium* contains a unique group of human pathogens. These organisms are difficult to stain with the usual laboratory stains, such as the Gram stain, but once they are stained with special stains they resist decolorization with acidified alcohol and are referred to as acid fast bacilli or AFB. Included in this genus are *Mycobacterium tuberculosis*, the cause of the chronic pulmonary disease tuberculosis; *M. leprae,* the cause of leprosy; and approximately twelve other human pathogens, collectively referred to as mycobacteria other than tuberculosis (MOTT) or atypical mycobacteria, that can cause a tuberculosis-like pulmonary

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disease or infections in other parts of the body. In addition to the organisms associated with human disease there are a variety of other *Mycobacterium spp.* that can cause disease in animals or live in the environment as saprophytes. Because of the differences in epidemiology, pathogenesis and treatment of *M. tuberculosis* and the other *Mycobacterium spp.*, this chapter will deal with only *M. tuberculosis*.

Tuberculosis is a chronic pulmonary disease that can disseminate to other parts of the body, including the skeletal system. Human remains from Egyptian mummies and prehistoric times reveal characteristic lesions of tuberculosis. Historic descriptions of this disease appear as early as 1000 B.C. Throughout the world there are approximately 10 million new cases of tuberculosis reported each year, with nearly 3 million deaths a year attributed to this disease. In the United States, the number of cases has been on the decline from 84,000 cases in 1953 to 22,255 cases in 1984. This decline prompted the Advisory Committee for the Elimination of Tuberculosis to publish a plan to eliminate tuberculosis from the United States by the year 2010. However, from 1985 to 1991 the decline in tuberculosis abruptly ended and there have been 39,000 more cases reported than would have been predicted by the trends through 1984. This change prompted the development of a national plan to combat multidrug-resistant tuberculosis which was published in the June 19, 1992 issue of *Morbidity and Mortality Weekly Report*. The summary of this report reads, in part:

> At no time in recent history has tuberculosis (TB) been as great a concern as it is today. TB cases are on the increase, and the most serious part of the problem is the recent occurrence of outbreaks of multidrug-resistant (MDR) TB, which pose an urgent public health problem and require rapid intervention.

MICROBIOLOGY

M. tuberculosis is a rod shaped organism that ranges from 0.3 to 0.6 μm in width and 1 to 4 μm in length. The cell wall is complex and unlike the cell wall of either Gram-positive or Gram-negative organisms. Lipids account for 60% of the dry weight of the cell wall. These lipids and the unique structure of the cell wall account for the acid fast properties of the organism and allow the organism to resist a number of adverse environmental conditions, such as drying and exposure to acids and alkalis. The cell wall also contains a number of materials that have a variety of biological effects. Two of these factors are Wax D and cord factor (trehalose-6,6′-dimycolate). Wax D has immunoadjuvant activity that enhances antibody production against proteins incorporated into an oil-Wax D-water emulsion, while cord factor elicits pulmonary granulomas, activates the alternative complement pathway, is toxic for mice, and damages mitochondrial membranes. The cell wall may also be responsible in part for the slow growth of the organism by slowing the diffusion of nutrients.

Growth on solid media takes from 10 to 25 days incubation at 37°C before colonies become visible. Specialized media, such as the egg based Lowenstein-

Jensen medium or the various Middlebrook synthetic media, are used to grow mycobacteria. Increased CO_2 tension enhances growth.

CLINICAL INFECTION

M. tuberculosis is primarily a human pathogen and the source of the organism is persons with active disease. Of all the *Mycobacterium spp.*, only *M. tuberculosis* and *M. leprae* are communicable from human to human. *M. tuberculosis* is acquired by the inhalation of organisms contained in droplet nuclei ranging in size from 1 to 10 μm. Nuclei of this size are produced by coughing, sneezing, or talking, and remain suspended in air for extended periods of time and are capable of reaching the lung alveoli and initiating infection. Droplets larger than 10 μm either settle from the air or do not penetrate into the alveolar spaces of the lung. Once the organisms are lodged on the alveolar surface they are phagocytized by the alveolar macrophages where they multiply intracellularly. Shortly after the initial infection an inflammatory reaction occurs and the alveoli fill with fibrin, desquamated macrophages and a few polymorphonuclear leukocytes. From this initial site the organism spreads to the hilar and mediastinal lymph nodes. From here they may spread into the blood stream and disseminate to other parts of the body. Organisms are cleared from the blood stream by the reticuloendothelial system, but multiplication continues in the lungs, lymph nodes and other tissues. Symptoms at this time are relatively non-existent or mild and the patient seldom seeks medical attention.

After 3 to 4 weeks the macrophages become activated by the lymphokines released by T-helper cells, organism multiplication becomes curtailed and the patient develops a cellular immunity and tuberculin hypersensitivity. At the first exposure site a tubercle develops. This is an aggregation of "giant" cells which are enlarged macrophages that resemble epithelial cells, hence the name epithelioid cells. These cells are surrounded by a ring of fibroblasts, macrophages, and lymphocytes. The few organisms present are found within the epithelioid cells. The central core of epithelioid cells will frequently disintegrate and undergo "caseous" or cheese-like necrosis.

The direction the disease takes at this juncture depends on a number of host and organism factors. In the majority of patients with intact immune systems and who are infected with relatively low numbers of organisms, the process is interrupted and the caseous lesions dry by inspissation, and calcium is deposited over the years. These calcified lesions become known as *Ghon complex* and can be demonstrated on radiographs and at autopsy. In other patients these lesions may not completely calcify and may serve as the focus for reactivated disease that occurs later in life as the patient's health may be affected by other conditions, such as alcoholism, malignancies or diabetes. About 5 percent of the patients will develop the symptoms of typical tuberculosis in less than 2 years after the primary infection, while in other individuals 20 or more years may pass before the development of reactivation disease. For other patients, however, the disease can progress rapidly from the

primary infection to active tuberculosis. For dark-skinned races, individuals exposed to a large concentration of organisms and persons with immunosuppresive disease, such as HIV infection, progression to active disease occurs at a more rapid rate.

Active tuberculosis is characterized by the formation of lung cavities that develop as a result of the caseous necrosis. If the necrosis erodes through a bronchus, the organism can spread throughout other areas of the lung. Contiguous spread to the pleural space and the pericardium can occur and pleurisy and pleural effusions are not uncommon findings in tuberculosis. Hematogenous spread occurs when necrosis develops within the lymphatics or when large numbers of organisms reach the bloodstream leading to the development of miliary tuberculosis, which is characterized by the formation of tubercles and calcified lesions throughout the body. Meningitis can also occur in disseminated disease and is more common in children with disseminated disease than in adults. Hematogenous spread can lead to the formation of tubercles in the kidney and the development of renal tuberculosis.

In patients with AIDS the disease rapidly progresses and frequently disseminates in the majority of patients. In these patients the histological lesions lack the characteristic granuloma, but rather have high concentrations of organisms, a predominance of polymorphonuclear leukocytes and relatively few epithelioid cells, lymphocytes, or macrophages. Survival of HIV infected individuals is usually limited to a few months after diagnosis.

As with primary disease, patients with active tuberculosis may display no symptoms until the disease is quite advanced. The primary symptoms depend on the organ system involved; however, the constitutional symptoms most frequently associated with tuberculosis are malaise, easy fatigue, anorexia, unexplained weight loss, fever, and drenching night sweats. Cough and sputum production varies with the degree of lung involvement, but in the advanced stages of cavitary disease there is a chronic cough with production of mucopurulent sputum that may contain blood. Radiographic findings include three primary characteristics: confluent or cavitary disease in the apical or posterior segments of the upper lobes; a homogeneous, confluent infiltrate or cavity; and evidence of bronchogenic spread. However, these symptoms are not diagnostic for tuberculosis and may be seen in fungal diseases or with various malignancies.

DIAGNOSIS

The diagnosis of tuberculosis involves clinical symptoms, skin testing, AFB stains, and isolation and identification of *M. tuberculosis* from clinical specimens.

The skin test is referred to as the tuberculin test and has its origin in the work of Robert Koch at the turn of the nineteenth century. The antigen used for the skin test is PPD (purified protein derivative) which is obtained by the ammonium sulfate precipitation of broth cultures of *M. tuberculosis*. The most precise method of

administering the test is the Mantoux test, in which a standard dose of 0.1 ml of 5 TU (Tuberculin Unit) of PPD is injected intracutaneously. After 48 to 72 h the area of enduration is measured. Reactions of less than 4 mm are considered negative, while reactions of 5 mm or greater require interpretation according to the chart shown in Figure 1. A positive skin test does not mean that the person has tuberculosis at the time of the test, nor does it imply that the patient is immune to the disease but does indicate an immunological exposure to the organism. In a patient with symptoms of tuberculosis and a normal immune system a positive test may be the first positive diagnostic result available to the clinician. Furthermore, if the PPD status of the patient has changed from negative to positive the diagnosis of

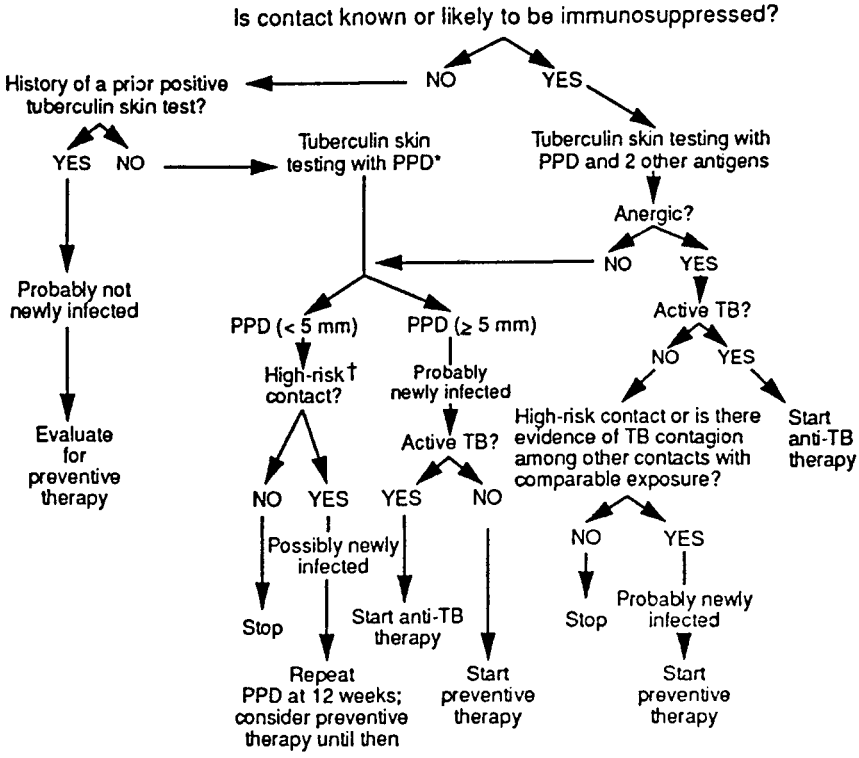

Figure 1. Guidelines for the interpretation of the tuberculin skin test.

Notes: *PPD = purified protein derivative.

†Members of the immediate family, close social contacts, or others who shared the same indoor environment with an infectious TB patient for substantial periods.

Source: From MMWR 41(RR-11), p. 39

tuberculosis is more likely and the patient can be placed on therapy to prevent the development of active disease.

For isolation and identification of mycobacteria in clinical specimens it is necessary to send multiple samples to the laboratory. Generally, three sputum samples collected on three separate days will detect 95 percent of the culture positive patients. Gastric aspirates are recommended in children and patients who have difficulty in expectorating. Urine, cerebral spinal fluid, and biopsy samples of lymph nodes and other body sites are also used for the diagnosis of extrapulmonary tuberculosis.

Specimens are processed in the laboratory with NaOH and N-acetyl-L-cysteine to digest the mucus and free the organism from its intracellular location and to kill other microorganisms that may be present in the specimens. After processing, AFB stains such as the Kinyoun's or auramine O stain, are performed on the processed specimens. The sensitivity of the AFB stain is less than 50 percent but the results are usually available in less than 24 h after collection of the specimen and a positive stain indicates a heavy organism burden and therefore a potentially more contagious patient. However, a positive AFB stain does not indicate that the organisms are alive nor does it indicate which species of mycobacteria is present.

The processed sample is also used to culture the organism. Since *M. tuberculosis* may take 2 to 8 weeks to grow on solid media, most modern laboratories are also using a broth culture system developed for the BACTEC 460 instrument. The media used with this system contain ^{14}C-labeled substrates which the mycobacteria metabolize to $^{14}CO_2$ that is detected by the BACTEC instrument. In the author's laboratory, the time to detect *M. tuberculosis* is shortened from 23 days to 11 days with the BACTEC system. The BACTEC 460 can be used to identify *M. tuberculosis* within 3 to 5 days after detection. Nucleic acid probes can also be used with the growth in the BACTEC vials to make a positive identification of *M. tuberculosis*. The BACTEC instrument can also be used for determining the antimicrobial susceptibility of *M. tuberculosis*. In the majority of situations, the final identification and susceptibility testing of *M. tuberculosis* can be available in 20 to 25 days with the BACTEC system, compared to the one to six months necessary for conventional tests.

TREATMENT

Prior to 1990 the majority of *M. tuberculosis* strains were sensitive to isoniazid (INH), rifampin, streptomycin, and ethambutol and the majority of resistant strains were seen in patients who did not complete an adequate therapeutic regimen or who came from areas of the world that had a high number of resistant strains. Therapy was usually INH and rifampin for 6 to 12 months. However, there has been an increase in the resistance of tuberculosis to these drugs. In 1991, 33 percent of the tuberculosis cases in New York City were caused by organisms resistant to at least one drug and 19 percent were resistant to both INH and rifampin. From 1990

Table 1. Regimen Options for the Treatment of Tuberculosis in Children and Adults

			Initial Treatment Phase			Continuation Treatment Phase		
Option	*Indication*	*Total Duration of Therapy*	*Drugs**	*Interval and Duration*		*Drugs**	*Interval and Duration*	*Comments*
1	Pulmonary and extrapulmonary TB in adults and children	6 mos	INH RIF PZA EMB or SM	Daily for 8 wks		INH RIF	Daily or two or three times wkly[†] for 16 wks	• EMB or SM should be continued until susceptibility to INH and RIF is demonstrated. • In areas where primary INH resistance is <4%, EMP or SM may not be necessary for patients with no individual risk factors for drug resistance.
2	Pulmonary and extrapulmonary TB in adults and children	6 mos	INH RIF PZA EMB or SM	Daily for 2 wks, then	Two times wkly[†] for 6 wks	INH RIF	Two times wkly[†] for 16 wks[§]	• Regimen should be directly observed. • After the initial phase, EMB or SM should be continued until susceptibility to INH and RIF is demonstrated, unless drug resistance is unlikely.
3	Pulmonary and extrapulmonary TB in adults and children	6 mos	INH RIF PZA EMB or SM	3 times wkly[†] for 6 mos[§]				• Regimen should be directly observed. • Continue all four drugs for 6 mos.[¶] • This regimen has been shown to be effective for INH-resistant TB.

Table 1. Continued

Option	Indication	Total Duration of Therapy	Initial Treatment Phase: Drugs*	Initial Treatment Phase: Interval and Duration	Continuation Treatment Phase: Drugs*	Continuation Treatment Phase: Interval and Duration	Comments
4	Smear- and culture-negative pulmonary TB in adults	4 mos	INH RIF PZA EMB or SM	Follow option 1, 2, or 3 for 8 wks	INH RIF PZA EMB or SM	Daily or two or three times wkly† for 8 wks	• Continue all four drugs for 4 mos. • If drug resistance is unlikely (primary INH resistance <4% and patient has no individual risk factors for drug resistance), EMB or SM may not be necessary and PZA may be discontinued after 2 mos.
5	Pulmonary and extrapulmonary TB in adults and children when PZA is contraindicated	9 mos	INR RIF EMB or SM**	Daily for 8 wks	INH RIF	Daily or two times wkly† for 24 wks§	• EMB or SM should be continued until susceptibility to INH and RIF is demonstrated. • In areas where primary INH resistance is <4%, EMB or SM may not be necessary for patients with no individual risk factors for drug resistance.

* EMB = ethambutol; INH = isoniazid; PZA = pyrazinamide; RIF = rifampin; SM = streptomycin.

†All regimens administered intermittently should be directly observed.

§For infants and children with miliary TB, bone and joint TB, or TB meningitis, treatment should last at least 12 months. For adults with these forms of extrapulmonary TB, response to therapy should be monitored closely. If response is slow or suboptimal, treatment may be prolonged on a case-by-case basis.

¶Some evidence suggests that SM may be discontinued after 4 months if the isolate is susceptible to all drugs.

**Avoid treating pregnant women with SM because of the risk for ototoxicity to the fetus.

Note: For all patients, if drug-susceptibility results show resistance to any of the first-line drugs, or if the patient remains symptomatic or smear- or culture-positive after 3 months, consult a TB medical expert.

Source: From Anon. (1994) MMWR 43(RR-13), 67.

through 1992 the CDC investigated nine outbreaks of MDR tuberculosis in hospitals and prisons in Florida and New York. In these outbreaks there was a high prevalence of HIV disease, a mortality rate of 72 to 89 percent, a short (4 to 16 weeks) time between diagnosis and death, and a high rate of transmission of MDR-tuberculosis to health care and correctional facility workers (17 cases). As a result of these outbreaks, the CDC and the American Thoracic Society published in 1993 the recommendations for initial tuberculosis therapy (Table 1). The therapy is divided into two main categories, treatment of patients with and without HIV infection. Because of the problems associated with certain patients taking their medication and the subsequent development of antimicrobial resistance, the recommendations strongly suggest that patients be given their medications in a directly observed therapy (DOT) situation. DOT not only ensures that the patients are taking adequate therapy but also lessens the development of drug resistant tuberculosis and reduces the number of treatment failures.

REMAINING ISSUES

The emergence of MDR tuberculosis and the dangers of transmission to health care workers have raised a number of issues concerning the rapid diagnosis of tuberculosis, the factors associated with TB transmission, and the therapy of MDR tuberculosis. Hospitals are being required to review their patient isolation procedures, the structure of their air handling units, and their employee protection methods. Rooms which house either confirmed or suspected cases of tuberculosis must have negative pressure, at least 6 to 8 changes of room air per hour, and the exhausted air must be filtered before being recirculated to other parts of the hospital or be totally exhausted to the outside. Employee health measures, such as yearly tuberculin skin testing and providing of adequate protective equipment, are being implemented and enforced by the majority of health care facilities. It will take time before all of these measures are in place for all health care facilities.

Another major factor in the transmission of tuberculosis is the general health of individuals and the conditions in which they live. Generally, tuberculosis is seen in impoverished environments. The increase in homeless persons and their crowding in inadequately ventilated shelters, the overcrowding in the intercities, the increased migration from foreign countries, and the increase in numbers of AIDS cases have contributed to the increase in number of tuberculosis cases in the United States. Historical evidence shows that these factors will need to be addressed before the current increase in tuberculosis cases can be stopped. The decline in number of tuberculosis deaths from 500/100,000 in 1900 to less than 50/100,000 in 1950 occurred prior to the development of effective antimicrobial therapy and was due largely to the changes in socioeconomic conditions.

Rapid diagnostic methods, such as DNA amplification by the polymerase chain reaction, are being developed with the goal of 24 to 48 h turn around time for mycobacterial specimens. However, these methods are neither completely reliable

nor readily available for all hospital laboratories. Until the time when these tests are available the control of tuberculosis will still depend on the awareness of the initial health care provider and the prompt institution of effective therapy and evaluation of patient contacts. And finally, there needs to be the development of newer drugs that can be used to treat the MDR tuberculosis.

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Chapter 10

Mycobacterium avium-intercellulare (MAI)

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INTRODUCTION

Mycobacterium avium and *M. intracellulare* are acid fast bacilli that are grouped together as the *Mycobacterium avium* complex (MAC). MAC are members of a larger group of mycobacteria currently referred to as "nontuberculous mycobacteria" (NTM) or "mycobateria other than tuberculosis" (MOTT). Other pathogenic mycobacteria included in this group are *M. kansasii, M. fortuitum, M. chelonae,*

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M. abscessus, M. xenopi, M. malmoense, M. terrae, M. simiae, M. gordonae, M. scrofulaceum, M. haemophilum and *M. marinum.*

M. avium, the avian tubercle bacillus, was first described as the causative agent of disease in chickens in 1890. Episodic cases of human disease due to *M. avium* were described in Europe and North America in the middle part of this century. Pulmonary disease due to MAC was first described in 1943 in a middle-aged miner from Minnesota who had a clinical picture of silicotuberculosis (Feldman et al., 1943). A series of patients with pulmonary disease caused by nontuberculous mycobacteria was reported from the Battey Hospital in Georgia in 1957 (Crowe et al., 1957). The organism isolated from these patients was first given the name "Battey bacillus" and later speciated as *M. intracellulare*. Bacteriologists found that the biological properties of *M. avium* and *M. intracellulare* were almost indistinguishable, so these organisms are now referred to collectively as *M. avium-intracellulare* (MAI) or *M. avium* complex (MAC).

MAC was originally placed in Runyon's group III as a slow-growing, nonchromogenic mycobacteria. Colonies may be white or yellow, smooth or rough, opaque or transparent, and flat or domed. Nonpigmented and translucent colony types may be more resistant to antimicrobials and more virulent than other types (Inderlied et al., 1993).

EPIDEMIOLOGY

MAC are environmental pathogens. Several epidemiologic studies have shown that MAC can be isolated from soil, dust, animals, and aerosols of both fresh and salt water. An epidemiologic study of Navy recruits using delayed hypersensitivity skin testing revealed a greater incidence of infections due to MAC in recruits from the southeastern United States than from other parts of the country (Edwards et al., 1969). However, since the AIDS epidemic, MAC infections have been documented in all 50 states.

Recently it has been suspected that there is an increase in the incidence of disease attributed to MAC in immunocompetent patients as well as in patients with AIDS. The best estimate of the incidence of MAC made prior to the AIDS epidemic was 1.3 cases/100,000 general population. In contrast, disseminated MAC infection occurs in 15–40 percent of HIV-infected patients in the USA. Disease caused by MAC has been reported with increasing frequency in recent years, and MAC is now isolated more frequently than *M. tuberculosis* in the mycobacteriology laboratory. The majority of this increase is a direct result of the AIDS epidemic.

MAC is composed of 28 serovars that are assigned to the two species. Types 1–6, 8–11, and 21 are *M. avium*, and types 7, 12–20, and 25 are *M. intracellulare*. Types 1, 4, 8, and 9 are the most common types of MAC causing infection in the United States (Wolinsky et al., 1992). MAC organisms are ubiquitous in nature. They are widely distributed and have been isolated from fresh and salt water, soil, chickens, pigs, dogs, cats, insects, and dairy products. The specific environmental sources

that contribute to infection and disease in humans are not clear, since isolates from local environmental sources have been shown to differ significantly from human isolates in the same geographic location. Both the respiratory and gastrointestinal tracts have been proposed as portals of entry for MAC. It appears that, at least in the HIV-infected patient population, the gastrointestinal tract is the most common site of colonization and source of dissemination (Havlik et al., 1993). Unlike infection with *M. tuberculosis*, person-to-person transmission has not been documented with MAC.

CLINICAL PRESENTATIONS

Pulmonary Infections

The majority of non-AIDS patients with pulmonary MAC disease have underlying lung disease or other predisposing factors. These factors include bronchiectasis, tobacco-induced chronic bronchitis, and emphysema, prior tuberculosis, prior chest irradiation therapy, inorganic dust exposure, and fibrotic disorders including idiopathic fibrosing alveolitis and rheumatoid arthritis. A series of patients from Philadelphia with pulmonary MAC disease but without apparent underlying disorders has been reported (Prince et al., 1989). At the National Jewish Medical and Research Center, an apparent increase in the incidence of thoracic skeletal anomalies, including ankylosing spondylitis, pectus excavatum, and scoliosis has been observed among patients with pulmonary MAC. Mitral valve prolapse has also been a common finding in this group of patients. It has been postulated that these anomalies may be markers for an underlying defect in mucociliary clearance that predisposes to mycobacterial colonization and infection. Other conditions associated with excessive mucus production (e.g., chronic bronchitis), abnormally thick and tenacious secretions (e.g., cystic fibrosis) or reduced mucus clearance (e.g., bronchiectasis) may also predispose to pulmonary infection by providing a favorable environment for bacterial proliferation, adherence to airway epithelium and invasion of lung tissue.

Local pulmonary cellular defenses such as alveolar macrophages are also being considered as a link to MAC infection. In the immunocompetent patient, MAC infection is primarily limited to the lung. In contrast, MAC usually presents as a disseminated infection in HIV-infected patients and rarely as a primary lung infection. It is likely that dissemination of MAC infection in immunocompromised patients is due to a defective cellular immune response network that compromises the ability of macrophages to control intracellular growth of mycobacteria. To extend this hypothesis to the non-AIDS patient with pulmonary MAC infection, one could question the existence of abnormalities in macrophage function that might increase the susceptibility of certain patients to infection. There is limited data to support this hypothesis.

Patients with advanced pulmonary MAC infection usually present with a prolonged history of cough, malaise, intermittent fevers, and weight loss. Patients with bronchiectasis and MAC infection may experience increased sputum production and hemoptysis. For the clinician to distinguish between MAC colonization and infection and between stable and progressive MAC pulmonary disease, a history and physical exam, chest radiographs and CT scans and serial sputum smears and cultures for mycobacteria are required.

Pulmonary MAC produces a wide variety of radiographic abnormalities. Many chest radiographs of patients with pulmonary MAC infection are similar to those seen with classic tuberculosis, showing unilateral or bilateral upper lobe infiltrates associated with cavitary lesions. (See Figure 1.) A subset of patients exhibit bilateral nodular disease, mainly in the mid to lower lung fields. This pattern is more common in women. At National Jewish, we have observed a series of female patients that presented with pulmonary MAC infections associated with coarse, saccular bronchiectasis of the right middle lobe and lingula. Although there was usually evidence of infection in other areas of the lung, disease was predominantly localized to these two regions. Most of these patients did not have upper lobe cavitary disease.

Sputum cultures are necessary to establish a diagnosis of pulmonary MAC infection. The patient should collect several sputa over a period of days to weeks to submit to the laboratory for AFB smear and culture examination. If the patient is unable to produce sputum specimens, bronchoscopy with or without bronchoalveolar lavage, transbronchial biopsy or open lung biopsy may be necessary to establish the diagnosis. The laboratory can provide semiquantitative data on the numbers of organisms seen on sputum smear as well as the numbers of colonies growing on culture medium from processed sputum specimens. Since MAC are slow-growing organisms, the laboratory may not see growth for one to two weeks after the specimen is plated. Most laboratories also use a combination of the BACTEC® radiometric culture system (Becton-Dickinson Diagnostic Instrument Systems, Sparks, CA.) and DNA probe analysis (e.g., Gen-Probe, San Diego, CA) for rapid identification of these organisms (Ellner et al., 1988). This approach has reduced identification time from 14 to 21 days to 7 to 10 days. Development of the polymerase chain reaction (PCR) method for direct detection of MAC in clinical specimens is being investigated but has not progressed as rapidly as the PCR test for *M. tuberculosis* (Eisenstein et al., 1990).

Treatment of pulmonary MAC disease is difficult and controversial. At National Jewish we believe that a multi-drug antimicrobial regimen combined with aggressive pulmonary hygiene is the treatment of choice. Surgical resection of diseased portions of lung is also useful in selected patients if disease is primarily limited to a single lobe or if total destruction of one lung has occurred (Pomerantz et al., 1996). Surgery should not be performed until the medical regimen has been used for at least one to two months.

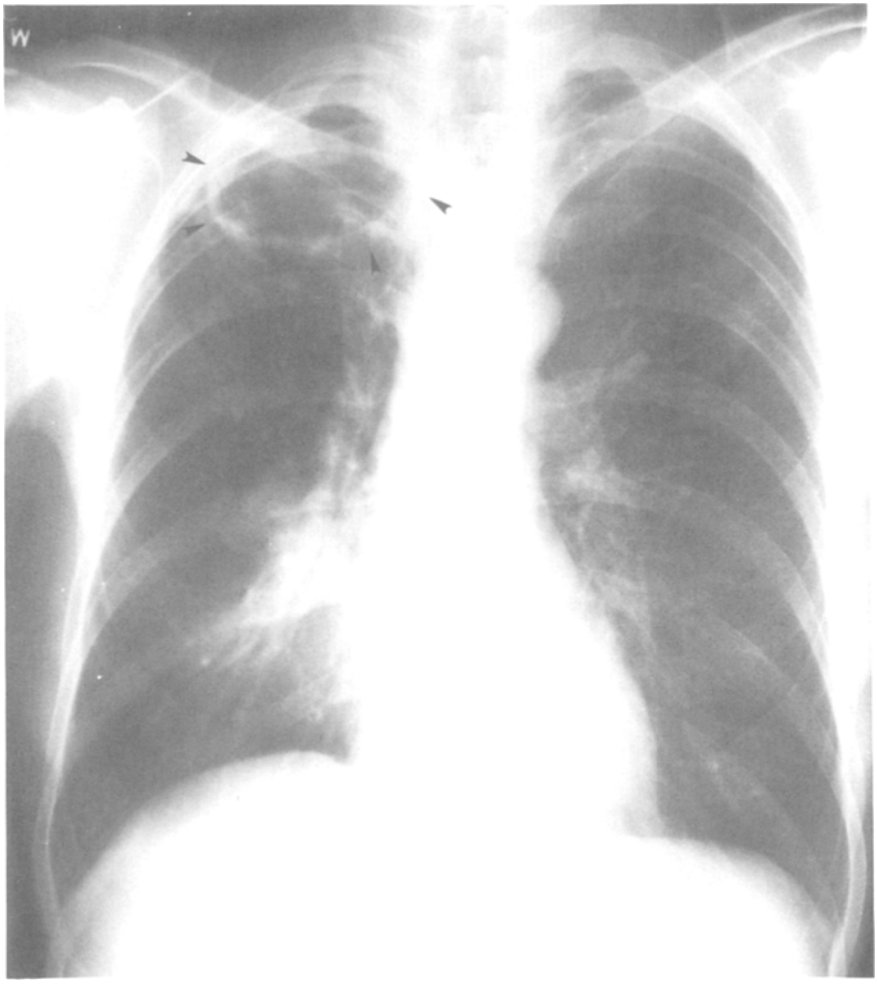

Figure 1. An example of a chest x-ray of a patient with MAC infection presenting similarly to a case of classic tuberculosis. This film demonstrates a large, right upper lung field cavitary lesion (the circular defect seen in the upper aspect of the x-ray of the patient facing the reader) and thickening of the apical pleura.

The role of drug susceptibility testing to define the antibiotic treatment regimen for patients with MAC infection is controversial. One study of a small number of patients showed a direct correlation between susceptibility testing results *in vitro* and response to treatment (Horsburgh et al., 1987). In 1985, the Committee on *M. intracellulare* Disease recommended that initial therapy for pulmonary MAC infection should include isoniazid, rifampin, and ethambutol, with an initial 2 to 4

month phase of streptomycin therapy (Iseman et al., 1985; ACCP Consensus Conference, 1985). These recommendations were made prior to development of the new semisynthetic macrolides such as clarithromycin and azithromycin. The current treatment approach at National Jewish for the patient with pulmonary MAC infection includes the use of 3 to 5 oral antimicrobials (based on susceptibility testing data), usually including rifampin, ethambutol, clarithromycin and clofazamine. An aminoglycoside (e.g., streptomycin, kanamycin or amikacin) is often added to the regimen depending upon susceptibility testing results, the extent of the infection and the role and timing of surgery in the treatment plan. Treatment is prolonged (18–24 months) to attempt to reduce the incidence of clinical relapse and requires frequent monitoring and communication between the patient and physician to detect and manage medication-related side-effects.

Extrapulmonary MAC Disease

Prior to the AIDS epidemic, extrapulmonary MAC disease was very uncommon. However, with the appearance of AIDS, the incidence of disseminated MAC disease has risen dramatically. For the sake of clarity, discussion of these problems is divided into two parts—the first on localized, single-site extrapulmonary infection, the second on multifocal or disseminated disease.

Localized Nonpulmonary MAC Disease

Localized, nonpulmonary MAC disease is usually manifested as infection at sites of direct inoculation into soft tissue or bone or as cervical lymphadenitis that likely results from infection via the oropharynx. Patients with localized MAC infections are usually not immunocompromised, so a complicated immune system evaluation is not warranted. Management of these localized infections usually consists of surgical debridement and prolonged, multi-drug chemotherapy. As with pulmonary MAC, the choice of antimicrobials is based on culture results and susceptibility testing. An empiric combination of drugs to treat soft tissue MAC infections is rifampin, ethambutol, clarithromycin, and clofazamine. This regimen can be modified after drug susceptibility testing is completed. A parenteral aminoglycoside may be used during the initial phase of therapy depending upon the extent of the infection. Total duration of therapy is usually 18 to 24 months.

MAC infection causes cervical lymphadentits primarily in children aged 1 to 5 years (Schaad et al., 1979). It typically presents as a painless, unilateral, cervical-submandibular lymph node enlargement. Occasionally lymph nodes will suppurate and drain or ulcerate. Lymph node biopsy shows caseating or noncaseating granulomas. Acid fast bacilli will be detected on histopathologic study in about 50 percent of cases. Culture identification of the pathogen causing cervical lymphadenitis is important, since management of MAC infection is vastly different from that of *M. tuberculosis* infection. Surgical excision of the infected lymph node(s) is usually curative in children with MAC-induced lymphadenitis. Chemotherapy with

isoniazid and rifampin to cover for possible TB infection may be used while awaiting culture results. If the causative organism is identified as MAC and NOT *M. tuberculosis*, these medications can be discontinued. If the cultures confirm TB infection instead of MAC, a complete course of anti-TB therapy is indicated. There are rare cases of progressive disease in which MAC lymphadenitis requires antimicrobial therapy.

Disseminated or Multifocal Extrapulmonary MAC Disease

Patients with either multifocal or disseminated MAC (DMAC) disease have some type of immune deficiency. There have been reports of younger patients with multifocal osteomyelitis without other organ involvement or known predisposition to other infections (Horsburgh et al., 1985). These patients appear to have ill-defined abnormalities of cellular immunity, including failure of macrophages to limit intracellular replication of bacteria and absence of cytokine responses that induce macrophage antimicrobial function. There have also been reports of DMAC infection in patients with hairy-cell leukemia (Weinstein et al., 1978) as well as a possible X-linked deficiency of CD45R0 cells in a child with disseminated MAC infection who responded to interferon gamma therapy (Holland et al., 1994).

Most patients with DMAC infection are also infected with the human immunodeficiency virus (HIV). DMAC has been associated with more than 90% of opportunistic infections in AIDS patients in developed countries. In a prospective study of 1,006 HIV-infected patients, the incidence of MAC bacteremia was 21% at 1 year and 43% at 2 years after diagnosis of HIV infection. In this study, MAC bacteremia was associated with $CD4^+$ lymphocyte counts $<100/mm^3$; however, the majority of AIDS patients with DMAC have $CD4^+$ counts $<500/mm^3$ (Benson, 1994a).

Symptoms among HIV-infected adults with DMAC include fever, night sweats, diarrhea, and abdominal pain. A recent study reported a significant association between fever, weight loss, and diarrhea and the presence of DMAC infections in patients with AIDS. The physical findings most commonly reported included weight loss, intraabdominal lymphadenopathy (detected by abdominal imaging procedures) and hepatosplenomegaly (Havlik et al., 1992). Children with DMAC appear to have findings similar to those observed in adults. The laboratory abnormalities described most often in these patients are severe anemia (hematocrit <25%) and an elevated serum alkaline phosphatase.

Both the respiratory tract and gastrointestinal tract appear to be portals of entry and sources for dissemination of MAC in AIDS patients (Havlik et al., 1993). Diagnosis of DMAC is usually made by recovering organisms from specimens of blood (using lysis centrifugation techniques), bone marrow, or normally sterile tissues. Bacteremia may be intermittent. Therapy of DMAC in AIDS patients should be instituted as soon as a positive culture is obtained.

Treatment of DMAC in AIDS patients is important because it has been shown to reduce mycobacterial load, to alleviate symptoms and to prolong survival (Horsburgh et al., 1991). The initial therapeutic regimen should include at least two antimycobacterial drugs to prevent the emergence of drug resistance. If a patient is experiencing moderately severe symptoms, it is appropriate to initiate therapy with two to three oral drugs and intravenous amikacin. The oral drugs of choice include clarithromycin, azithromycin, rifampin, rifabutin, ethambutol, ciprofloxacin, and clofazamine (Benson, 1994b). Most clinicians currently choose to initiate therapy with one of the semisynthetic macroloide antibiotics, clarithromycin or azithromycin, and two other agents. A few weeks of therapy may be required before these patients experience symptomatic improvement. Since bacterial cure may not accompany clinical response and since relapse of infection after therapy is common, life-long suppressive therapy with at least two drugs is usually required for these patients.

Prophylaxis of DMAC is recommended in AIDS patients whose $CD4^+$ cell counts are $<100/mm^3$. Rifabutin is approved for prophylaxis against MAC infection. However, clarithromycin or azithromycin are currently preferred for this use because of reduced interactions with antiretroviral drugs such as the protease inhibitors.

SUMMARY

MAC infection has several clinical manifestations. This is one of the most common opportunistic pathogens in AIDS patients with $CD4^+$ cell counts less than 50 cells/mm^3. MAC also causes pulmonary, soft tissue, and bone or joint infections in immunocompetent patients. When repeatedly isolated from the sputum in a patient with a compatible clinical picture or when isolated from a normally sterile site, MAC should be considered as a pathogen and treated with a minimum of three antimycobacterial drugs. In certain cases of extensive, localized infection, surgery may be a necessary adjunct to medical therapy. Childhood lymphadenitis caused by MAC is usually cured by simple excision without a requirement for antimicrobial therapy. In contrast, MAC infection in AIDS patients is often disseminated and requires prolonged (if not life-long) therapy with multiple medications. Antibiotic prophylaxis may be useful for prevention of MAC infections in AIDS patients with low $CD4^+$ cells counts.

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Chapter 11

Hansen Disease

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INTRODUCTION

The first accurate description of Hansen disease appeared in about 600 B.C. in the writings of the Indian physician Sushruta, although references to diseases resembling it can be found in Indian and Chinese records from as early as 1400 B.C (Browne, 1975). Hansen disease probably entered the Western world in the third century B.C., spread slowly through the Mediterranean region and Europe, reached epidemic proportions in Western Europe in the 12th and 13th centuries, and then,

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gradually disappeared as living conditions and nutrition improved. Today, Hansen disease afflicts about 5.5 million persons worldwide, with most cases being found in tropical and subtropical regions—India, Africa, Southeast Asia, and Central and South America (Noordeen et al., 1992). In the United States, there are fewer than 200 new cases annually, and most are in the foreign-born population.

Hansen disease is a chronic, infectious disease that primarily affects the peripheral nervous system, skin, and mucous membranes of the upper respiratory tract, especially nasal mucosa (reviewed in Ridley, 1988; Bryceson and Pfaltzgraff, 1990). In advanced disease, the eyes, testes, muscle, liver, spleen, and bone marrow can also be affected. The involvement of the peripheral nervous system in this disease leads to the development of crippling deformities of the hands and feet in 20–30% of patients, while involvement of the eyes can lead to blindness. In fact, Hansen disease is the leading cause of crippling of the hand in the world today. The World Health Organization estimates that 2 to 3 million individuals have deformities due to Hansen disease, including many persons who have completed chemotherapy and are considered cured of the infection, such that rehabilitation and physical therapy are major components of Hansen disease treatment and control programs (Noordeen et al., 1992). A consequence of these deformities is the psychological and social stigma that has historically been associated with Hansen disease.

DIAGNOSIS

In general, Hansen disease is not a difficult disease for the clinician to diagnose. Diagnosis is usually based on clinical observation of the signs and symptoms of Hansen disease and a histologic examination of skin lesions for the presence of acid-fast bacilli and a characteristic inflammatory response. The hallmark signs of Hansen disease are: (1) skin lesions, (2) anesthesia within the lesions or in the distribution of a large peripheral nerve, and (3) enlarged peripheral nerves. The individual clinical manifestations of Hansen disease can resemble those of other diseases of the skin and nerves including cutaneous leishmaniasis, fungal infections (e.g., tinea versicolor), lupus erythematosus, lupus vulgaris, and sarcoidosis. However, the combination of a chronic skin disease and peripheral nerve involvement should always lead to the consideration of Hansen disease.

Besides the histologic examination of biopsy specimens, there currently are no reliable, rapid laboratory diagnostic tests for Hansen disease. Routine hematologic tests are of little diagnostic value, and many Hansen disease patients have false-positive serologic tests for syphilis or autoantibodies. Tests to detect characteristic antigens (phenolic glycolipid I or PGLI) or antibodies (anti-PGLI) can help confirm the suspicion of this disease, but these tests are not sufficiently specific or sensitive for definitive diagnosis nor are they commercially available. The lepromin skin test (the Mitsuda reaction) is useful for classifying Hansen disease patients but is of

little diagnostic value because many uninfected persons produce positive lepromin reactions.

ETIOLOGIC AGENT

It is generally accepted that Hansen disease results from an infection with *Mycobacterium leprae*, although this has not been rigorously proven because *M. leprae* has not yet been cultivated *in vitro* despite the fact that it was the first pathogenic bacterium to be associated with a particular disease. It was in 1873 that Armauer Hansen first observed rod-shaped bacteria in tissue biopsy specimens, and in 1874 he proposed that they were the cause of the disease that now bears his name (Hansen, 1874).

M. leprae bacteria are Gram-positive, acid-fast rods ranging from 1 to 8 μm in length and 0.2 to 0.5 μm in width (reviewed in Shinnick, 1991). (Acid-fastness refers to the ability to retain the color of certain dyes, usually carbol fuchsin, following treatment with mild acid.) Several properties of the bacterium may contribute to features of the disease: (1) *M. leprae* grows very slowly (generation time of about 12 days), which may influence the length of time from infection to the appearance of clinical symptoms or the chronic nature of the infection, (2) *M. leprae* grows best at 30°C, which may play a role in its affinity for cooler parts of the body such as skin and the paucity of skin lesions on the warmer parts of the body, (3) *M. leprae* is an obligate intracellular pathogen which replicates primarily in macrophages and Schwann cells, which may play roles in persistence, immune responsiveness, and tissue damage, (4) *M. leprae* is surrounded by a layer of mycolic acids and phenolic glycolipids, which may help it resist the bactericidal activities of macrophages, and (5) *M. leprae* is the only bacterial pathogen capable of entering peripheral nerves, which may play a role in the loss of nerve function and generation of disabilities.

Humans are the primary host and reservoir for *M. leprae*, even though naturally acquired *M. leprae* infections have been observed in armadillos, chimpanzees, and Mangabey monkeys (Walsh et al., 1981). The mode of transmission of *M. leprae* from an infected person to a susceptible one remains unknown although it appears that infection occurs predominantly by the respiratory route. The primary portal for exit appears to be nasal secretions which in some patients can contain as many as 10^8 bacteria per ml (Davey and Rees, 1974). Other routes of transmission such as skin-to-skin contact may be responsible for some cases, and a few cases have resulted from accidental inoculation of susceptible individuals by needleprick or by tattooing.

THE CLINICAL SPECTRUM

Most persons who are infected with *M. leprae* recover naturally. Only a few develop disease, and symptoms usually appear two to four years after infection, but

incubation periods as short as three months or as long as forty years have been observed. The earliest lesion usually is an ill-defined, slightly hypopigmented macule with or without associated sensory loss. This form of the disease, called indeterminant leprosy, may heal spontaneously (50–75% of patients) or may remain indeterminant or may progress to one of the common clinical forms of the disease.

The established types of Hansen disease form a continuous spectrum which can range from a single lesion with no detectable bacteria to multiple lesions containing large numbers of bacteria, up to 5×10^9 organisms per gram of tissue. The five-stage Ridley-Jopling classification system (Ridley and Jopling, 1966) places a patient in this spectrum based on clinical and histopathologic findings and also provides information on the infectiveness of the patient, long-term prognosis, and the types and likelihood of complications. The polar forms of the disease (tuberculoid, lepromatous) are clinically stable while the intermediate forms (borderline tuberculoid, borderline, borderline lepromatous) are relatively unstable and may progress toward either polar form. Table 1 summarizes some of the clinical and histological characteristics of the polar forms of Hansen disease (also reviewed in Kaplan and Cohn, 1986; Ridley, 1988; Sieling and Modlin, 1992).

Tuberculoid (abbreviated TT) patients have one or a few skin lesions with well-defined, raised, erythematous margins. The lesions are usually hypopigmented in persons with dark skins and copper colored in persons with light skins. The lesion is anesthetic, and solitary peripheral nerves are commonly enlarged. Histologically, the lesion is a well-ordered granuloma containing epithelioid cells, multinucleated giant cells, and large numbers of lymphocytes. There are very few, if any, acid-fast bacilli present. The lepromin skin test produces a positive Mitsuda reaction in TT patients.

Borderline tuberculoid (BT) lesions resemble tuberculoid lesions, but are smaller and more numerous with less-well defined edges. The lesions contain fewer epithelioid cells and multinucleated giant cells, and a few acid-fast bacilli are usually present. BT patients have positive Mitsuda reactions.

Borderline (BB) patients have numerous lesions of various sizes. The lesions contain immature epithelioid cells, macrophages, and T-cells, but no multinucleated giant cells. Many bacilli are present. The Mitsuda reaction is variably positive in BB patients.

Borderline lepromatous (BL) patients have large numbers of papules, plaques, or nodules. The lesions contain numerous acid-fast bacilli and undifferentiated macrophages, but no epithelioid or multinucleated giant cells. A few bacilli are also found in nasal secretions and these patients are considered infectious. The Mitsuda reaction is negative in BL patients.

Lepromatous (LL) patients have extensive cutaneous involvement with numerous, small, bilaterally symmetrical, erythematous macules, papules, or nodules. Sensory loss is symmetrical and often affects the extremities. The

Table 1. Clinical, Histologic, and Immunologic Spectra of Hansen Disease

	Ridley-Jopling Classification	
Feature	*Tuberculoid*	*Lepromatous*
Number of lesions	few	many
Histology		
bacteria	–/+	+++
macrophages	few	many
lymphocytes	many	few
epithelioid cells	many	none
Nerve Involvement	+	+
Antibody Response	+/–	+++
Cellular Immune Response		
Mitsuda Reaction	+++	—
$CD4^+/CD8^+$ ratio	1.9:1	0.6:1
$CD4^+$	+	+/–
Cytotoxic $CD8^+$	+	—
Suppressor $CD8^+$	—	+
TH1 subset (IFN, IL-2)	+	+/–
TH2 subset (IL-4, IL-10)	+	+++

lesions contain large numbers of foamy macrophages and acid-fast bacilli. Bacillemia is common. Because nasal secretions from LL patients contain large numbers of bacilli, these patients are considered the most infectious. The Mitsuda reaction is negative in LL patients.

REACTIONAL STATES

About half of Hansen disease patients will experience clinically evident inflammatory reactions, which are responsible for much of the tissue damage associated with the disease. The two general classes of these reactions are reversal reactions and erythema nodosum leprosum.

Reversal reactions can complicate all three borderline categories. Existing lesions develop erythema and swelling, new lesions may appear, and there is a shift towards tuberculoid histology (a so-called upgrading reaction). Major symptoms include fever and edema and erythema of the lesions which may lead to ulceration. An associated neuritis is common and often involves swelling of the infected nerves

and loss of motor function. Reversal reactions are histologically similar to delayed-type hypersensitivity reactions.

Erythema nodosum leprosum (ENL) occurs in lepromatous and borderline lepromatous patients, most frequently in the first two years of treatment. Major symptoms include tender inflamed nodules, fever, and lymphadenopathy. Frequently, ENL is associated with polyarthralgia and painful neuritis that can result in loss of peripheral nerve function. Histologically, ENL is characterized by polymorphonuclear cell infiltration and deposits of IgG and complement, and hence it resembles an Arthus reaction.

THE IMMUNOLOGIC SPECTRUM

Interestingly, the clinical and histologic spectrum is not related to the genetics of the bacterium, but rather to the immune responsiveness of the host. Histocompatibility alleles appear to play a role in determining what type of disease develops, but not in susceptibility to infection. Also, the distribution of patients along the spectrum varies in different populations. For example, lepromatous cases are more common in Caucasians (30–50% of all cases) than in Africans (<5% of all cases) (Fine, 1982).

Tuberculoid disease is characterized by a strong cellular immune response to the *M. leprae* bacilli, and the tuberculoid lesions contain a predominance of $CD4^+$ T-cells of the T-helper-1 (Th1) subset (Table 1). The Th1 subset is defined by the production of cytokines thought to be involved in generating protective immunity against intracellular pathogens. These cytokines include interferon-gamma, interleukin-2 (IL-2), and GM-CSF (granulocyte-macrophage colony stimulating factor). In tuberculoid leprosy, there is only a weak antibody reponse to *M. leprae* antigens. In contrast, lepromatous disease is characterized by a lack of a T-cell response to *M. leprae* antigens and a strong antibody response to *M. leprae* antigens. The lepromatous lesions contain numerous $CD8^+$ suppressor T-cells which produce IL-4, IL-5, and IL-10. These cytokines are thought to down-regulate the protective $CD4^+$ T-cell response, and *in vitro*, this suppressor activity can be abrogated by the addition of neutralizing antibodies to IL-4. Incidentally, this immunosuppression is quite specific for *M. leprae* in that the patients are not more susceptible to other infections or to neoplasia.

Reactional states result from changes in the immune response to the *M. leprae* bacilli. In reversal reactions, there is an influx of $CD4^+$ and cytotoxic $CD8^+$ T-cells into the BT, BB, or BL lesions, an augmented cell-mediated immune response to *M. leprae* antigens, and killing of the bacilli. The cytokine profile of the lesion also moves toward a Th1 response with production of large amounts of interferon-gamma. Since the reversal reaction is quite similar to a delayed-type hypersensitivity reaction, it is usually treated with corticosteroid therapy.

Immunologically, erythema nodosum leprosum resembles an Arthus reaction with evidence of T-cell involvement. Here, there also is an influx of $CD4^+$ T-cells,

but the cytokine profile is more reminiscent of a Th2 response than a Th1 response in that large amounts of IL-4 are produced which biases the response towards antibody production. Tissue damage appears to be mediated by local immune complex deposition and complement fixation. Another important feature of ENL is that large amounts of TNFα are produced, which may play a role in the immunopathology of this reaction as well as the accompanying fever. Interestingly, the administration of a drug that specifically down-regulates TNFα production (i.e., thalidomide) alleviates the ENL symptoms (Sampiao et al., 1992).

TREATMENT

Effective chemotherapy can halt the progression of the disease and can rapidly render the patient noninfectious. The modern era of chemotherapy began in 1943 with the introduction of sulfones, and dapsone was the mainstay of treatment for the next 4 decades. However, by the early 1980s, an alarming number of cases resistant to dapsone therapy had been reported. To combat the emergence of the dapsone-resistant organisms, patients are now treated with combinations of two or three drugs (WHO, 1988). For patients who have few lesions and few bacteria per lesion (paucibacillary cases, usually TT and BT patients), the World Health Organization recommends a six month treatment regimen including dapsone and rifampin. For patients who have many lesions or many bacteria per lesion (i.e., multibacillary cases, usually BL and LL patients), a two year treatment regimen including dapsone, rifampin, and clofazimine is recommended.

There is no commercially available, effective vaccine against Hansen disease.

SUMMARY

Hansen disease is still a significant public health problem in many parts of the world, and as the mobility of the world's population increases, the likelihood that a U.S. physician will encounter a case of Hansen disease will increase. Despite the broad spectrum of immunologic, histologic, and clinical manifestations of this disease, a diagnosis of Hansen disease should be considered whenever a combination of skin lesions and sensory loss occur. Effective chemotherapeutic regimens with two or three drugs rapidly render the patient noninfectious, and help prevent the disabilities and crippling deformities associated with this disease.

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Chapter 12

Antimicrobial Therapy

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INTRODUCTION

The modern era of antimicrobial chemotherapy began with the discovery of the sulfonamides during the 1930s. This work was followed by the discoveries of streptomycin, para-aminosalicylic acid (both drugs are used for tuberculosis), penicillin, and a host of other agents. The term "antibiotic" was once reserved for agents derived from natural sources, such as penicillin. "Antibiotics" were distinguished from the chemically synthesized antibacterial agents, such as the sulfonamides. The proliferation of semi-synthetic antibiotic derivatives, however, has blurred this distinction. Therefore, for practical purposes, the terms "antibiotic," "antibacterial," and "antimicrobial agent" are frequently used interchangeably. The term antimicrobial agent is broader in scope, and includes agents that are devoid of antibacterial activity, but are active against some other microscopic organism, such as fungi or viruses.

GENERAL PRINCIPLES

The proper use of antimicrobial agents rests on several principles. First, there is no substitute for an accurate diagnosis. Sometimes, the diagnosis of an infectious process is very specific to a single causative organism, or "pathognomonic." More often, the patient's constellation of signs and symptoms is suggestive of several possible diagnoses, with each having several potential etiologies. Therefore, a careful medical history must be obtained from the patient. The history should include the onset of the symptoms, the pattern of the symptoms, any recent travel, any recent drug or antibiotic use, any recent hospitalizations, and whether such a problem has occurred previously. The drug history may suggest non-infectious etiologies, such as drug fever, or may suggest the emergence of resistant bacteria. Such information is critical to properly identifying the patient's problem and designing an effective treatment plan.

Second, samples from the infected site(s) and contiguous sites (such as blood or urine) should be obtained, as indicated. Whenever possible, the samples should be collected before the start of antimicrobial therapy. Samples collected after therapy has begun may not yield positive smears or cultures, despite the fact the organisms still reside in the patient. Sometimes, repeat culturing of the infected site is required to make the diagnosis. Once samples are obtained, orders to the clinical laboratory must be specific for each test to be performed or type of organism to be sought. For example, a sputum sample from a patient with a possible "pneumonia" may need to be examined for the presence of bacteria, mycobacteria, fungi, protozoa, and viruses. Since multiple stains, cultures, or other tests may be required to detect these diverse organisms, the requisition for the clinical laboratory should be as specific as possible. Otherwise, the default test, such as a Gram's stain and a bacterial culture, may be performed to the exclusion of the other tests. Sufficient material must be provided to the laboratory in order to complete the battery of tests

requested. Otherwise, some priority must be stated so that the most likely organisms are the ones pursued first by the clinical laboratory.

Preventive Therapy

The use of antibiotics prior to infection is called "preventive therapy," "prophylaxis" or "chemoprophylaxis." One example is the use of antibiotics just before and immediately after surgical procedures on uninfected tissues. The goal is to prevent infection should any bacterial contamination of the wound occur during the procedure. Similarly, the use of antibiotics before and after dental procedures in patients with valvular heart disease is another example. Knowing that bacteria are present in the oral cavity, and knowing that most dental procedures can produce transient bacteremia, antibiotics are given to inhibit or kill the bacteria before they can invade the damaged heart valve. In other cases, "preventive therapy" is used after contamination or infection has occurred, but before active disease has been established. This type of therapy actually represents empiric treatment of an evolving infectious process. Examples include the use of antibiotics soon after animal bites or other traumatic injuries, and the use of isoniazid in patients who are skin test positive for infection with *Mycobacterium tuberculosis* but who do not have active disease. In these situations, it is hoped that early intervention will prevent the more serious consequences of active disease.

Empiric Therapy

Once disease is present, as evidenced by signs and symptoms such as fever, pain, cough, and so on, antibiotic therapy should begin promptly. Sometimes, it is not possible to immediately collect samples from the site of infection (such as a deep-seated abscess), or sometimes the patient may present while on antimicrobial therapy. Therefore, a definitive microbiological diagnosis may not be possible, or at best it may be delayed. The need to collect "clean" (antibiotic-free) samples must be weighed against the risk to the patient from delayed antimicrobial therapy. This is a clinical judgment that must be individualized.

Because some patients will require treatment before a complete diagnosis is established, the clinician must develop some best guesses as to the cause of the problem. Once these differential diagnoses are established, empiric therapy may be directed against the most likely cause (or causes) of the apparent infection. Empiric therapy is greatly facilitated by current statistics from the clinical microbiology laboratory regarding the organisms isolated most frequently at a given center, and their susceptibility patterns. The data are most useful if classified by hospital unit (intensive care unit versus general surgery ward, etc.) and source (blood, sputum, urine, etc.). The susceptibility data for a given organism should be displayed for several antimicrobial agents. The hospital's antibiotic susceptibility patterns can be updated annually, or more often if appropriate. With this knowledge, the clinician can tailor therapy for specific situations. For example, a burn unit may have a high

rate of methicillin-resistant *Staphylococcus aureus*, whereas a dermatology outpatient clinic may not. Knowing where the patient likely acquired the infection can profoundly affect the selection of the correct antimicrobial agent.

Clinical Microbiology

The clinical microbiologists can greatly assist the clinician in directing antimicrobial therapy. First, they can narrow the spectrum of potential pathogens by staining and culturing the biological samples obtained from the patient. Identification of the infecting organism should generally include the genus and species, and in some cases, the subspecies. As more sophisticated methods of speciation are developed, the taxonomy of microorganisms changes over time. Therefore, unfamiliar organism names may represent new classifications of "old friends." It is incumbent upon the clinician to obtain more information from the laboratory if he or she is not familiar with the isolated species.

Susceptibility testing is undertaken to provide the clinician with additional information about potentially useful drugs with which to treat the patient. One older method used for bacteria is the "Kirby-Bauer" disk diffusion method (reviewed by Dudley, 1992). The patient's infecting organism is first isolated into a pure culture. Next, a standard suspension of the organisms is made, and this is spread evenly across the surface of an agar plate. Several antibiotic-impregnated paper disks are placed on this "lawn" of the patient's infecting organism, and incubated for about a day. The larger the zone of clearing (the area without bacterial growth) around the disk, the more susceptible the organism is to that drug. Thus, the test gives a qualitative result ("susceptible" or "resistant") or at best, a semi-quantitative result.

Quantitative tests include the agar and broth dilution methods. The most common method for bacteria is the broth microdilution test. The plates used in this test are now mass-produced and sold to clinical microbiology laboratories. Each plate contains perhaps three dilutions each of several different antibiotics, all at standardized volumes. Each antibiotic concentration is placed in a small well on a pre-formed plastic tray. The clinical microbiologist inoculates each well with a standardized volume of a specific dilution of a pure-cultured organism. If the number of organisms delivered into the wells is too large, the antibiotics may appear to be less active, producing the so-called inoculum effect. The plates are incubated for 18 to 24 hours, and the endpoint is the presence or absence of turbidity in each well. Turbid wells indicate that the organism is not susceptible to that concentration of the antibiotic, whereas clear wells indicate growth inhibition or death of the organisms. The clear wells can be sub-cultured onto agar plates for an additional 24 hours to determine if any organisms survived a given drug dilution. The minimal inhibitory concentration (MIC) is the lowest concentration of a given antibiotic that inhibits the growth of the organism. The minimal bactericidal concentration (MBC) is the lowest concentration of the antibiotic that kills 99.9% of the viable organisms.

When an antibiotic is tested against large numbers of several different species of microorganisms, its activity is often described in terms of an MIC_{50} and an MIC_{90}. These are the concentrations of the drug that inhibited 50% and 90%, respectively, of each species tested. Many antibiotics show greater activity against either Gram-positive or Gram-negative organisms, while a few are considered "broad-spectrum," inhibiting a wide array of aerobic and even some anaerobic organisms. Some drugs are only active against fungi or other organisms. If the MIC_{90} of a drug is well below the concentration that could be delivered safely to the site of infection in a patient, then the antibiotic may be tested in animals and, eventually, in human clinical trials.

The clinician must be aware of the limitations of *in vitro* susceptibility testing. First, these tests often work better against rapidly growing, "typical" organisms than against slower growing isolates, certain drug resistant forms, mucoid strains, fastidious species, or anaerobes. It is possible to get false "susceptible" or false "resistant" results. Unexpected results should prompt the clinician to visit the laboratory to see the isolate and to discuss any unusual aspects of it with the clinical microbiologists. They may be able to recommend other methods of testing that may provide more accurate results. Sometimes, the isolate will need to be sent to a specialized reference laboratory for additional testing.

Second, *in vitro* results only suggest which drugs might be useful. Additional factors, including the site of infection, any prior history of treatment, the pharmacokinetic profile of the drug, and the patient's history of drug intolerance all must be considered before the first dose of an antibiotic is given. Additional factors to be included are the presence of hepatic or renal dysfunction in the patient, pregnancy, lactation, and the willingness of the patient to adhere to the prescribed regimen. Only when all of these factors are considered can the "right" prescription be written.

Combination Therapy

Whenever possible, a single antibiotic should be chosen that best meets the patient's needs. This approach limits the patient's exposure to potential adverse reactions, and limits potential opportunities for the development of antibiotic resistance. For selected organisms, combination therapy appears to be more effective (*Pseudomonas aeruginosa*) or has been shown to be essential for cure (*M. tuberculosis*). In the latter case, monotherapy in the face of active tuberculosis disease virtually guarantees the development of antibiotic resistance. In other cases, such as traumatic wounds to the abdomen, the spectrum of potential pathogens is too broad for most antibiotics to cover. In specific circumstances, two or even three drugs may be necessary to cover for Gram-positive, Gram-negative, atypical, and anaerobic organisms until patient-specific culture and susceptibility data are available.

When more than one antibiotic is used, the potential exists for multiple drug interactions (detailed by Moellering, 1995). On the whole body level, there may be

pharmacokinetic interactions, such as binding in the gut and decreased drug absorption, or competition for excretory pathways with increased serum concentrations. On the microbiological level, two or more drugs may be tested together against an organism to determine whether or not they work well together. The drugs may act synergistically, in an additive fashion, or they may antagonize each other's effects. Synergy may be defined as a four-fold reduction (two doubling dilutions) of the MIC of each antibiotic in combination, relative to the MIC for each single agent. Such combinations may result in more rapid killing of the organism in the patient, and would therefore be desirable. A four-fold increase in each MIC is considered two-way antagonism, an undesirable interaction. Other interactions, such as between "bacteriostatic" agents like tetracycline or chloramphenicol and "bactericidal" agents such as penicillin or gentamicin, may result in decreased killing by the "cidal" agents. Therefore, combinations that may reduce the efficacy of one or more of the antibiotics should be avoided whenever possible.

Animal Models

Antibiotics are generally tested in animal models, such as the mouse model, prior to testing in humans. These studies provide data regarding the pharmacokinetics, toxicity, and efficacy of an antibiotic under specific conditions. The doses used, particularly in the acute toxicity studies, are many-fold higher than the doses that ultimately will be used in humans. Therefore, toxicities seen under these conditions may or may not ever be seen in humans. Also, a given animal model (such as the rabbit endocarditis model) can only approximate the human condition. While it may be instructive to review animal model data, it is important to realize that animals typically display altered pharmacokinetic profiles relative to man, which in turn may alter the dose-response relationships. The "scale-up" from animals to humans is imprecise at best. Therefore, animal model data forms an important but insufficient basis for making therapeutic decisions in humans.

THE USE OF ANTIBIOTICS IN HUMANS

Human Investigations

The testing of antibiotics in humans is divided into four phases. Phase I studies involve the early pharmacokinetic and safety studies in healthy volunteers. Exceptions occur when the drug is in very short supply, or when the potential toxicities preclude giving the drug to healthy volunteers. In such cases, the Phase I data will be collected simultaneously with the Phase II clinical data. Phase II studies typically are open-label trials of the drug in patients who have the target disease state. Different sizes and frequencies of dosing may be tried in an effort to find an acceptable balance between efficacy and toxicity. The most effective doses are then used in the Phase III trials, which most often are double-blind, randomized trials

comparing the new drug to the "gold standard" therapy for the disease. Finally, Phase IV studies are post-marketing surveillance studies that look for specific toxicities or potential new uses that could not be readily studied in the earlier phases. Sometimes, as with certain anti-HIV (human immunodeficiency virus) drugs, conditional approval is granted by the Food and Drug Administration (FDA) pending further outcome studies. This allows a potentially useful drug to be used by a broader group of patients prior to defining its ultimate place in therapy. If the drug proves to be effective for a life-threatening disease, more patients can be helped at an earlier date. The risk lies in giving a potentially useless drug to patients, who may suffer toxicities while deriving no benefit. Thus, "fast-tracking" the approval of new drugs remains a controversial approach to the introduction of new therapies.

Pharmacokinetics

The pharmacokinetic profile of the agent, primarily determined in Phases I and II, plays a significant role in determining an antibiotic's place in therapy. First, the drug must have physiochemical properties that lend themselves to the creation of effective dosage forms. Some drugs are too acid labile to be given by mouth. In such cases, pro-drugs that combine a stable chemical group to the labile drug, or special dosage forms that display pH-dependent drug release, may be used. For the acutely ill patient, particularly if he or she presents with nausea and vomiting, the intravenous route of administration is preferred. Once the patient has responded to treatment, consideration can be given to completing the course of therapy with a less expensive oral dosage form.

Some drugs are too hydrophobic to be given intravenously or intramuscularly, at least without special solubilizing agents. This property also will dictate where in the body the drugs may go. Drugs that are ionized at pH 7.4 are not likely to pass the "blood-brain" barrier, or the blood-cerebrospinal fluid (CSF) barrier. Such agents may be of little utility in the treatment of meningitis, unless they can be injected directly into the CSF. Devices such as Rickham and Omaya reservoirs are designed to deliver drugs directly into the ventricles, allowing diffusion of the drug wherever the CSF flows. Clearly, the site of infection must always be considered when selecting an antibiotic.

A related topic is that of "tissue penetration." Because of their differing physiochemical properties, antibiotics may penetrate more or less well into specific fluids or tissues, such as ascites, alveolar lining fluid, or bone. Further, some antibiotics, like the β-lactams, do not penetrate well into phagocytic cells such as macrophages, and this prevents them from killing bacteria that are engulfed within these cells. Finally, the infected tissue may be damaged or its blood supply may be compromised, such as in calcified tuberculous lesions, diabetic foot ulcers, or burns. In these situations, drugs that would normally reach the sites of infection may be unable to reach the area. Surgical débridement is frequently an essential element in

the cure of a patient. While various studies have been designed to evaluate the "tissue penetration" of antibiotics, there are many technical limitations to these studies (reviewed by Nix et al., 1991a and 1991b). In the clinical situation, it is very difficult to know how much antibiotic is present in an active form within a specific patient's lesion.

Additional properties of the drug must be considered relative to the patient's condition (see also Moellering, 1995, and Barriere, 1992). Agents that are cleared predominantly by the kidneys are more difficult to use in patients with renal failure. Given a choice, it may be easier to give a drug that is hepatically cleared under those circumstances. Of note, the human liver appears to retain some or most of its ability to clear drugs until the very end stages of cirrhosis. Still, renally cleared agents may produce more predictable serum concentration profiles in patients with liver disease. Other host factors, including extremes of age, pregnancy, lactation, obesity, amputations, and immune deficiencies may alter both the pharmacokinetic profiles and the risk-benefit ratios for specific antibiotics. Consult the recommended readings at the end of the chapter for specific dosing guidelines.

Pharmacodynamics

The pharmacokinetic profile of an antibiotic can be analyzed in combination with the *in vitro* susceptibility pattern of the antibiotic (reviewed by Drusano, 1995). Several "pharmacodynamic" parameters can be derived from such analyses (Figure 1). The ratio of the maximum concentration (Cmax) or "peak" to the static MIC of the organism gives one measure of the potency of the drug. In Figure 1, the Cmax is 2.0 μg/ml and the MIC, shown by the broken line, is 0.5 μg/ml. Therefore, the Cmax:MIC ratio is 4. With some agents, maximizing this parameter results in better killing of the organism. This is particularly true for the aminoglycosides and the fluoroquinolones, agents that attack targets within the bacteria. Increasing the Cmax:MIC ratio may also extend the time that it takes a culture of the organism to resume log-phase growth. This recovery lag has been termed the post-antibiotic effect, or PAE. In theory, drugs that display a PAE can be dosed less frequently. This is not only more convenient, but may result in less toxicity. Studies are underway to put these concepts into clinical practice.

Closely related to the Cmax:MIC ratio is the portion of the area under the serum concentration-time profile (AUC) that remains above the MIC. In Figure 1, the total AUC for the drug, shown by the curved solid line, is 12.82 μg*hr/ml. The portion above the MIC is 5.18 μg*hr/ml, or 40.4% of the total AUC. This hybrid parameter reflects, in part, both the Cmax:MIC ratio and the time that the drug remains in the body. Several studies suggest that maximizing this parameter is also desirable for the aminoglycosides and the fluoroquinolones.

Another measure of the drug's potential usefulness is the time that serum concentrations remain above the MIC. In Figure 1, the serum concentration reaches the MIC of 0.5 μg/ml at 0.5 hr and falls below the MIC at 10 hr. The total time

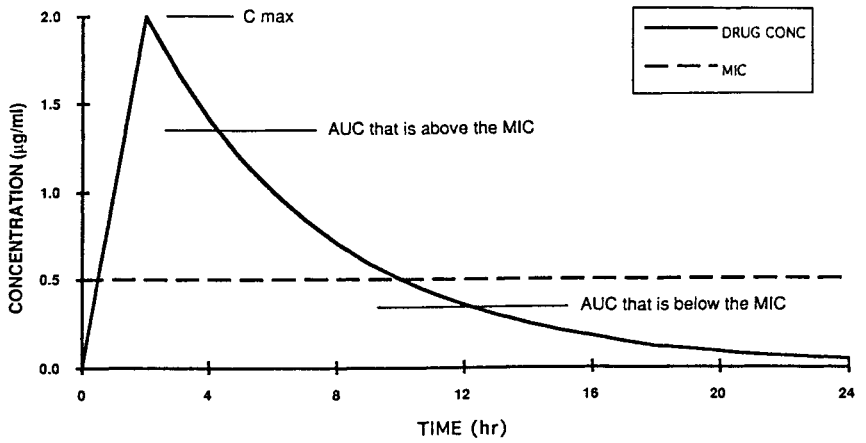

Figure 1. A hypothetical serum concentration-versus-time curve, displaying the maximum serum concentration (Cmax), the area under the curve (AUC), and the relationship of these parameters to the minimal inhibitory concentration (MIC).

above MIC is 9.5 hr, or 39.6% of the 24 hour dosing interval shown. Extending the time above the MIC reduces the time that the organism has to resume log-phase growth. For cell-wall active agents such as the β-lactams (penicillins, cephalosporins, and related drugs), best results may be seen when the serum concentrations remain at least 4 times the MIC. Therefore, β-lactams with long serum half-lives appear to be desirable. For severe infections (such as *Pseudomonas aeruginosa* pneumonia), it may be advantageous to give the β-lactams by continuous intravenous infusion. Again, further studies are required to refine this approach to therapy.

Therapeutic Drug Monitoring (TDM)

Serum drug concentrations can help the clinician adjust the drug dose to the individual patient's needs (discussed by Evans, 1992, and by Jusko, 1992). Depending on the clinical situation, it may be reasonable to increase the serum concentrations in order to achieve greater effect. This must be balanced against the increase of certain adverse effects. The assay used should be sensitive (able to detect the drug at physiologically important concentrations) and specific (not affected by other drugs or metabolites present in the serum). The assay should be reproducible over time, and the clinical pharmacology laboratory should maintain quality control records for each assay reported. Effective assay techniques include radioimmunoassay (RIA), enzyme immunoassay (EMIT), fluorescence polarization immunoassay (TDx), high-performance liquid chromatography (HPLC), gas chromatography (GC), and high-performance capillary electrophoresis (HPCE). Each assay has specific advantages (such as specificity or speed) and disadvantages (such as

complexity or cost). The clinical pharmacology laboratory typically will chose one technology after considering these various factors.

When performing TDM, it is critical to accurately record the amount of the dose, the time of the dose, and the time of sample collection. This information will describe the conditions during the test, and will allow for rational dose adjustment. Except in situations where the patient is acutely toxic and the clinician wants to see if the reaction is drug-related, "random" samples (no times of dose or sample collection) are generally not useful.

INTRODUCTION TO THE ANTIBIOTIC CLASSES

This section serves to introduce the reader to the more commonly used antimicrobial agents. Only major features of the drugs will be presented. This section is not intended to be a complete listing by class nor an exhaustive description of the spectrum, pharmacokinetics, or toxicity of these drugs. Because of the large number of potential indications, Tables 1 and 2 summarize the major features of the antibacterial agents. Information about the antimycobacterial drugs and the antifungal drugs is summarized below. For the interested reader, detailed information about these drugs can be obtained from the recommended reading list at the end of this chapter.

Antibacterial Agents: Cell Wall Active Agents

The penicillins, cephalosporins, and glycopeptides such as vancomycin head the list of antibiotics that interrupt cell wall synthesis. In most but not all circumstances *in vitro*, this results in bacterial cell death.

Penicillins

The penicillins are a family of agents that began with penicillin and gradually expanded to include broad-spectrum agents like azlocillin and piperacillin. They are widely used because they are generally safe and well tolerated. Penicillin G (the current purified form of the original penicillin mixture), its salts, and the acid-stable phenoxypenicillin derivatives share a similar spectrum of activity. They are active against most Gram-positive aerobes as well as many species of facultative and strict anaerobes, including *Clostridium perfringens*, the cause of gas gangrene. They are not active against the anaerobe *Bacteroides fragilis*, however. Penicillin derivatives are also active against many *Neisseria* species (including the cause of gonorrhea) and *Treponema pallidum*, which causes syphilis. Organisms that produce β-lactamase are generally resistant to the penicillin derivatives, and other organisms with altered target sites may be either "tolerant" (MBC ≥32 times higher than the MIC) or resistant.

Methicillin and the isoxazolyl penicillins (oxacillin, nafcillin, dicloxacillin, and flucloxacillin) are resistant to hydrolysis by Staphylococcal β-lactamases, and

Table 1. Indications and Primary Mechanisms of Action for the Antibacterial Agents

Drug Class/Specific Drugs	*Primary Indications*	*Other Indications*	*Primary Target*
Aminoglycosides			
amikacin	most Gram (–) bacteria	combination therapy:	protein
gentamicin		Enterococcal infections	synthesis
tobramycin			
streptomycin	Mycobacterium tuberculosis	*Brucella*	
	Mycobacterium avium	*Yersinia pestis*	
		combination therapy: Enterococcal infections	
Cephalosporins			
"1st generation"	most Gram (+) bacteria	*Escherichia coli*	cell wall
ex. cefazolin		*Klebsiella pneumoniae*	synthesis
"2nd generation"	most Gram (+) bacteria	some Gram (–) bacteria	
ex. cefoxitin		some members of class: most anaerobes	
"3rd generation"	most Gram (–) bacteria	many Gram (+) bacteria	
ex. cefotaxime		some anaerobes	
Clindamycin			
	most anaerobes	many Gram (+) bacteria	protein synthesis
Chloramphenicol			
	some Gram (+) bacteria	*Rickettsia*	protein
	some Gram (–) bacteria	*Salmonella*	synthesis
Glycopeptides			
vancomycin	most Gram (+) bacteria	*Clostridium difficile* diarrhea	cell wall
teicoplanin	especially: methicillin-resistant Staphylococci	many Gram (+) anaerobes	synthesis

continued

Table 1. (Continued)

Drug Class/Specific Drugs	*Primary Indications*	*Other Indications*	*Primary Target*
Macrolides			
erythromycin	many Gram (+) bacteria	*Bordetella pertussis* *Chlamydiae* *Legionella* Mycoplasma	protein synthesis
azithromycin clarithromycin	many Gram (+) bacteria	some Gram (–) bacteria *Mycobacterium avium*	
Metronidazole			
	most anaerobes *Gardnerella vaginalis*	*Entamoeba histolytica* *Giardia lamblia* *Trichomonas vaginalis*	DNA synthesis
Penicillins			
penicillin G	many Gram (+) bacteria (except β-lactamase producers)	many anaerobes *Treponema pallidum*	cell wall synthesis
oxacillin, etc.	most *Staphylococci*	*Streptococci*	
ampicillin, etc.	many Gram (+) bacteria (except β-lactamase producers)	Enterococcal infections *Escherichia coli* *Haemophilus influenzae*	
piperacillin, etc.	most *Pseudomonas aeruginosa*	most Gram (–) bacteria some anaerobes	

Quinolones			
ciprofloxacin, ofloxacin, lomafloxacin	most Gram (–) bacteria	some Gram (+) bacteria	DNA gyrase
Tetracyclines			
tetracycline	*Chlamydiae*	*Brucellosis*	protein
doxycycline	*Richettsia*	Mycoplasma	synthesis
minocycline	*Yersinia pestis*	*Treponema pallidum*	
Trimethoprim-Sulfamethoxazole			
	Nocardia	*Bordetella pertussis*	purine
	Pneumocystis carinii	*Legionella*	synthesis
	Burkholderia cepacia	many Gram (+) bacteria	
	Shigella	many Gram (–) bacteria	
	Stenotrophomonas maltophilia		

Table 2. Routes of Administration, Routes of Excretion, Dosing in Renal Failure, and Major Adverse Effects of the Antibacterial Agents

Drug Class/Specific Drugs	*Route of Administration*	*Primary Excretion*	*Dosing in Renal Failure*	*Adverse Effects by Drug Class*
Aminoglycosides				
amikacin	IV, IM	renal	reduced	nephrotoxicity
gentamicin	IV, IM	renal	reduced	ototoxicity
tobramycin	IV, IM	renal	reduced	renal cation wasting
streptomycin	IM, (IV, not FDA approved)	renal	reduced	hypersensitivity
Cephalosporins				
"1st generation" ex. cefazolin	choose based on oral or IV dosing	renal	reduced	hypersensitivity GI upset, diarrhea hematological toxicity
"2nd generation" ex. cefoxitin	most IV some oral	renal	reduced	bleeding disorders interstitial nephritis
"3rd generation" ex. cefotaxime	most IV some oral	renal, hepatic	reduced	
Clindamycin				
	oral, IV	hepatic, renal	potentially reduced	GI upset pseudomembranous colitis
Chloramphenicol				
	oral, IV	hepatic, renal	potentially reduced	bone marrow suppression aplastic anemia gray baby syndrome
Glycopeptides				
vancomycin	IV (oral for C. difficile)	renal	reduced	red-man syndrome
teicoplanin	IV	renal	reduced	ototoxicity nephrotoxicity?
Macrolides				

erythromycin	oral, IV	hepatic	usually reduced	GI upset
azithromycin	oral	hepatic	unchanged	hypersensitivity
clarithromycin	oral	hepatic		drug interactions
				ototoxicity
				hepatotoxicity
Metronidazole				
	oral, IV	renal, hepatic	reduced	metallic taste
				GI upset
				neurotoxicity
Penicillins				
penicillin G	oral, IV	renal	reduced	hypersensitivity
oxacillin	oral, IV	renal, hepatic	reduced	GI upset, diarrhea
ampicillin	oral, IV	renal	reduced	hematological toxicity
piperacillin	IV	renal, hepatic	reduced	bleeding disorders
				interstitial nephritis
				CNS toxicity on overdose
Quinolones				
ciprofloxacin	oral, IV	renal, hepatic	reduced	GI upset
ofloxacin	oral, IV	renal	reduced	CNS excitation
lomafloxacin	oral	renal	reduced	hypersensitivity
				interstitial nephritis
				cartilage damage?
Tetracyclines				
tetracycline	oral, IV	renal	reduced	GI upset
doxycycline	oral	nonrenal, renal	reduced	superinfections
minocycline	oral	renal	reduced	hypersensitivity
				photosensitivity
				teeth staining
				hepatotoxicity
Trimethoprim-Sulfamethoxazole				
	oral, IV	hepatic, renal	reduced	hypersensitivity
				GI upset
				hematological toxicity

therefore are useful agents for many skin and soft-tissue infections. They retain some but not all of the activity of penicillin against other Gram-positive organisms. Ampicillin, amoxacillin, and a series or related derivatives expand penicillin's coverage to include some Gram-negative bacteria such as *Escherichia coli* and *Haemophilus influenzae*. Hence, they are frequently used for urinary tract, upper respiratory tract and middle ear infections. Ampicillin and the related drugs are also useful agents against many *Enterococci*, which are Gram-positive organisms, and against *Listeria monocytogenes*. When used to treat *Enterococci*, ampicillin is frequently combined with the aminoglycosides streptomycin or gentamicin. Because ampicillin and amoxacillin are susceptible to the β-lactamases produced by some of the organisms above, they may be combined with β-lactamase inhibitors such as sulbactam and clavulanic acid in order to maintain their efficacy.

Carbenicillin, ticarcillin, and the newer drugs mezlocillin, azlocillin, and piperacillin are active against *Pseudomonas aeruginosa* and many *Enterobacteriaceae* species, while retaining moderate activity versus Gram-positive organisms and many anaerobes. These agents are frequently combined with aminoglycosides in the treatment of severe Gram-negative or mixed infections.

Among the various categories of penicillin derivatives above, both oral and intravenous dosage forms can be found. Therefore, they can be used in a variety of settings. Exceptions include the anti-pseudomonal penicillins, which are primarily given intravenously. Most penicillins are renally cleared, so the doses or frequencies of administration must be reduced in patients with renal dysfunction. Their most prominent toxicities include hypersensitivity reactions, ranging from mild rashes to anaphylactic shock. Less commonly, interstitial nephritis and bone marrow suppression may occur. Penicillins are generally considered safe to administer to pregnant women, if indicated.

Cephalosporins

The cephalosporins, like the penicillins, are β-lactam antibiotics, and similarly disrupt the construction of bacterial cell walls. "First generation" cephalosporins, including cephalothin, cephalexin, and cefazolin, are active against many Gram-positive bacteria, *E. coli*, and *Klebsiella pneumoniae*. They are more resistant to Staphylococcal β-lactamases than penicillin and ampicillin, and are often used for the prevention of wound infections during surgery. Cephalosporins are not active against methicillin-resistant *Staphylococci*, and they lack the activity of penicillin and ampicillin versus *Enterococci*.

"Second generation" cephalosporins such as cefamandole, cefoxitin, cefuroxime, and ceforanide expand the Gram-negative spectrum of the first generation drugs. However, unlike the "first generation" drugs, their spectrums are much more variable. Cefoxitin is particularly stable against β-lactamases, and has very good activity against anaerobes, including many *B. fragilis* isolates. Cefoxitin and newer

derivatives are often used for mixed infections, such as abdominal wounds or pelvic infections.

"Third generation" cephalosporins are truly broad-spectrum agents. Cefotaxime, ceftriaxone, ceftazidime, and related drugs inhibit many Gram-positives, Gram-negatives including most *Enterobacteriaceae*, and many anaerobes. Only a few, such as ceftazidime, have useful activity versus *Pseudomonas aeruginosa.* In addition, many Gram-negatives such as *Enterobacter* sp. produce large amounts of β-lactamase when exposed to these drugs. Therefore, "third generation" cephalosporins must be used cautiously in settings where resistant organisms are likely to be found, such as intensive care units. "Third generation" cephalosporins have proven to be very useful for the treatment of various Gram-negative central nervous system (CNS) infections, and are also useful against β-lactamase producing *N. gonorrhoeae.*

The pharmacokinetic and toxicity considerations for the cephalosporins are, in general, similar to those described above for the penicillins. A series of other β-lactam drugs, including penems, carbapemes (imipenem/cilastatin and meropenem) and monobactams (aztreonam) share many properties with the broad-spectrum penicillins and cephalosporins. A complete listing and description of these agents are beyond the scope of this chapter.

Glycopeptides and Related Drugs

The glycopeptide antibiotics include vancomycin and teicoplanin, with daptomycin being a related compound. In the United States, vancomycin is the member of this class used most commonly. Glycopeptide activity is largely restricted to the Gram-positive bacteria, including many Gram-positive anaerobes. They are primarily used in the treatment of methicillin-resistant *Staphylococcus aureus, S. epidermidis*, and ampicillin-resistant *Enterococci.* When used to treat *Enterococci*, vancomycin may be combined with streptomycin or gentamicin. Vancomycin has also been given orally, where it remains in the gut to eliminate *Clostridium difficile*, a cause of antibiotic-associated diarrhea and pseudomembranous enterocolitis.

In general, vancomycin is reserved for situations where penicillins or cephalosporins are not adequate. It is given most commonly by intravenous infusion. Elimination of vancomycin is primarily through the kidneys, so dosage reduction must be considered in patients with renal dysfunction. This may be accomplished by extending the dosing interval, which directly addresses the problem of slower clearance. Vancomycin may cause hypotension with or without an upper body rash, particularly if the drug is infused rapidly (red man's or red neck syndrome). Other hypersensitivity reactions may also occur. Vancomycin has been associated with oto-and nephrotoxicity, although clear cause and effect relationships are difficult to establish for the latter. Less experience has been accumulated with teicoplanin; it may show a toxicity profile similar to vancomycin.

Antibacterial Agents: Intracellular Poisons

A large number of these drugs attack bacterial protein synthesis, while a few classes have unique target sites. For example, the quinolones inhibit bacterial DNA gyrase, while trimethoprim and the sulfonamides interrupt purine synthesis and, in turn, DNA synthesis. Bear in mind that our understanding of antibacterial mechanisms of action is not complete. The proposed mechanisms may not fully explain the demonstrated effects of the drugs, and further research may reveal additional targets.

Aminoglycosides

After 50 years of use, the aminoglycosides remain central agents within this broad category of drugs. The aminoglycosides are generally bactericidal in a concentration-dependent manner. Gentamicin, tobramycin, netilmicin, and amikacin are most commonly used for Gram-negative infections. Streptomycin, kanamycin, and amikacin are also useful agents against *M. tuberculosis, M. avium*, and other mycobacteria. In combination with penicillin, ampicillin, or vancomycin, streptomycin and gentamicin may be used to treat enterococcal infections. The aminoglycosides are devoid of anaerobic coverage, and must be combined with other agents in complicated situations, such as abdominal wounds.

The major limitations of the aminoglycosides are nephrotoxicity (typically non-oliguric acute tubercular necrosis) and ototoxicity (hearing loss or vestibular toxicity). Various studies have suggested that either elevated peak or trough concentrations may be associated with the development of these toxicities. In this author's view, consistently elevated trough concentrations (from too frequent dosing) and long courses of therapy are most likely to lead to toxicity. Age is a factor from the standpoint that older patients have decreased renal function. For most patients over 65 years of age, these renally cleared drugs should be given every 12 to 24 hours. Large, daily doses of these drugs are being studied in an effort to maximize efficacy while minimizing trough concentrations. This approach is likely to gain broader acceptance, but it may not be applicable in all cases where an aminoglycoside is used.

Quinolones

The quinolones changed from being an obscure class of urinary tract agents into mainstream antimicrobials with the introduction of the fluoroquinolones. Like the aminoglycosides, they are generally bactericidal in a concentration-dependent manner. Ciprofloxacin, ofloxacin, and related agents are considerably more potent and display more favorable pharmacokinetic profiles than their predecessors. Most of the available quinolones are more active against Gram-negative bacteria, including most *Enterobacteriaceae*, *Salmonella* sp., *Shigella*, and *P. aeruginosa*. Their moderate activity against Gram-positive organisms has not allowed them to chal-

lenge penicillins or cephalosporins in that area. Newer quinolones are likely to show markedly improved Gram-positive spectrums. The quinolones are not useful clinically against difficult anaerobic infections, and like the aminoglycosides, should be combined with other agents when such coverage is needed.

The quinolones vary in the percentage of dose cleared hepatically, while nearly all are at least partially cleared renally. Conveniently, most can be administered both orally and intravenously. They are well-tolerated drugs, with gastrointestinal (GI) intolerance and CNS excitation being the most common adverse reactions. Renal toxicity, photosensitization, and other hypersensitivity reactions occur less frequently. These drugs have been shown to affect weight-bearing joint development in animals, but this has not occurred frequently in humans. In limited situations, these drugs have been used safely in children when the potential benefits clearly outweighed the potential risks.

Macrolides

Erythromycin is the mainstay of this class of antibiotics. Under most conditions, these agents are considered bacteriostatic, although they occasionally appear to be bactericidal. Newer entries include clarithromycin and the chemically related azithromycin. The newer agents combine improved pharmacokinetics with better activity versus Gram-negative bacteria and mycobacteria, especially *M. avium*. Erythromycin remains a useful alternative to penicillins and cephalosporins for non-life-threatening Gram-positive infections, and many atypical infections caused by *Bordetella pertussis* (whooping cough), *Mycoplasma pneumoniae*, and *Legionella* sp. Erythromycin is often used when Gram-positive coverage is needed in a penicillin-allergic patient.

The macrolides are generally given by mouth, although erythromycin can be given intravenously. The major limitation of the macrolides has been significant gastrointestinal (GI) upset upon oral administration. The newer agents appear somewhat better in this regard. Rashes, ototoxicity, hepatotoxicity, and hepatic enzyme inhibition, leading to reduced clearance of other drugs, are occasional problems with the macrolides.

Tetracyclines

The tetracyclines are generally bacteriostatic, and because of widespread use, many common bacteria are resistant to these agents. The tetracyclines remain important drugs for atypical infections, including *Chlamydiae, Mycoplasma pneumoniae, Rickettsia, Yersinia pestis* (plague), and as reserve agents for *Treponema pallidum* (syphilis). They are occasionally used for nontuberculous mycobacterial infections, and are useful in combination therapy for pelvic inflammatory disease (PID) in women.

Tetracycline can cause GI upset, rashes, and staining of developing teeth. It should be avoided in pregnant women for the latter reason, and because of

hepatotoxicity seen in that setting. Minocycline is prone to causing CNS effects such as dizziness, and doxycycline is often used instead.

Trimethoprim-sulfamethoxazole

The sulfonamides were the first class of synthetic antimicrobial agents, but as a class are less frequently used today. Sulfamethoxazole, in combination with trimethoprim, remains an important exception. Alone, the sulfonamides are generally bacteriostatic, but with trimethoprim, frequently bactericidal. Trimethoprim-sulfamethoxazole is a reasonable alternative for some Gram-positive and many Gram-negative infections, *B. pertussis, Legionella* sp., *Salmonella* sp. Trimethoprim-sulfamethoxazole remains the primary treatment for difficult infections caused by *Pseudomonas cepacia, Xanthomonas maltophilia, Shigella, Nocardia* and *Pneumocystis carinii*. The last organism is a significant opportunistic pathogen in patients with AIDS. Their major limitation is hypersensitivity reactions, which occur much more frequently in HIV-infected patients.

Metronidazole and clindamycin

Metronidazole and clindamycin are drugs used primarily for anaerobic infections. Metronidazole is primarily active against strict anaerobes, such as *B. fragilis*, while clindamycin is also active against microaerophillic and many aerobic Gram-positive organisms. Metronidazole is also used to treat *Entamoeba histolytica, Trichomonas vaginalis, Giardia lamblia*, and *Gardnerella vaginalis*, while both drugs are alternative agents for *Clostridium perfringens* (gas gangrene). Additional information about these drugs is provided in the tables.

Chloramphenicol

Chloramphenicol was a very important drug historically for the treatment of bacterial meningitis. Its role in these infections has been diminished by the advent of third-generation cephalosporins, which are as active and potentially less toxic. Chloramphenicol is an alternative agent for infections caused by *Pseudomonas cepacia*, *Salmonella* sp., and *Rickettsia*. Bone marrow suppression and aplastic anemia, although relatively uncommon, dampen enthusiasm for this drug. Also, a newborn's liver lacks the ability to metabolize chloramphenicol rapidly. If doses are not reduced, accumulation of the drug can result in fatal circulatory collapse, the so called "gray baby syndrome."

Antimycobacterial Agents

The antimycobacterial drugs are used to treat tuberculosis, *M. avium* infection, leprosy, and variety of related infections. A brief overview will be presented here (detailed by Iseman, 1993, and Bass et al., 1994). *Isoniazid* (INH) and rifampin are the two most potent agents against *M. tuberculosis*, and are central to current 6

month "short course" treatment regimens. When combined with *pyrazinamide* (PZA) for the first two months of the 6 month course, cure rates can approach 100%. Most patients receive these drugs orally, although parenteral forms of isoniazid and rifampin are available. While rifampin is a broad-spectrum antibiotic, INH and PZA are primarily and exclusively active, respectively, against *M. tuberculosis*.

All three drugs undergo some degree of hepatic metabolism prior to excretion (see Peloquin, 1991 and 1993). The primary toxicities with isoniazid are hepatotoxicity and peripheral neuritis. The latter may be suppressed by the administration of 10–20 mg daily of vitamin B6 (pyridoxine). Rifampin can cause hepatotoxicity, and may discolor urine and other secretions reddish-orange. Less commonly, rashes, thrombocytopenia, and renal failure may occur. Patients experiencing the latter two toxicities should not receive rifampin again. Intermittent doses, especially above 900 mg, may precipitate a flu-like syndrome. Rifampin is a potent hepatic enzyme inducer, resulting in many significant drug interactions. Pyrazinamide can also cause hepatotoxicity, and the accumulation of uric acid may result in arthralgias, but usually not true gout.

Ethambutol is another commonly used antimycobacterial drug, and is effective against many species. It is usually given orally, although a parenteral form is available in Europe. Ethambutol is renally cleared, and doses must be reduced in the presence of renal dysfunction to avoid ocular toxicity. The drug may increase serum uric acid concentrations, and hypersensitivity reactions occasionally occur.

Para-aminosalicylic acid (PAS) was replaced by ethambutol as a commonly used antituberculosis drug because of its frequent GI toxicity. A new granule form of the drug substantially improves GI tolerance, making it easier to use this drug for INH- or rifampin-resistant *M. tuberculosis*. PAS is not active against most other mycobacteria.

Streptomycin, *kanamycin*, and *amikacin* all may play a role in the treatment of mycobacterial diseases, and were discussed above with the other aminoglycosides.

Capreomycin, a polypeptide, appears to share a similar mechanism of action and spectrum of toxicities with the aminoglycosides. Frequently, it retains *in vitro* activity when an isolate of *M. tuberculosis* displays resistance to one or more of the aminoglycosides. All of these agents are given parenterally, which is the reason they are usually not drugs of first choice.

Cycloserine, *ethionamide*, *prothionamide*, *thiacetazone*, and *viomycin* are agents reserved for multidrug-resistant tuberculosis (MDR-TB). The last three drugs are seldom used in the United States. They are less potent than INH and rifampin, and are prone to higher rates of adverse effects, although they can be used effectively if used cautiously. Because achieving this balance requires extensive experience, MDR-TB is best managed by mycobacterial disease specialists. *Clofazimine* and *dapsone* are drugs useful against leprosy, along with rifampin and ethionamide. Occasionally, clofazimine is used against *M. tuberculosis* or *M. avium*, and dapsone has been tested experimentally for a variety of infectious and non-infectious inflammatory conditions.

The quinolones *ciprofloxacin* and *ofloxacin* display useful activity versus INH- and rifampin-resistant isolates of *M. tuberculosis*, and ciprofloxacin is sometimes used to treat *M. avium*. These and other quinolones are occasionally active against other mycobacteria, and are being studied in the treatment of leprosy.

Antifungal Agents

Susceptibility testing with fungi is particularly difficult, and the interpretation of such results remains somewhat controversial (see Carver, 1992). Therefore, while the clinician should take full advantage of the clinical laboratory's capabilities, the final decision regarding the use of a specific agent should not rest on susceptibility data alone.

Nystatin was an early polyene antibiotic found to be active *in vitro* against fungi. Today, it is used topically against limited, superficial fungal infections. *Amphotericin B*, a more potent polyene antibiotic, attacks sterols in the cell membrane of fungi. It is generally considered fungicidal, and remains the primary agent for serious fungal infections. Amphotericin B is active against the majority of fungal isolates, and has been used extensively against *Aspergillus* sp., *Candida* sp., *Coccidioides immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*. Amphotericin B is limited by its significant toxicity, including headache, fever, chills, and even rigors during intravenous infusions, nephrotoxicity, renal cation wasting, and anemia. Therefore, patients receiving this drug must be monitored carefully. Empiric dosing guidelines have been proposed for most fungal infections, and many try to cap doses at specific gram amounts in order to prevent severe or irreversible renal damage. However, in patients with cyclic or persistent immunosuppression, these dosing limits may have to be exceeded. Research continues with liposomal forms of amphotericin B in an effort to improve patient tolerance. These formulations eliminate the colloidal suspension of water-insoluble amphotericin B with sodium desoxycholate, and in turn may reduce some of the adverse reactions.

Clotrimazole and *econazole* are imidazole derivatives that are used to treat superficial fungal infections. *Miconazole* and *ketoconazole* are more potent imidazole derivatives that offered clinicians some alternatives to amphotericin B. Neither drug, however, is as effective as amphotericin B for most applications, and are generally limited to non-life-threatening infections. Miconazole is solubilized in a polyethoxylated castor oil vehicle (Cremaphor EL) prior to intravenous administration, and this vehicle causes irritation at the site of infusion. Ketoconazole, which is given orally, is an alternative agent for the treatment of *C. immitis* and *H. capsulatum* infections. It requires low gastric pH for dissolution and absorption. Ketoconazole frequently causes GI distress, and is a potent hepatic enzyme inhibitor. This enzyme inhibition blocks hydroxylation of natural steroids, blunting the adrenal stress response, and also leads to significant drug interactions.

The next generation of triazole antifungal agents, including *itraconazole* and *fluconazole*, has moved these drugs into the treatment algorithms for serious fungal

infections. While amphotericin B remains the primary agent for many acute treatment regimens, the triazoles are becoming the drugs of choice for many forms of "maintenance therapy." In this setting, patients continue to receive antifungal agents to prevent the recurrence of fungal disease until their immune systems are able to effectively defend them. In cancer patients, the drugs may be stopped once the bone marrow recovers following chemotherapy. In patients with AIDS, however, the drugs may need to be continued for life. Itraconazole, like ketoconazole, requires low gastric pH for absorption, but appears to have a less potent effect on hepatic enzymes. In contrast to ketoconazole and itraconazole, fluconazole is less dependent on low gastric pH for absorption and more dependent on renal clearance for elimination. It also is capable of causing drug-interactions because of inhibition of cytochrome P-450 function in the liver.

Griseofulvin is an oral agent used for superficial fungal infections. 5-Fluorocytosine is another oral agent that may be used in combination with amphotericin B for the treatment of cryptococcal meningitis. It displays dose-dependent bone marrow suppression. Because it is renally cleared, its dose must be reduced in patients with renal dysfunction, including those who develop nephrotoxicity from amphotericin B.

SUMMARY AND CONCLUSIONS

Clearly, a large number of factors must be considered before prescribing an antimicrobial agent. It is not possible to detail all of these issues in a single chapter. Nevertheless, the above account should provide the clinician with the basic tools needed to address infectious diseases. As the reader enters clinical practice, it may be useful to revisit this chapter to take inventory of the issues that one will have to face. Many excellent texts are available that provide extensive information about the various drugs described above. These texts should be consulted before prescribing these antimicrobial agents in a patient.

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Chapter 13

Mechanisms of Resistance to Antibacterial Agents

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INTRODUCTION

Antibiotics are substances that are naturally produced by microorganisms to inhibit other species, providing a competitive advantage to the survival of the producing cell. The terms "antimicrobial agent" and "antibacterial" encompass antibiotics, as well as synthesized or modified compounds, that have the same effect. Survival principles dictate that antibiotic-producing microorganisms must logically be re-

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sistant to their own antimicrobial products. It is known, for example, that the most clinically important mechanisms of resistance to aminoglycosides are present in the members of the genera that naturally produce these compounds, *Streptomyces* and *Micromonospora* (Davies, 1986). The resistance mechanisms, either enzymatic inactivation of the aminoglycoside or alteration of the drug's ribosomal target, may have originated in this, or another native soil, genus and the responsible gene may have moved horizontally into species which are pathogenic for humans. Once present in the infecting organism, these traits offer no survival advantage until the cell is faced with the onslaught of an antimicrobial agent. The resistance trait, even if present in an extremely rare proportion of cells, may then enable the survival of the infecting organism, and then may proliferate, spread by inheritance or by horizontal movement on plasmids or phages, and become endemic within an institution or community. The extent of such entrenched resistance problems within hospitals appears to be proportional to the frequency of use of antimicrobials. A notable example of this problem that gave birth to the need to study and control resistance is penicillinase-producing *Staphylococcus aureus*. Until the discovery of penicillin, the prognosis of patients with serious staphylococcal infections was poor. Soon after the introduction of this potent antibiotic, however, an enzyme, now referred to as β-lactamase, that would inactivate penicillin, was isolated in the early 1940s from penicillin-resistant staphylococci (Kirby, 1944). By the end of the 1940s more than one-half of the *S. aureus* isolates in British hospitals were reported to be penicillin-resistant due to β-lactamase (Barber and Rozwadowska-Dowzenko, 1948). It remains uncertain as to whether this trait originated in the penicillin-producing mold or within the staphylococcal cell, perhaps as a genetic mutation affecting one of the drug's target cell wall proteins. For any given bacterial species and antimicrobial agent ("drug/bug") combination, resistance is now a factor. The ability to study and control the emergence and spread of resistance justifies the concerted efforts of epidemiologists, laboratorians, and primary physicians.

In general, an understanding of the mode of action of antimicrobial agents at the cellular level is essential for the characterization of resistance mechanisms. Most clinically used antibacterial agents may be classified according their distinct modes of action, which include inhibitory effects upon the synthesis of proteins, cell wall, folic compounds, or nucleic acids. Antibacterial classes and their modes of action were reviewed more extensively in the preceding chapter.

Inhibitors of protein synthesis (translation) include the aminoglycoside-aminocyclitol compounds, tetracyclines, chloramphenicol, and the macrolide-lincosamide-streptogramin (MLS) compounds. The aminoglycoside-aminocyclitol class includes more than 150 compounds including commonly used drugs such as gentamicin, tobramycin, and amikacin. Although aminoglycosides are one of the largest antimicrobial classes as well as one of the oldest (streptomycin was discovered in 1944), the exact mechanism of action has not been entirely elucidated. These drugs, however, are known to inhibit translation at the bacterial ribosome and most are considered bactericidal. Tetracyclines also act at the ribosomal level. This class

of translation inhibitors is also one of the older drug classes, introduced in 1947 with the discovery of chlortetracycline, and remains as one of the most frequently used antimicrobic classes worldwide. Chloramphenicol was admitted to clinical use in 1950 and is known to be a specific and effective inhibitor of polypeptide chain elongation due to its affinity for peptidyl transferase. Erythromycin, a 14-member ring lactone macrolide, was introduced in the early 1950s as the first MLS antibiotic. This class exerts cidal effects upon translation at the 50S ribosomal subunit.

Two major classes of bacterial cell wall inhibitors are the β-lactams and glycopeptides, both of which are considered bactericidal. Subclasses of β-lactams include the bicyclic penicillins and cephalosporins, and the monocyclic monobactams. These compounds possess in common a four-sided β-lactam ring in their structure and all act by acylation of membrane enzymes involved in cell wall synthesis. Like the β-lactams, the glycopeptides (vancomycin and teicoplanin) also inhibit cell wall synthesis, but specifically through their interference of the transglycosylase reaction during the assembly of the peptidoglycan.

The sulfonamides and trimethoprim are folic acid pathway inhibitors that affect different steps in the conversion of hydroxymethylpteridine along with *p*-aminobenzoate into N^5N^{10}-methylene tetrahydrofolate. Since these compounds have affinity respectively for dihydropteroate synthetase and dihydrofolate reductase, their combined use has offered a distinct synergistic advantage over the efficacy of each drug alone in the therapy of a broad range of bacterial infections.

Bacterial nucleic acid inhibitors include those that affect DNA replication and those that inhibit RNA polymerase and, therefore, transcription. Fluoroquinolones, the most common of the DNA replication inhibitors, inhibit DNA gyrase and at higher concentrations may also affect another related DNA enzyme, topoisomerase type I. Nalidixic acid, a non-fluorinated quinolone, was the first gyrase inhibitor released for clinical use and was approved in 1962 for treatment of urinary tract infections. Rifamycins, the most notable of which is rifampin, are hydrophobic antibiotics that inhibit DNA-dependent RNA polymerase, a metabolically essential enzyme responsible for the synthesis of messenger RNA.

The degree of efficacy of any antibacterial agent at the cellular level is dependent upon two primary factors. First, the compound must be successfully transported from the external environment of the cell across surface layers, and delivered to its site of action while still retaining its biologically-active molecular structure. Secondly, the drug's site of action must be accessible and unaltered. Resistance to a particular antibacterial drug is usually attributable to one of three primary mechanisms. The general categories of these mechanisms include exclusion (including impermeability or export of the drug prior to its effects), enzymatic modification of the drug to an inactive or less active form, and alteration of the drug's target site. In some instances, however, there is evidence for an interplay of more than one of these general mechanisms. For example, enzymatic modification(s) to a drug may alter its transport while not appreciably affecting its biologic activity at the target site.

EXCLUSION

Considering the complexity and diversity of the bacterial cell surface layers, antimicrobial agent exclusion, or impermeability, is perhaps the most common mechanism of resistance. Since antimicrobial agents are not beneficial imports for target cells, uptake is not usually a natural process, and therefore exclusion is often a passive and intrinsic mechanism of resistance. One of the most basic of these intrinsic factors may be external slime or capsular layers that simply "trap" the drug disabling its internal movement. Some capsules are composed of lipopolysaccharides possessing a net negative charge which repels cationic antimicrobial agents. The uptake of aminoglycosides, which are strongly basic molecules, begins with their electrostatic attachment to the outer membranes of susceptible cells. Although the ribosome is the primary site of action of these drugs, disruption of the net charge across the outer membrane is believed to contribute to cellular death. Following attachment, aminoglycosides must be actively transported into the cytoplasm by a process coupled to oxidative phosphorylation (Taber et al., 1987). The endogenous absence of a cytochrome-mediated electron transport chain, for example in obligately anaerobic bacterial species, may be regarded also as intrinsic drug exclusion. Drug specific factors such as hydrophobicity, primary or secondary molecular structure, or net charge also may affect drug passage into the cell. Poor interaction between the lipid-rich outer membrane of gram-negative bacilli and vancomycin, a hydrophilic molecule, at least partially explains the intrinsic permeability barrier to the efficacy of this drug/bug combination. Even in susceptible organisms, a very small proportion of a drug's molecules actually penetrates the bacterial cell, and therefore, other factors not related to the bacterium itself may contribute to this type of resistance. Foremost among these factors is the stability of the compound in biologically active and nontoxic concentrations during its pre-entry residence in the cell's immediate environment.

Evidence that exclusion has become a non-intrinsic, or acquired, mechanism of resistance in normally susceptible organisms comes usually in three forms. First, the phenotypic levels of resistance are typically lower than those mediated by other mechanisms, such as enzymatic inactivation or altered target. Secondly, cross resistance to other drugs in the same or even different classes may be more evident if impermeability is a factor. Finally, since mutations that decrease antibacterial drug uptake usually also affect uptake of favorable nutrients, there is often an overall decrease in the health of the bacterium. These phenomena are most often the result of alterations either in porins, proteinaceous channels in outer membranes through which exogenous substances are transported, or in carrier molecules. Organisms that typically grow on minimal media containing a single carbon source, such as succinate or raffinose, may become nonviable when mutations affecting uptake or nutrients or drugs arise. As many as five different mutations in outer membrane protein (*omp*) genes, all of which may affect antimicrobial permeability have been detected in a single strain of *Escherichia coli* (Hancock, 1984). Since these traits

are typically recessive, they must replace their corresponding wild type genes in the haploid genome to become expressive. Once established, this type of resistance usually becomes a stable and constitutive trait passed only horizontally during normal cellular reproduction. At least one of these recessive mutations affects the *omp*F gene, consequently conferring high-level resistance to multiple drug classes (chloramphenicol, tetracycline, and fluoroquinolones) in a single event, and has been termed the *mar* (multiple antibiotic resistance) locus (George and Levy, 1983; Cohen, et al., 1989). Several loci that reduce quinolone permeation have been described. Mutations in the Gram-negative *nalB* locus primarily affect uptake of nalidixic acid, but have little affect on newer quinolones. Production of the wild type ompF protein is also decreased in mutants of *E. coli* resistant to norfloxacin or ciprofloxacin. The genes involved, *nfxB*, *norB*, *cfxB*, and *norC*, appear to affect binding of quinolones to intact bacteria and to reduce levels of the porin ompF protein. While the *norC* mutation also causes alterations in lipopolysaccharide, *nfxB* and *cfxB* are regulatory mutations that control expression of ompF at the transcriptional level that lead to pleiotrophic resistance similar to the *mar* mutations (Wolfson and Hooper, 1989).

Although studies of resistance in the mycobacteria have lagged behind other genera, the complexity of the cell surface suggests the presence of a natural barrier to the penetration of many compounds that are active in other genera. The sudden re-emergence of tuberculosis in the mid 1980's, however, was accompanied by an alarming increase in the prevalence of drug-resistant *Mycobacterium tuberculosis* followed by intensive efforts to elucidate resistance mechanisms and to develop novel effective antituberculosis drugs. Despite the appearance of strains that are resistant to as many as seven normally effective antituberculosis compounds (e.g., isoniazid, rifampin, ethambutol, streptomycin, kanamycin, pyrazinamide, and ethionamide), the traits conferring these phenotypes are believed to have been acquired in a stepwise fashion suggesting an interplay of more than a single resistance mechanism. Drug exclusion has not yet been shown to play an important role in resistance to primary antituberculosis drugs. Mechanisms of resistance to pyrazinamide (PZA) and isoniazid (INH) in many clinically resistant *M. tuberculosis* isolates are associated with mutations in gene encoding pyrazinamidase (converts pyrazinamide to its active form) and catalase-peroxidase, respectively. Altered drug targets as presented later in this chapter are responsible for most clinical resistance to rifampin and streptomycin in the mycobacteria.

A less rigid definition of exclusion that includes any mechanism whereby drugs are prevented from reaching their cellular target, would permit the inclusion of two related antibacterial resistance mechanisms, active efflux and drug blocking. Among clinically useful compounds, tetracyclines and MLS agents are affected the most by active efflux. The drug, unaltered but perhaps in less than normal concentrations, is permitted entry into the cell, but then is actively "pumped" back out into the cell environment before reaching its target. Tetracycline efflux involves an inner membrane protein, "Tet" (43,000 daltons), which is common to tetracycline resis-

tance classes A through E in Gram-negative bacteria (Levy, 1988). Resistance classes K and L, both of which are of principal importance in Gram-positive cocci, also involve at least one efflux protein. Active efflux of 14-membered macrolides from *Staphylococcus epidermidis* has also been reported. As with most tetracycline efflux genes, the MLS gene, *erpA*, is carried on a plasmid and codes for a membrane protein (LeClercq and Courvalin, 1991a). In contrast with tet classes A through E which are of widespread clinical importance, the distribution of the *erpA* trait among clinical isolates is apparently low.

The blocking mechanism implies the unusual presence of a cellular product in resistant strains that prevents the drug from reaching its site of action. Substances with this effect have been detected in some isolates resistant to tetracycline or vancomycin. The most widespread tetracycline resistance determinant, *tetM*, encodes a protein which has similarity to ribosome elongation factors, but which protects the ribosome from tetracycline. The broad host range and generally wide distribution of *tetM* is at least partially attributable to its typical location on promiscuous transposons such as Tn*916*. A similar mechanism, encoded by the *tetO* gene, has been described in *Campylobacter* species (Levy, 1988). Proteins present in the cytoplasmic membrane of some vancomycin-resistant enterococci harboring the *vanA* gene have likewise been shown to perhaps block access of glycopeptides to their pentapeptide target. Since the vanA protein possesses affinity for D-alanyl-D-alanine, it may prevent binding of glycopeptides to molecules that have terminal residues containing *N*-acyl-D-alanyl-D-alanine. The *vanA* gene may be carried on mobile plasmids and has been shown to be inducible by vancomycin, and to a lesser extent by teicoplanin (Arthur and Courvalin, 1993). (See Table 1.)

Table 1. Antibacterial Drug Exclusion Mechanisms

Mechanism	*Affected Drug Class(es)*	*Typical Organism(s)*
Intrinsic impermeability		
Transport deficiency	Aminoglycosides	Anaerobic bacteria
Natural barrier	Most classes	Gram-negative bacilli
Drug property (e.g., charge or hydrophobicity)	Rifampin, glycopeptides	Gram-negative bacilli, Gram-positive cocci
Altered outer membrane protein	β-lactams, chloramphenicol, quinolones, tetracyclines, trimethoprim	Gram-negative bacilli, Gram-positive cocci
Efflux	Tetracyclines	Gram-negative bacilli, Gram-positive cocci
	Macrolides	*Staphylococcus* sp.
Target blocking		
Pentapeptide	Glycopeptides	*Enterococcus* sp.
Ribosome	Tetracyclines	Gram-negative species, Gram-positive cocci

ALTERED TARGET

Antibacterial resistance mediated by the altered target mechanism implies that the drug is unchanged, but fails to inhibit the bacterium due to modification, loss, or overproduction of a specific vital target molecule. Among the most clinically important of these target alterations are: (1) N^6-dimethylation of the 23S ribosomal RNA conferring MLS resistance, (2) enzymatic modification of 16S rRNA or changes in ribosome-associated proteins conferring aminoglycoside resistance, (3) decreased affinity of essential cell wall synthetic enzymes for β-lactam compounds, and (4) modification or overproduction of enzymes required for either the folate pathway (conferring resistance to sulfonamides or trimethoprim), DNA replication (novobiocin or quinolone resistance), or transcription (rifamycin resistance).

At least eight classes of MLS resistance determinants (*erm* genes) that encode ribosomal methylation have been characterized, and most are associated with plasmids and/or transposons. These genes, found only in Gram-positive organisms, demonstrate variable degrees of nucleotide sequence relatedness which suggests a possible common ancestral origin, possibly in an MLS-producing soil *Streptomyces* sp. or *Arthrobacter* sp. Whether these genetic loci are constitutive or inducible does not relate to the nature of the structural *erm* gene, but rather to sequences upstream that control messenger RNA structure. Molecules, such as erythromycin, which are positive inducers of the methylase gene, act by altering stem-loop structures in the mRNA, a process referred to as attenuation control (Leclercq and Courvalin, 1991a; 1991b).

Ribosomal changes that confer aminoglycoside resistance have the greatest clinical impact upon streptomycin and spectinomycin. Phenotypic levels of resistance tend to be considerably higher by this mechanism than by others, for example, enzymatic modification. Pathogenic species which are affected the most by altered ribosome aminoglycoside resistance are *Neisseria gonorrhoeae, Mycobacterium tuberculosis, Enterococcus faecalis*, and *Staphylococcus aureus*, and once acquired, the traits are constitutive and chromosomally encoded.

When considered together as a collective mechanism, altered penicillin-binding proteins (PBPs) play a major role in β-lactam resistance. The term "PBP", however, is somewhat of a misnomer because reduced affinity or quantity of these proteins impacts β-lactams in addition to penicillin. These alterations may be manifested as PBPs that are normal in function but with either reduced levels of production or reduced drug affinity. Organisms for which clinical β-lactam resistance may be attributable to PBP alterations include *E. coli, Staphylococcus* species, *Enterococcus* species, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Bacteroides fragilis, N. meningitidis*, and *N. gonorrhoeae*. Most data suggest that more than a single PBP change within a given species may be associated with resistance and the changes reflect levels of susceptibility, patterns of resistance, or both. Among the staphylococci, however, the appearance of a novel protein, PBP 2′ (PBP 2a) encoded by the *mecA* gene, has

been closely associated with resistance to penicillinase-resistant penicillins (e.g., methicillin) and is subject to sophisticated regulation by factors some of which may be plasmid-encoded (Georgopapadakou, 1993).

Resistance to sulfonamides and trimethoprim is most often mediated by alterations in folate pathway enzymes that are targeted by these drugs. The alteration may be production of a normal enzyme (e.g., dihyropteroate synthetase or DHFR) but in quantities that overwhelm available concentrations of drug. Low-level trimethoprim resistance (MIC $\leq$ 100 μg/ml) in staphylococci is reportedly due to the overproduction of DHFR. Synthesis of an alternative enzyme that functions as DHFR but which is not affected by trimethoprim confers high-level resistance (MIC > 1000 μg/ml) to this drug (Lyon and Skurray, 1987). Since folate metabolites are synthesized using these alternative enzymes, this mechanism of resistance to antifolate drugs is often referred to as "bypass" resistance.

Mutations which affect either subunit A or subunit B of DNA gyrase may be responsible for quinolone resistance. High-level resistance to nalidixic acid, as well as resistance to newer 4-fluoroquinolones (e.g., norfloxacin) in *E. coli* has been reported to be associated with *gyrA* (*nalA*) mutations encoding the A subunit. Conversely, mutations affecting the gyrase B subunit may be more important in *P. aeruginosa* (Inoue et al., 1988). The relative importance of exclusion versus altered target mediated resistance to quinolones and rifamycins among clinically important organisms remains largely unknown. It is known, however, that amino acid substitutions, deletions, or insertions in the β subunit of RNA polymerase encoded by the *rpoB* gene have been associated with rifampin resistance in *Mycobacterium* species as well as *E. coli* (see Table 2) (Telenti et al., 1993; Musser, 1995).

Table 2. Altered Antibacterial Drug Targets

Target	*Drug Class(es)*	*Organism(s)*
Ribosome		
23S subunit methylation	Macrolides, lincosamide streptogramin (MLS)	Gram-positive cocci
16S subunit effects	Aminoglycosides, aminocyclitols	Gram-positive cocci, Gram-negative bacilli, *Mycobacterium* sp.
β-lactam binding proteins (PBPs)	Most β-lactams	Gram-negative species, Gram-positive species, *Bacteroides* sp.
Altered enzyme	Sulfonamides, trimethoprim, rifampin, quinolones	Gram-negative species, Gram-positive species, *Mycobacterium* sp.

DRUG MODIFICATION

Serious problems of drug resistance leading to treatment failures may be caused by modification of antibacterial drugs by enzymes produced by resistant organisms. There are two underlying genetic factors that have exacerbated the problems associated with enzymatic drug modification. First, drug modification genes, more often than for other classes of resistance mechanisms, tend to be located upon transferable genetic elements, principally plasmids and transposons, that often have broad host ranges. Secondly, especially for β-lactamases, subtle changes in the enzyme's structural gene sequence that lead to minor amino acid substitutions often have profound effects resulting in extensions of a particular enzyme's substrate (e.g., drug) profile (Philippon et al., 1989; Jacoby and Medeiros, 1991). β-lactamases, aminoglycoside-modifying enzymes (AMEs), and chloramphenicol acetyltransferase (CAT) are the most studied classes of drug modification enzymes, although drug modification has been described for MLS drugs and tetracyclines, and theorized for glycopeptides. Enzymatic modification has not yet been reported as a mechanism of resistance to sulfonamides, trimethoprim, the quinolones, or the antituberculosis drugs.

The first β-lactamase classification scheme, reported in 1973 by Richmond and Sykes (Richmond and Sykes, 1973), and expanded in 1976 by Sykes and Matthew (Sykes and Matthew, 1976), categorized these enzymes primarily on substrate profile, enzyme inhibitors, and the net electric charge (isoelectric point) associated with the enzyme. These schemes were updated by Bush in 1989 and again in 1995 to include additional biochemical parameters such as kinetic data, molecular weight, and susceptibility of the enzyme to more recently introduced β-lactamase inhibitors (e.g., clavulanic acid, tazobactam, or sulbactam) (Bush, 1989; Bush et al., 1995).

β-lactamases may have evolved from penicillin-binding proteins, creating an ironic "prey becoming the predator" scenario. Currently, β-lactamases may be found in almost any bacterial genus examined, although many are of no clinical consequence. β-lactamases belonging to Bush Class 1 (Richmond and Sykes Class 1), or CEP-N Class, are not susceptible to 10 μM concentrations of clavulanic acid, and are produced by most strains of enterobacteria and *P. aeruginosa*, but usually at inconsequentially low constitutive levels. Typical of the loci encoding these enzymes is the wildtype *ampC* locus in the *E. coli* genome. In some instances the Class 1 β-lactamase gene may be induced, or stably derepressed, to produce enzyme in sufficient quantity to confer resistance to a broad range of β-lactam drugs (Livermore, 1987; Livermore, 1995).

The first plasmid-mediated β-lactamase reported in gram-negative bacilli, TEM-1, was shown to be structurally similar to the original penicillinase found in *S. aureus* in the 1940s (East and Dyke, 1989). Examples of other β-lactamases that are related to TEM (Bush Class 2), at least to the extent that they are susceptible to inhibition by clavulanic acid, include SHV (and variants), OHIO-1, TLE, K1, CTX,

CEP, LXA, ROB and CAZ. Most of these enzymes are constitutively produced and plasmid encoded. At least 36 variants of TEM-1, some of which are capable of hydrolyzing even the most recently introduced β-lactams, have been described so far. During the last 15 years more than 40 new β-lactamases capable of conferring resistance to third generation cephalosporins have been described (Jacoby and Medeiros, 1991; Bush, 1995). Attempts to overcome this proliferation with novel compounds that either inhibit the enzyme or resist enzymatic inactivation, will more than likely exert positive pressure on the emergence of additional TEM variants. Perhaps even more alarming, although not unexpected, are recent reports of TEM variants that are less susceptible to β-lactamase inhibitors (Blazquez et al., 1993; Bush, 1995).

Gram-negative β-lactamases are translated cytoplasmically, then transported across the inner membrane to the periplasm which is the site of drug interaction. A "leader" peptide (usually 23 amino acid residues in length), attached to the amino terminus of the cytoplasmic enzyme, is required for transport and is cleaved to yield the mature periplasmic β-lactamase. Kinetic interaction and subsequent hydrolysis of the β-lactam substrate, and therefore the primary and secondary structure of the enzyme, are the most important factors in the expression of β-lactamase mediated resistance. Other unusual factors, such as hyperproduction of enzyme, reduced drug uptake via altered outer membrane proteins, reduced PBP binding capacity, or an interplay of several factors, undoubtedly influence the levels and patterns of resistance to β-lactams.

Hydrolysis of the β-lactam ring is a biochemical mechanism of action that is common to all β-lactamases. Aminoglycoside-modifying enzymes (AMEs), however, are classified according to three distinct effects upon the drug molecule: adenylation (the enzymes are abbreviated "ANT" or "AAD"), acetylation ("AAC"), or phosphorylation ("APH") (Shaw et al., 1993). Within the three AME classes, there are at least 50 variants, many of which are plasmid encoded. Most, if not all, AMEs are constitutively produced and remain in the cytoplasm which is their site of drug interaction (Bryan, 1984). Some AMEs do not completely inactivate their substrate, but only modify the molecule, for example by altering secondary structure, which may only reduce its efficiency of transport or ribosome interaction. Specific enzymes are designated according to the site of modification on the drug molecule. Resistance to kanamycin, for example, may be conferred by ANT(4′), which adenylates the hydroxyl group at the fourth carbon of the single-prime ring moiety attached to the basic deoxystrentamine ring (or streptidine if streptomycin) at its fourth carbon. One notable exception to AMEs that exert single-site effects is the bifunctional (AAC[6′]-APH[2″]) enzyme, described originally in *Enterococcus faecalis* (Ferretti et al., 1986) and now found often associated with transposons in staphylococci and enterococci. Some AMEs loci are known to be shared between Gram-positive and Gram-negative species, and aminoglycoside usage is well documented as a risk factor in the establishment of AME gene pools within clinical institutions (Larson et al., 1986; Levine et al.,1985; Buisson et al., 1990). More than

a single AME locus is often carried on a common conjugative plasmid along with other resistance genes, for example, extended spectrum β-lactamase genes creating the threat of simultaneous multidrug resistance emerging in normally susceptible organisms.

Laboratory characterization of AMEs is traditionally performed by a method that exploits the affinity of aminoglycosides for phosphocellulose paper, and which requires radioisotopes and various cofactors that simulate the enzyme's natural environment (Davies et al., 1971). Assays which identify specific AME genes using DNA probes or amplification by the polymerase chain reaction, however, are now used in research more frequently than the phosphocellulose binding assay.

Enzymatic acylation is a clinically important mechanism of resistance to chloramphenicol and related compounds (Shaw, 1983). Several differences exist in chloramphenicol acetyl transferases (CATs) from Gram-negative bacilli and those found in Gram-positive cocci, although both families of enzymes achieve the same end result and point mutations in loci of the two families can complement one another. Gram-negative CATs tend to be constitutively produced and found in association with other resistance loci on larger (> 20 kilobase) plasmids, whereas in staphylococci CAT loci have been shown to be inducible and occur usually in the absence of other resistance markers on small (< 5 kilobase) plasmids. These enzymes are extruded into the Gram-negative cell environment but are typically confined to the Gram-positive cell cytoplasm. They are readily detectable using a commercial kit which utilizes acetyl coenzyme A as a substrate. A sulfhydryl

Table 3. Enzymatic Inactivation of Antibacterial Agents

Enzyme	*Substrate(s)*	*Organism(s)*
β-lactamase		
Plasmid mediated	penicillins (amino-, ureido-, carboxy-), first and second generation cephalosporins	Gram-negative species, *Staphylococcus* sp.
Extended spectrum	Most β-lactams except carbapenems, monobactams, cephamycins	Gram-negative bacilli
Constitutive chromosomal	Most β-lactams except imipenem	Gram-negative bacilli
Carbapenemase	Imipenem	Gram-negative bacilli
Aminoglycoside modifying enzymes (AMEs)	Aminoglycosides, aminocyclitols	Most bacteria
Chloramphenicol acetyltransferase (CAT)	chloramphenicol, fusidic acid	Gram-negative bacilli, Gram-positive cocci
MLS acetyl transferase	streptogramin	*Staphylococcus* sp.
MLS hydrolase	streptogramin	*Staphylococcus* sp.
MLS esterase	erythromycin	*Escherichia coli*
MLS phosphotransferase	Macrolides	*Escherichia coli*

moiety, exposed upon deacylation of the kit substrate by CAT, reacts with 5,5′-dithiobis-2-nitrobenzoic acid to yield a yellow color (Table 3).

TOLERANCE AND PERSISTENCE

Microorganisms are considered tolerant to a bactericidal agent when they are inhibited by normally effective concentrations of the drug but yet evade its killing action. Even though the drug's MIC reflects normal susceptibility levels, its MBC (minimum bactericidal concentration) is unusually elevated (Handwerger and Tomasz, 1985). The MBC is routinely defined as the drug concentration that is required to kill at least 99.9% of the susceptibility test inoculum. When this amount of drug is ≥ 32 times the MIC (MBC/MIC ratio is ≥ 32), the organism is considered genotypically tolerant. The mechanism of tolerance to β-lactams involves defects in the organism's autolytic processes. After binding and acylation of essential PBPs in the bacterial plasma membrane by the β-lactam, death of the cell normally ensues when covalent bonds in the cell wall are hydrolyzed by a group of ubiquitous enzymes called autolysins or murein hydrolases. Tolerance most likely is due to changes in these enzymes, the levels of their production, or in controls of their enzymatic activities. A second type of tolerance, referred to as phenotypic tolerance, relates more to environmental (e.g., growth) conditions than to genetic defects in the organism. Factors such as pH, serum levels, cation concentration(s), inoculum growth state and/or size, and the presence of certain autolytic inhibitors may enhance antimicrobial tolerance. Persistence, a phenomenon associated most often with *in vitro* susceptibility studies of *S. aureus*, refers to the survival of a small proportion (< 0.1%) of cells in the presence of normally cidal drug concentrations, presumably due to their physiologic state. Since β-lactams affect only exponentially growing cells undergoing active cell wall synthesis and autolytic processes, cells that are present in a stationary growth phase may persist and, therefore, escape death. Persistence and tolerance must be carefully considered when performing and interpreting *in vitro* susceptibility tests. Even when test conditions such as medium, ionicity, pH, and inoculum are optimal, correlation of *in vitro* susceptibility data to expected therapeutic outcomes for a given drug/bug combination is only approximate.

THE IMPACT OF ANTIMICROBIAL RESISTANCE

For any form of chemotherapy the worst possible outcome is treatment failure, and for antimicrobics this failure is most often caused by drug resistance in the infecting organism. Drug resistance is becoming so pervasive, and challenges to overcome resistance so great, that research and development programs for new antimicrobics may be in jeopardy. If the entry of new drugs does not maintain pace with resistance problems, attitudes regarding infection control will require dramatic changes. Future empirical therapy may rely more upon combination or rotational antimicro-

bic regimens, acceptance of increased risks of untoward drug effects, immune modulators, or vaccine development.

Although drug resistance has been found in virtually every microbial species that has been targeted, there are at least seven areas of infectious diseases where resistance has exerted perhaps the greatest impact. These include nosocomial infections, bacterial meningitis, enteric diseases, sexually transmitted diseases, tuberculosis, parasitic diseases, and resistant human immunodeficiency virus (HIV) infections. Among nosocomial pathogens, the staphylococci, particularly methicillin-resistant *S. aureus* (MRSA) continue to pose perhaps the greatest treatment challenge. As the second most common nosocomial pathogen, as many as fifty percent of *S. aureus* isolates from large teaching hospitals are resistant to at least one normally effective antistaphylococcal drug, including an alarmingly high incidence of resistance to recently introduced fluoroquinolones. The only remaining drug to treat some multidrug resistant staphylococci is vancomycin, and concern regarding the development of resistance to this drug is valid, considering the presence of vancomycin resistance genes on plasmid among the enterococci and the tendency of these two genera to share plasmid encoded resistance traits.

Ranking above *S. aureus* in its prevalence of nosocomial infections is *E. coli* which accounts for approximately twenty percent of nosocomial infections, principally targeting the urinary tract. The ubiquitous distribution of *E. coli* both in the environment and as part of the normal bacterial flora on healthy humans makes this species a likely conduit for resistance genes into health care institutions. Multidrug resistance plasmids are shared among several nosocomial Gram-negative opportunists, and the continued use of newer drugs, such as ceftazidime or amikacin in surgical wards and/or intensive care units, exerts selective pressure toward the dissemination of these plasmids as well as the broadening of their resistance profiles. Another major contributor to the spread and entrenchment of resistance among Gram-negative bacilli is the family of transposons related to Tn*21*. These elements facilitate the shuttling of a wide variety of resistance genes, including those encoding various β-lactamases, AMEs, and CAT. The Tn*21*-like transposons apparently encode an unusually active integrase gene enabling their recombination-independent movement among plasmids and genomes of many Gram-negative species (Mercier et al., 1990).

Therapy with antimicrobial agents exert positive pressure on the emergence of resistant organisms through either the selection of endogenously resistant species or resistant variants or by facilitating the acquisition of resistant organisms from the natural environment. Since plasmids or transposons do not (yet) play a role in the development of drug resistance in *M. tuberculosis*, selection of resistant variants (secondary resistance) and epidemic spread of these variants (primary resistance) are the key mechanisms for emergent resistance in this species. The frequency of mutations causing phenotypic resistance in *M. tuberculosis* varies from 10^{-7} (one in ten million cells) for ethambutol to 10^{-10} for rifampin. The frequency of resistance to both compounds simultaneously is, therefore, 10^{-17}. While it is

obvious that multidrug therapy of tuberculosis greatly reduces the probability of resistance development, failure to complete the prescribed regimen (usually six months in otherwise healthy individuals) often leads to reactivation of the disease and often the proliferation of the rare resistant mutant. An alarming one-third of 466 tuberculosis cases surveyed in New York City in 1991 were caused by drug resistant *M. tuberculosis* and the strongest predictor of the presence of resistant organisms was a previous history of antituberculosis therapy (Freiden et al., 1993). Solutions to problems of antimicrobial drug resistance reside in the concerted efforts of laboratories in which resistance is studied *in vitro*, epidemiologists who must identify and control outbreaks involving resistant organisms, the pharmaceutical industry which must develop safe antimicrobics that maintain their effectiveness, and primary infectious disease physicians who must carefully choose treatment regimens. Experts from these four disciplines must have knowledge of the underlying resistance mechanisms at the microbial cellular level in order for the pursuit of these solutions to be successful.

SUMMARY

The discovery more than fifty years ago that microorganisms naturally produced substances that inhibited other microorganisms introduced the antibiotic era. It has become evident, however, that bacteria are capable of developing resistance to almost all antibacterial agents. An understanding of the genetic and biochemical mechanisms that explain resistance is important for treatment decisions, new drug development and epidemiologic control of the spread of resistance. There are three major classes of antibacterial resistance mechanisms—drug exclusion, altered target, and enzymatic drug modification. Within each of these groups some mechanisms may be intrinsic, others arise by chromosomal mutations that are passed only to cellular offspring, and others may be rapidly disseminated among different strains and species on mobile genetic elements that may encode multiple drug resistance. The survival of the antibiotic era will require that advances in rapid diagnosis and characterization of these mechanisms be used in conjunction with carefully chosen treatment regimens, infection control practices, and pharmaceutical development strategies.

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Chapter 14

Lyme Disease

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INTRODUCTION

Lyme disease (LD) is a multi-system inflammatory disease caused by the spirochete, *Borrelia burgdorferi*, spread by the bite of infected Ixodes ticks. In 1975, a group of researchers at Yale University described Lyme arthritis, an outbreak of "juvenile rheumatoid arthritis" in three small towns in Connecticut (Steere et al., 1977). Five years later, this arthritis was linked to a preceding outbreak, known as erythema migrans (EM) and tick bite. The first report of EM in the Americas was in 1970 in Wisconsin (Scrimenti, 1970). "Lyme arthritis," as it was first named, has become known as "Lyme disease," in part because of the association with cardiac disease, but also because approximately 10% of untreated patients had a neurologic syndrome essentially identical to a tick-borne neurologic syndrome in Europe, known as Bannwarth's syndrome. The multi-system, inflammatory nature of Lyme disease, a multi-focal epidemic disease, has thus been established.

EPIDEMIOLOGY

By 1990, Lyme disease was identified as an epidemic disease in the Northeast (from Massachusetts through Pennsylvania, with cases described as far south as Georgia); the northern Midwest (primarily Minnesota and Wisconsin), and in northern California and Oregon. Ninety percent of the cases reported in the United States are from these three regions, and the total number has steadily increased each year. Most cases occur between April and October, although even in the Northeast and Midwest, some cases are acquired in November and December.

After *B. burgdorferi* was identified as the etiologic agent of Lyme disease in the United States, studies established that the same organism was the cause of EM in Germany, Austria, and Scandinavia. In addition, two other skin lesions reported in Europe from the late nineteenth century, acrodermatitis chronica atrophicans (ACA) and lymphadenosis benigna cutis (LABC), and tick-borne meningopolyneuritis, or Bannwarth's syndrome, were established as being caused by infection with the same organism.

Lyme disease is worldwide in distribution, including Africa, Asia, and Australia. The primary vector in each area is an Ixodes tick, *Ixodes scapularis* in the Northeast, Midwest, and Southeast, *I. pacificus* in California, *I. ricinus* in Europe, and *I. persulcatus* in Asia, and possibly *I. hyocyclus* in Australia. (Previously it was thought that the vector in the Northeast and Midwest was a separate species, *I. dammini*- what we now know is actually *I. scapularis*, so do not be confused by the older literature which refers to *I. dammini*). The disease is primarily spread by the nymphal stage of the tick.

The eggs of *I. scapulari* are laid in the leaf clutter at the floor of the forest in the fall and hatch in the spring, with the resulting larvae emerging and seeking a blood meal. They typically get this meal from a white footed field mouse. If the mouse is infected, the larva, which is almost always born uninfected, can then acquire *B.*

burgdorferi; mice can carry the organism for long periods of time without any untoward effects and so are reservoirs of the infection. Once the larva gets its meal it drops off and undergoes a metamorphosis emerging in the spring as a nymph. They lie in wait for a host by clinging to the underside of a blade of grass. Nymphs are a bit more active than the larvae in getting a blood meal; most get a meal from a mouse, but any animal which exhales carbon dioxide and is warm can be identified by the tick as a suitable host. In this way an infected tick can latch onto a mouse, a dog, a fox, a horse, or a human for its blood meal. The blood meal lasts for about 48 hours and it is only at the end of the feeding process that the infection is passed. The reason for this is that the organism is dormant in the gut of the tick. After the start of feeding it becomes more active and begins to proliferate. It is only after the increase in numbers of organism that *B. burgdorferi* migrates to the salivary glands and is passed into the host. Thus, the best evidence suggests that it takes a long time for the infection to be passed to the host. The nymphs are actively seeking a host in the spring, summer, and early fall— this is therefore the time of year that Lyme disease is most prevalent.

Once the nymph has fed, it drops off and undergoes its final metamorphosis, emerging in the fall as the adult. The adults typically get their blood meal from deer (thus, the tick that spreads Lyme disease in the Northeast and Midwest is known as the deer tick). Once the adults mate, the adult dies (having, in a Darwinian sense, completed his life's responsibility) and the female drops off to lay her egg mass in the leaf clutter; then she too dies. Adults can feed on humans and can spread disease, but they are large enough that people can feel or see them and they are active in the fall and winter, when people are not out-of-doors very much and are reasonably well-clothed. In California, where one can still wear shorts in November and December, a risk of acquiring Lyme disease still exists at that time of year. As you can see, understanding the life cycle of Ixodes ticks explains a large part of the timing of Lyme disease.

Sufficient new information has been generated to hold five international convocations and fill four volumes (Steere et al., 1984; Stanek et al., 1986; Benach and Bosler, 1988; Skoldenberg and Stiernstedt, 1991); the fifth such meeting was held in June 1992, in Arlington, Virginia (Sigal, 1994a).

CLINICAL DESCRIPTION OF LYME DISEASE

Terminology

Lyme disease (LD) can cause damage to a number of organ systems (Sigal, 1988; Steere, 1989). *Early localized LD* usually occurs within a month of inoculation with *B. burgdorferi* (Steere et al., 1983; Benach et al., 1983). The clinical syndrome includes EM, a marker for LD, and associated symptoms, often described as resembling a "summer cold." *Early disseminated LD*, which includes cardiac and/or neurologic disease, usually occurs two to three months after the infection.

Late LD includes arthritis and/or chronic neurologic manifestations; these late features of LD may occur years after EM. Early disseminated or chronic LD may occur in the absence of any preceding history suggestive of earlier LD.

Early Localized Lyme Disease

This occurs about a week after tick bite (range 1 to 30 days, median 7 days) and consists of EM and associated symptoms: fever, fatigue, malaise, headache, stiff neck, arthralgia, and myalgia. About 50 to 70% of patients will have EM and, of these, 50% of patients will have multiple lesions. Only about 30% of patients recall the tick bite; the nymphal stage tick is about the size of a sesame seed and so is easily overlooked. Regional (occasionally generalized) lymphadenopathy may occur. There may be pain on neck flexion, conjunctivitis, sore throat, temporomandibular joint pain, and hepatosplenomegaly and/or right upper quadrant tenderness (Steere et al., 1983a). In the absence of EM, nothing about this symptom complex is remarkably different from a viral infection. No combination of these findings is specific or even strongly suggestive of LD in the absence of EM.

Laboratory studies, like erythrocyte sedimentation rate, complete blood count, and liver function tests, are abnormal in between 20 and 50% of cases, but are not specific and so are not helpful in diagnosing LD (Steere et al., 1983a). Early in disease, specific serologic tests may be negative and seroconversion may not occur for up to 8 weeks (Craft et al., 1984; Russell et al., 1984). *B. burgdorferi* has been cultured from biopsy specimens taken at the outer border of the erythema and has been grown from the blood of such patients (Steere et al., 1983).

Early Disseminated Lyme Disease

Two to three months following the onset of EM, about 10 to 15% of untreated patients with LD will develop neurologic complications. Meningoencephalitis, meningitis, cranial nerve (especially facial) palsies, and peripheral neuropathies may occur, alone or often in combination (Reik et al., 1979; Pachner and Steere, 1985; Pfister et al., 1986), often accompanied by fatigue, malaise, headache, and photophobia; fever is usually absent. Mild encephalopathy, including difficulty with concentration and memory, and irritability or emotional lability may occur. These neurologic findings are identical to those described as Bannwarth's syndrome (tick-borne meningopolyneuritis) in Europe in the early part of this century (Bannwarth, 1941). A cerebrospinal fluid lymphocytic pleocytosis is found, with elevated protein, but normal glucose levels (Reik et al., 1979; Pachner and Steere, 1985). The meningitis of LD is indistinguishable from that of enterovirus, virtually epidemic in the early fall. *B. burgdorferi* has been grown from the cerebrospinal fluid of patients with Lyme meningitis (Steere et al., 1983).

Neuropathic changes have been found on nerve conduction testing (Pachner and Steere, 1985), with changes of an axonopathy seen in one third of patients with peripheral neuropathy (Halperin et al., 1988). Nerve biopsies have shown heavy

epineural vessel infiltration with mononuclear cells (Duray, 1977; Camponovo and Meier, 1986; Vallat et al., 1987; Halperin et al., 1988; Kristoferitsch, 1988), but the organism has never been found. Vasculitis, but no organisms, was seen in one case (Camponovo and Meier, 1986); luminal obliteration of perineural vessels without vasculitis was found in another (Duray, 1977). Immune complexes, immunoglobulin, and infiltration with mononuclear cells (Duray, 1977; Camponovo and Meier, 1986; Vallat et al., 1987; Halperin et al., 1988; Kristoferitsch, 1988) complement have not been seen in biopsy specimens (Camponovo and Meier, 1986; Halperin et al., 1988).

Vascular disease caused by *B. burgdorferi* may be the underlying mechanism for the reported cases of cerebrovascular disease (May and Jabbari; 1990) and changes compatible with vasculitis have been seen on angiographic study of one patient with LD central nervous system disease (Midgard and Hofstad; 1987).

Many patients with encephalopathic symptoms have abnormalities on electroencephalography (Pachner and Steere; 1985) and reversible neuropsychiatric testing abnormalities have been documented (Halperin et al., 1988); in some patients small plaques have been found on magnetic resonance imaging (suggesting foci of inflammation or demyelination), occasionally resolving after antibiotic therapy (Halperin et al., 1988). Our experience has been that there are often widespread, often severe, abnormalities on both neuropsychologic testing and quantitative electroencephalography, some of which may resolve after therapy (Sigal, L.H., Fiedler, N., Willard-Mack, J., and Chabot, R., unpublished observations).

Cardiac disease occurs in 8 to 10% of previously untreated patients, in the same time period as, and occasionally coincident with, neurologic LD. Atrioventricular conduction defects, mild congestive heart failure, and ST and T wave changes compatible with myopericarditis have been reported (Steere et al., 1980). Reversible (Sigal, 1993a) and rarely fatal (Marcus et al., 1985) myocarditis has been reported, as has a chronic congestive cardiomyopathy related to *B. burgdorferi* (Stanek et al., 1990). The multi-focal damage documented in electrophysiologic studies (Steere et al., 1980; Reznick et al., 1986) is the likely explanation for multiple levels of heart block often seen, occasionally in rapid succession. Focal myonecrosis and a sparse interstitial infiltrate of polymorphonuclear cells and lymphocytes was described in one report of myocardial biopsies (Duray, 1977), while *B. burgdorferi*, myonecrosis, and perivascular mononuclear cell infiltration was found in others (Reznick et al., 1986). The finding of organisms within the myocardium (Marcus et al., 1985; Stanek et al., 1990) suggests that direct invasion occurs in Lyme myocarditis.

Late Lyme Disease

Arthritis is the classic late feature of LD (Steere et al., 1979), and the first feature of LD described fifteen years ago. A summary of the experience with 55 patients

who got LD before the efficacy of antibiotics had been established, and therefore did not receive antibiotic therapy, has been published. This is the only group of patients where the natural evolution of Lyme arthritis can be defined (Steere et al., 1987). Prospective study of these patients revealed that 44 (80%) experienced articular problems over the course of a six year period, including: ten patients (18%) with arthralgias with or following EM (1 day to 8 weeks [mean, 2 weeks] after the EM); twenty-eight patients (51%) with polyarthritis, often migratory, 4 days to 2 years after the onset of EM (mean, 6 months); half of whom reported preceding migratory arthralgias; and six patients (11%) with chronic, usually mono-articular, Lyme arthritis (most often the knee), onset 4 months to 4 years after EM (mean, 12 months). Five of these six patients had either arthralgia or intermittent arthritis prior to developing chronic synovitis. Migratory polyarthritis is the complaint which first brought "Lyme arthritis" to light in 1975 (Steere et al., 1977).

The synovium resembles rheumatoid synovium (Duray, 1977; Steere et al., 1979; Johnston et al., 1985), although there are subtle differences in pathology. In LD, there are hypertropic and hyperplastic changes, focal necrosis, vascular proliferation, and chronic inflammatory cell infiltration. Mononuclear cell aggregates and lymphoid follicles with germinal centers may be present, suggesting that the synovium is the site of an immunologic reaction (Sigal, 1989; Sigal, 1993). As in syphilis, endarteritis obliterans may be seen (Johnston et al., 1985), not very surprising given that both LD and syphilis are due to spirochetes. *B. burgdorferi* has been seen rarely in or near synovial vessels (Johnston et al., 1985) or in synovial fluid (Snydman et al., 1986), and has been grown from synovial fluid (Schmidli et al., 1988).

Tertiary neuroborreliosis, a term which deliberately draws upon the clinical analogy with tertiary neurosyphilis, includes chronic encephalomyelopathy and neuropathy (Pachner, 1986; Pachner and Steer, 1986; Ackermann et al., 1988). Like tertiary neurosyphilis, late neurologic features of LD may develop insidiously over months or years, even in the absence of clinically apparent preceding LD. Subclinical infection thus may occur for long periods prior to the emergence of overt neurologic damage; this raises serious concerns about asymptomatic seropositivity, i.e., possible "latent LD," found in a significant percent of people in areas endemic for LD.

Studies of patients with tertiary neuroborreliosis are limited, and the true clinical spectrum of this aspect of LD is being defined. Claims that amyotrophic lateral sclerosis (Mandell et al., 1989), multiple sclerosis (Coyle, 1989), and Alzheimer's disease (Pappolla et al., 1989) are due to infection with *B. burgdorferi* have been laid to rest. Many cases of neurologic damage have been stated as due to *B. burgdorferi* infection merely because the patient has a positive blood test; such circumstantial evidence does not prove causality (Sigal, 1993b). Misdiagnosis of patients with other neurologic disorders as having LD is a serious problem in some geographic areas.

Other Possible Features of Disease

A number of other clinical conditions have been ascribed to *B. burgdorferi*. On the basis of seroepidemiologic and biopsy evidence, it has been suggested that cutaneous lesions, including morphea, lichen sclerosis et atrophicans, eosinophilic fascitis, and other fibrotic lesions, are due to *B. burgdorferi*, although the claims are by no means proven. Subclinical infection may be common in endemic areas, so that seropositivity may represent coincidence and no more. There is no doubt, however, that ACA and LABC are due to *B. burgdorferi*; these lesions are reviewed in a volume of the Clinics in Dermatology (Asbrink and Hovmark, 1993). Other cutaneous conditions, e.g., erythema nodosum, and non-cutaneous, e.g., ophthalmologic disease, mild hepatitis, and myositis, have been linked to infection with *B. burgdorferi*; if the relationships are true, they are very rare findings.

Pregnancy and Lyme Disease

Another area of major concern relates to *B. burgdorferi* infection complicating pregnancy. Shortly after the LD epidemic in the Northeast was reported, adverse outcomes of pregnancy complicated by LD began to appear. In a review of 19 pregnancies complicated by LD between 1976 and 1984, 14 normal births and 5 adverse outcomes were noted (Markowiz et al., 1986). Subsequent reports attributed toxemia of pregnancy and other cases of fetal anomalies and fetal demise to *B. burgdorferi* infection; these have been reviewed (Williams and Strobino, 1990). At this time, no incontrovertible proof has been found that infection with *B. burgdorferi* causes adverse pregnancy outcomes. The experience at a number of centers, including our own, has been that if treated properly LD represents no defined risk to the fetus. Recent studies have failed to demonstrate any link between LD and congenital neurologic defects or between LD and abortion (reviewed in Williams and Strobino, 1990).

OTHER MEANS OF TRANSMISSION

There is no evidence that LD can be passed by sexual or other intimate contact. There is evidence, however, that *B. burgdorferi* can survive in blood (Baron et al., 1989) and various blood products (Baranton and Saint-Girons, 1988), for as long as 6 to 8 weeks (Sigal, L.H., unpublished observations). One study demonstrated that the risk of getting LD from transfusion is minimal if present at all (Gerber et al., 1992). Other insects, e.g., flies, mosquitoes, other ticks, have been described as spreading LD, but Ixodes ticks are clearly the vectors in the vast majority of all cases.

DIAGNOSTIC TESTING IN LYME DISEASE

The diagnosis of LD can be aided by the proper use of serologic and cellular immune testing. However, LD remains a clinical diagnosis, to be considered once a carefully obtained history and physical examination suggest the diagnosis. In the absence of a set of well substantiated criteria for the diagnosis of LD, e.g,. the Jones criteria for rheumatic fever, there is no substitute for a careful history and physical done by a well prepared health care provider, confirmed, as needed, by laboratory evidence of preceding infection. Recall Bayes' theorem: if the *a priori* likelihood of a disease is low, the positive predictive value of a test is low; if the *a priori* likelihood is high, the positive predictive value is high. Stated in real English: Do not cast large nets into the uncharted ocean; you may not know what you will catch or what to do with it!

Enzyme-linked immunosorbent assay (ELISA) is the test most often used, although the technique is not standardized. ELISA measures antibody to a preparation derived from the entire organism and can result in false-positive results. Most striking is the possibility that patients with other spirochetal infections (other Borrelial infections, syphilis [due to *Treponema pallidum*], and occasionally leptospiral infections) will test falsely positive for LD. Also a potential problem relates to patients with diseases where there is non-specific activation of many clones of B cells, termed "polyclonal B cell activation," e.g., malaria, Epstein-Barr virus infection, subacute bacterial endocarditis, and other chronic inflammatory diseases. If a patient makes a very strong antibody response to a protein in another microbe which strongly resembles a component of *B. burgdorferi*, it is possible to test positive by ELISA. This is especially the case where patients have had recent infections with organisms which contain flagella; the components of these motility organelles are closely related, so that a strong humoral response to the flagellin of a Gram-negative bacillus may cross-react with *B. burgdorferi's* flagellin and cause a false-positive ELISA. Recall that antibodies are not absolutely specific for their targets. One of the wonders of the immune system is the fact that there is some degree of polyspecificity of antibodies; the antibody you make to one bacillus may cross-react with another and thereby render you immune to an organism to which you have not previously been exposed; the negative aspect of this is that diagnostic tests can sometimes be falsely positive. Finally, results of ELISA are usually expressed with respect to a normal control level, established by testing many uninfected normal people. If one defines the "upper limit of normal" in an assay as being greater than 95% of the normals tested, one will, by definition, state that 5% of "normals" are positive; think about this, because the concept is crucial to understanding why false positive tests are so common.

Immunoblot assays are widely used to confirm positive ELISAs and to identify "false positive" ELISA results, which occur in up to 5% of the normal population; criteria for immunoblot interpretation have been suggested (Dressler et al., 1993). Immunoblot measures antibody to individual components of the organism which

have previously been separated by electrophoresis. The technique is probably more specific for the diagnosis of LD if done and interpreted properly. An interesting problem in serologic testing is that not infrequently patients may remain seronegative for up to 6 to 8 weeks; inoculation of a protein antigen into a naive host usually results in antibody production within 2 weeks. Thus, some sort of immunomodulation induced by *B. burgdorferi* has been postulated, but not proven. Cellular testing has little value, except in looking for concentration of antigen-specific T cells in inflammatory fluids with respect to peripheral blood (Pachner et al., 1985; Sigal et al., 1986).

Polymerase chain reaction (PCR) detection of DNA derived from *B. burgdorferi* in blood, cerebrospinal or synovial fluid and urine is used clinically, although its role in diagnosis is as yet unclear (Nishio et al., 1993). PCR can detect as few as five or fewer copies of the genetic material of the organism. It depends on the use of a very specific nucleic acid "probe," which anneals to and assists in the copying of genetic material of the organism in the sample, through the use of a thermostable DNA polymerase. One limitation of this test is that it does not differentiate between live and dead organism, so that PCR positivity does not establish that the patient has active infection.

Culture of the organism is a relatively difficult procedure with low yield. Thus, at this time, attempts at culturing *B. burgdorferi* from clinical specimens is not an effective diagnostic tool in LD.

ANTIBIOTIC TREATMENT OF LYME DISEASE

Antibiotic therapy in early localized LD usually results in cure and prevents progression to later features. Oral therapy is recommended for early disease; even for severe cases, intravenous drugs are not necessary. The only comparative study of antibiotic therapy in early disease suggested that either penicillin or tetracycline was more effective than erythromycin (1000 mg in 4 divided doses for 10 days) in preventing progression; 20 days of tetracycline was no better than 10 days (Steere et al., 1983b). There are many antibiotic regimens for early LD and no evidence that one drug is superior to another: tetracycline, amoxicillin, ampicillin, and penicillin have been used with good success, at doses of between 1000 and 2000 mg per day, in 4 divided doses and doxycycline at 100 mg 2 or 3 times a day is also effective. The optimum duration of therapy has not been determined, although current practice is generally to treat for 3 to 4 weeks (Rahn and Malawista, 1991; Sigal, 1992). There may be remarkable worsening of signs and symptoms of disease (Steere et al., 1983b), accompanied by fever, chills, malaise, headache, and myalgia, in approximately 15% of patients, within one or two days of institution of therapy; all usually resolve within a day or so. This phenomenon was first described in syphilis and is known as the Jarisch-Herxheimer reaction. Such reactions may also be experienced early in the therapy of other Borrelial infections, and of Brucellosis, where the reaction may be life threatening. It appears that antibiotics disrupt the

organism and cause the liberation of Borrelial components. When the immune system encounters these proteins, polysaccharides, and lipids, a sudden systemic response occurs, with exacerbation of symptoms–the Jarisch-Herxheimer reaction.

Untreated EM spontaneously resolves (median of 28 days), although it may persist for up to 14 months. Progression to later disease is most frequent in patients with more serious early manifestations (Steere, 1989), but progression may follow mild or inapparent early LD (Reik et al., 1986).

Oral or parenteral antibiotics are effective in treatment of early disseminated LD; the suggested route, dose, and duration of therapy vary with the type of manifestations (Rahn and Malawista, 1991; Sigal, 1992). Spontaneous resolution of meningitis and facial palsy, as well as cardiac disease does not mean that therapy is not indicated, at the very least to prevent later manifestations of LD from occurring.

Lyme arthritis is treated with intravenous antibiotics: penicillin (20 million U/day in 6 divided doses), cefotaxime (3 G twice a day), and ceftriaxone (1 G twice a day); chloramphenicol (dose determined by body mass) is also effective (Rahn and Malawista, 1991; Sigal, 1992). In 55% of the cases of arthritis reported, penicillin treatment was successful (Steere et al., 1984). One study suggests that treatment with ceftriaxone is effective in patients who failed to respond to prior penicillin (Dattwyler et al., 1987). There is evidence to suggest that oral therapy (one month) may be effective (Liu et al., 1989), but these studies must be confirmed.

The most appropriate treatment of late neurologic LD is probably intravenous antibiotics, as used for arthritis. However, there are no studies to allow a definitive statement about this, or to know if late neurologic damage is totally reversible. There are anecdotes which claim slow, but impressive, resolution.

CONTROVERSIES IN TREATMENT OF LYME DISEASE

Based on the premise that *B. burgdorferi* is slowly growing, some have suggested therapy for late LD should include intravenous courses for much longer periods or prolonged (up to 18 to 24 months) oral "maintenance" therapy. There is no proof that these regimens are any more effective than the traditional approach noted above; there is certainly reason to believe that longer regimens are associated with more side effects (Genese et al., 1993) and more expense to the patient or his/her third party payer. The one indisputable fact is that the earlier a patient is treated, the less likely progression to later LD. The overwhelming majority of patients with treated early LD will experience a cure. The advisability of treating early has led some clinicians to rush to the diagnosis of LD, and to make LD "the diagnosis of exclusion" in many patients with poorly-defined symptoms; this practice is to be discouraged. Diagnosis of LD should be on the basis of a well defined differential diagnosis and full consideration of the other diagnostic possibilities.

PERSISTING SYMPTOMS AFTER TREATMENT OF LYME DISEASE

One argument made in favor of more prolonged therapy is that persistence of symptoms and occasional "progression" to later problems, often in the presence of persisting elevated levels of anti-*B. burgdorferi* antibody, represents persisting infection. The general experience has been that it may take 6 months or longer for arthritis to fully resolve after antibiotic therapy (Steere et al., 1984); persisting non-specific symptoms may occur after therapy of other features of LD. This may be due to the persistence of *B. burgdorferi*-derived antigens at the site of disease serving as a focus for prolonged inflammation (Sigal, 1989). Some patients develop symptoms *after* LD, including fibromyalgia, which are not due to ongoing infection and do not respond to further therapy (Sigal, 1990). Many patients referred to The Lyme Disease Center at Robert Wood Johnson Medical School have been subjected to many unnecessary courses of oral or intravenous therapy for symptoms not due to persisting *B. burgdorferi* infection, but which have been mistakenly attributed to LD (Sigal, 1990). It is important to recall that every complaint in a patient who has had LD (or in an individual with serum antibodies to *B. burgdorferi*) is necessarily due to *B. burgdorferi* infection (Sigal, 1994b). Post-LD fibromyalgia is a major contributing factor to the debility so often seen in such patients (Sigal, 1990; Sigal and Patella, 1992; Hsu et al., 1993; Dinerman and Steere, 1993). Potential mechanisms to explain persisting symptoms in patients with prior LD (Sigal, 1994b) and an analysis of the costs of treating patients with ill-defined complaints for LD (Lightfoot et al., 1993) have appeared recently.

RESPONSE OF LATER FEATURES OF LYME DISEASE

Fatalities due to *B. burgdorferi* infection are very rare, indeed. The only LD fatalities reported in the English language literature were due to carditis (coexisting babesiosis complicated the clinical picture [Marcus et al., 1985]) and possibly due to adult respiratory distress syndrome related to LD (Kirsch et al., 1988). There is a brief French report of a fatal case of Lyme meningoradiculitis, complicated by encephalitis and phrenic paralysis (Melet et al., 1986). Permanent heart block due to LD was reported from the Netherlands (deKooning et al., 1989), but most cases of conduction defect have been reversible (Sigal, 1994c). Lyme meningitis resolves with antibiotics (Steere et al., 1983c). Lyme arthritis has proven somewhat less responsive to antibiotic therapy. In the initial report, 55% of patients treated with intravenous penicillin showed a positive response (Steere et al., 1984); later studies suggest a better response rate with third generation cephalosporins, as above. Nonetheless, some patients have required other forms of treatment, including hydroxychloroquine (a remittive agent) and synovectomy (Steere, 1989).

HOW TO DEAL WITH THE ASYMPTOMATIC SEROPOSITIVE INDIVIDUAL?

A controversial issue in the management of LD is the status of asymptomatic people who have a positive test for antibodies to *B. burgdorferi*. It is not known how many, *if any*, of these people will ever experience LD. The policy at the Lyme Disease Center at Robert Wood Johnson Medical School is that if a true seropositive result is obtained in an individual without any preceding history of LD, oral therapy for one month, as for early localized LD, is given; this represents prophylaxis, for which there is no scientific proof of efficacy. The reasoning is as follows: If a patient is truly seropositive, the patient may well have been exposed and developed a subclinical infection. There is a small likelihood that the patient may have the appearance of a later feature of LD months to years in the future (analogous to latent syphilis). Thus, a course of antibiotics now may prevent later manifestations of the disease. Preventive medicine is the best brand of medicine, so this form of "prophylactic" therapy has been advocated by some researchers in LD.

HOW TO REMOVE A TICK

If a tick is found on someone, it should be removed with thin tweezers or forceps, using antiseptic precautions. Old wives tales suggest that kerosene, petroleum jelly, or a lit match or cigarette are useful in tick removal; these methods should be eschewed, as they may cause the tick to act as a syringe and regurgitate into the wound, causing transfer of *B. burgdorferi*. Even if an engorged tick is found, it is estimated that in an endemic area only 1% of tick bites actually transmit the disease (Shapiro et al., 1992). This suggests that prophylactic antibiotic therapy of all tick bites is not necessary; a cost-effectiveness analysis suggested that an incidence of infection after tick bite of less than 3.6% would not justify prophylaxis of all tick bites (Magid et al., 1992). Another concern: in endemic areas, people are bitten repeatedly throughout the season. If repeated prophylaxis of large numbers of people is given, the cost and potential morbidity of therapy would be tremendous; in some endemic areas, individuals would be on constant antibiotic prophylaxis from April to October for their repeated exposures. In addition, prophylactic therapy may give a false sense of security and lead individuals to abandon preventive techniques; only 30% of all tick bites in LD are recalled, so that personal measures represent the best prophylaxis. We suggest that if no rash or signs or symptoms suggestive of LD develop, the individual return for blood testing 6 to 8 weeks after the bite; if seropositive, then treatment with oral antibiotics can be given. Some suggest that a blood test be done at the time of the bite; seropositivity suggests prior exposure, and, if treatment of asymptomatic seropositivity is considered advisable, therapy could be started at that time.

CONCLUDING REMARKS

LD has been described by the press and broadcast media as the scourge of the 1990's; they speak of LD as being second only to AIDS as a public health problem in the U.S. Well meaning physicians have stated as fact their belief that LD is only rarely cured, that prolonged and repeated therapy is needed to suppress the ailment. Reports of clinical problems ascribed to *B. burgdorferi* infection appear in the medical literature, supported by only a positive serologic test. The tests practitioners use to document exposure to *B. burgdorferi* have been unfairly branded as nearly useless, because of cross-reactivity, inaccuracy (especially in early disease), and lack of standardization. The result has been that alarmed patients in endemic areas often feel that their physicians do a poor job of diagnosing and treating LD, the tests and therapeutic agents are profoundly flawed and LD represents a cause for alarm (Sigal and Taragin, 1990). Given the perception of LD as a mysterious, difficult to diagnose disease, with a poorly defined clinical spectrum, it is no wonder that many patients have seized upon LD as the ultimate explanation for all ills. It is no wonder that the concern about LD borders on hysteria.

Lyme disease has become a major health concern in a growing number of communities. Much has been learned about the disease, although much study is still needed. The problem is manageable, if we can convince our patients that Lyme disease is a cause for concern, not panic; vigilance, not hysteria. Application of scientifically proven facts and performance of further studies, rather than reliance on speculation and hearsay will help allay fears and improve the practice of medicine.

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Chapter 15

Syphilis

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INTRODUCTION

Syphilis is a disease of exceptional interest to the clinician, life scientist, public health specialist and medical historian. The agent of syphilis, *Treponema pallidum*, is a highly unusual bacterium which causes an extraordinary range of clinical manifestations by virtue of a special ability to avoid host defenses. The public health specialist faces the tantalizing prospect of a disease which should be a good candidate for wholesale eradication but which continues to flourish in many developing nations and is increasing in inner city areas of the United States. The medical historian has a wealth of themes; did sailors returning with Columbus introduce the disease to Europe or did venereal syphilis evolve from African yaws to reach the Americas as a result of the slave trade? How does the impact of widespread syphilis on Western society a hundred years ago compare with the impact of AIDS today? How was it that the course of untreated latent syphilis could be studied in Tuskegee County, Alabama, for 30 years after the introduction of penicillin?

ETIOLOGY

Treponema pallidum is a slender, highly mobile, tightly-spiralled, bacterium. Its similarity to the agents of the endemic, non-venereal treponematoses called yaws, endemic syphilis or bejel, and pinta is so close that some believe that all these diseases are caused by an identical organism. Treponemes are too slender for Gram staining and to see them through a light microscope requires the use of either a dark ground condenser or fluorescent antibody or the examination of fixed sections stained with silver salts. The study of *Treponema pallidum* has been greatly hampered by inability to grow large clonal populations in artificial media. At present it can only be grown in animal models such as the rabbit or in tissue culture. *Treponema pallidum* is easily killed by heat, cold, drying, antiseptics, and soaps. The heat sensitivity of *Treponema pallidum* was exploited by Jauregg who won a Nobel prize for using malariotherapy to help patients with neurosyphilis. The use of soap and antiseptics by patients can make it difficult to demonstrate spirochetes in syphilitic skin lesions. The outer surface of *Treponema pallidum* is composed largely of lipid. Recent work suggests that it is antigenically inert and lacks lipopolysaccharide. These unusual properties are thought to play an important part in the ability of *Treponema pallidum* to evade immunological attack and survive for long periods in humans.

PATHOGENESIS

T. pallidum gains entry often through microscopic abrasions of genital skin or mucous membranes. It has an ability to penetrate intercellular tight junctions not possessed by non-pathogenic treponemes. The inoculum required to establish

infection is small (of the order of 100 treponemes) and thus the risks of acquiring infection from a person with infectious mucocutaneous lesions are quite high (estimated at about 30%). Following invasion treponemes multiply locally and some are disseminated via the blood stream and lymphatics to reach the cerebrospinal fluid and lymph nodes. A striking feature of the host response is a lack of classical acute inflammation; despite the clustering of treponemes around blood vessels, the recruitment of neutrophils into lesions is not marked and margination and extravasation of neutrophils is not seen. An effective local immune response is always activated within the primary lesion from which all treponemes are cleared by macrophages. Both cell-mediated and humoral immune responses contribute to this but it is clear that in some patients a subpopulation of treponemes are able to evade immunological attack. While host defenses lead to resolution of the primary lesion and render the patient immune to fresh inoculations of treponemes, they are not sufficient to prevent proliferation of the treponemes that give rise to the lesions of secondary syphilis at distant sites. The characteristic histological feature of syphilis is an obliterative endarteritis accompanied by perivascular infiltration by lymphocytes, macrophages and plasma cells. In the primary lesion T helper cells outnumber T-suppressor cells; in secondary lesions the reverse occurs. The **gumma** of late syphilis is believed to represent a hypersensitivity reaction to treponemal infection and is characterized by a granulomatous reaction with caseation in which treponemes are very scanty.

CLINICAL

Transmission

T. pallidum is usually transmitted through sexual contact with a person infected within the past 2 years. It can also be transmitted through infected blood and across the placenta. Non-sexual transmission is the norm for the endemic, non-venereal treponematoses and may occasionally arise in syphilis.

The Course of Syphilis

Terms used to classify syphilis are set out in Table 1 and the major clinical manifestations seen during the three active stages of syphilis are summarized in Table 2. Also see Figures 1 through 4.

The primary chancre develops after an incubation period of 9 to 90 days and has to be differentiated from other types of genital ulcer, particularly the painful soft sore of chancroid. Untreated, a chancre lasts 1 to 6 weeks, healing usually without a scar. In the absence of treatment, infected individuals pass into a period of either early latency or develop stigmata of secondary syphilis (Table 2). The truncal rash is sometimes confused with pityriasis rosea. Untreated secondary syphilis is liable to regress and relapse once or more over the ensuing 2 years before taking the patient

Table 1. Terms Used in the Classification of Syphilis

Term	*Definition*	*Comment*
Early syphilis	Within the first two years of infection.*	Broadly separates infective patients from non-infective patients
	Encompasses primary, secondary and early latent syphilis.	
Late syphilis	Present for > 1 year	Rarely infectious, except occasionally to the fetus of an infected mother
Latent syphilis	Asymptomatic, with serological evidence or CSF findings indicative of continuing infection	May remain latent, resolve or progress. Female patients may pass the infection to offspring.
Early congenital syphilis	Occurring within the first year of life	
Late congenital syphilis	Presenting after the first year of life	

Table 2. Clinical Manifestations of Syphilis

Stage of Syphilis	*Main Manifestations*	*Less Common Findings*
Early syphilis		
Primary	Solitary, painless, indurated, genital ulcer (primary chancre—Fig. 1) with painless rubbery inguinal adenopathy	Multiple primary lesions, extragenital chancres on mouth, nipple, finger, anus, etc. Painful lesions, e.g., resembling anal fissure
Secondary	1. Condylomata lata (pink-grey hypertrophic papillomas) in moist skin folds, especially perineum (Fig. 2) and axillae 2. Non-itchy maculopapular rash on trunk, palms (Fig. 3) and soles 3. Mucous membrane lesions	Condylomata under breasts, in naso-labial folds, "moth-eaten" alopecia, follicular, papulosquamous, psoriasiform or pustular skin rashes, generalized lymphadenopathy, inflammatory lesions of liver, kidneys, brain, eyes, bones, and stomach
Late (tertiary) sypilis		
Neurosyphilis	1. Meningovascular lesions giving rise to aseptic meningitis and cerebrovascular lesions 2. Lesions of the dorsal columns of the spinal cord (tabes dorsalis) 3. General paralysis of the insane (GPI)—progressive paresis accompanied by psychiatric symptoms 4. Eye lesions including optic neuritis, uveitis and the Argyle-Robertson pupil (miotic, responsive to accommodative effort but not to light)	

continued

Table 2. Continued

Stage of Syphilis	*Main Manifestations*
Cardiovascular syphilis	Aneurysms of the thoracic aorta, coronary ostial stenosis, aortic regurgitation
Benign late syphilis	Gummatous lesions of skin, bone, liver, heart, brain, etc.
Congenital syphilis	
Early congenital syphilis	Abortion, premature labor, stillbirth, neonatal death. Skin lesions resembling those of secondary syphilis in adults. Bullous skin lesions (Fig. 4). Snuffles, hepatosplenomegaly, anaemia, painful periostitis causing pseudoparesis, failure to thrive
Late congenital syphilis	Interstitial keratitis, deafness, notched incisors (Hutchinson's teeth), knee effusions (Clutton's joints), saddle nose, neurosyphilis

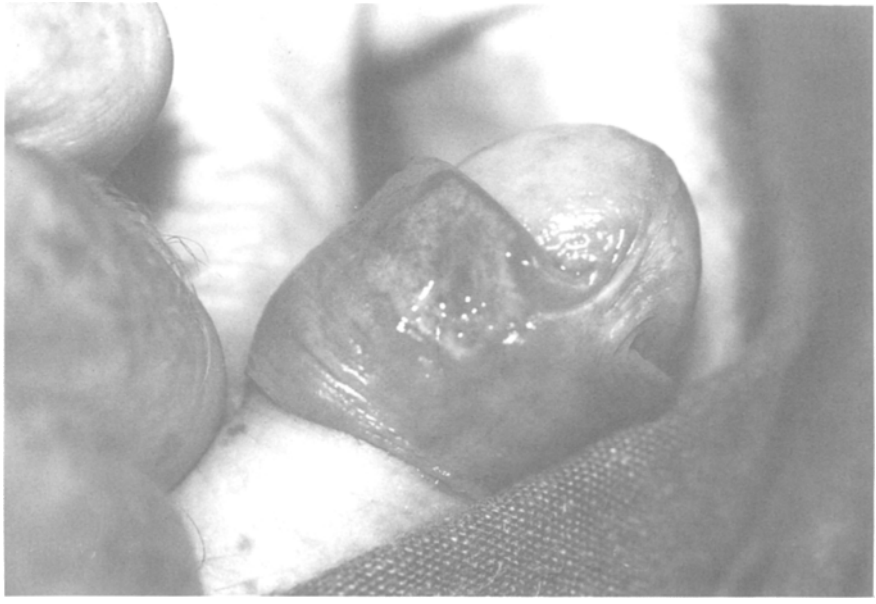

Figure 1. Primary chancre of syphilis. Lesion in Caucasian male which developed 3 weeks after a single exposure to a casual sexual partner in the tropics. The infection was also transmitted to the patient's regular partner. The first step in diagnosis is to try to demonstrate the presence of treponemes in the lesion as serologic tests may not yet be positive.

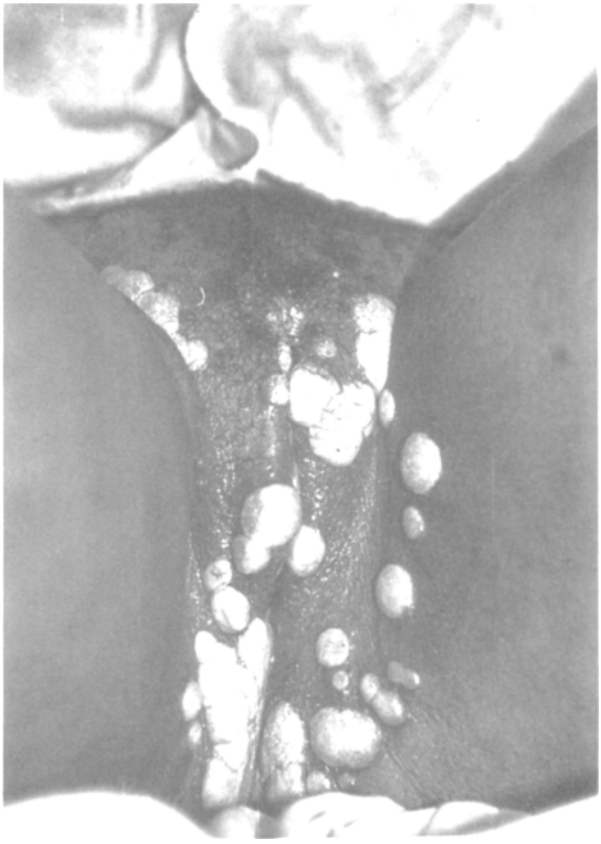

Figure 2. Multiple condylomata lata in a Papua New Guinean patient with secondary syphilis. No primary lesion reported; patient likely to have had a lesion of the cervix that passed unnoticed. Identical lesions are seen in yaws which was previously common in Papua New Guinea and was replaced about a decade after the mass treatment campaigns by a large epidemic of venereal syphilis. These are highly infectious lesions, teeming with spirochetes. Serologic tests should all be strongly positive.

into a period of late latency. The symptoms of late (tertiary) syphilis take years to appear. In the famous Oslo study of the course of untreated syphilis carried out early this century, 10% of patients developed lesions of the cardiovascular system, 7% developed lesions of the nervous system and 16% developed the so-called "benign" gummatous lesions of skin, bone and viscera (Clark and Danbolt, 1964).

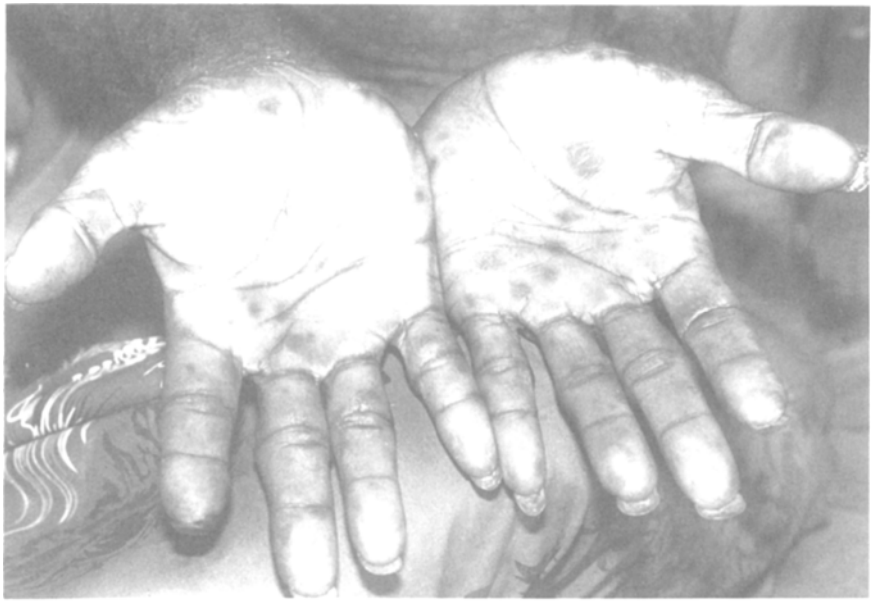

Figure 3. Lesions of the palms of the hands in a patient with secondary syphilis.

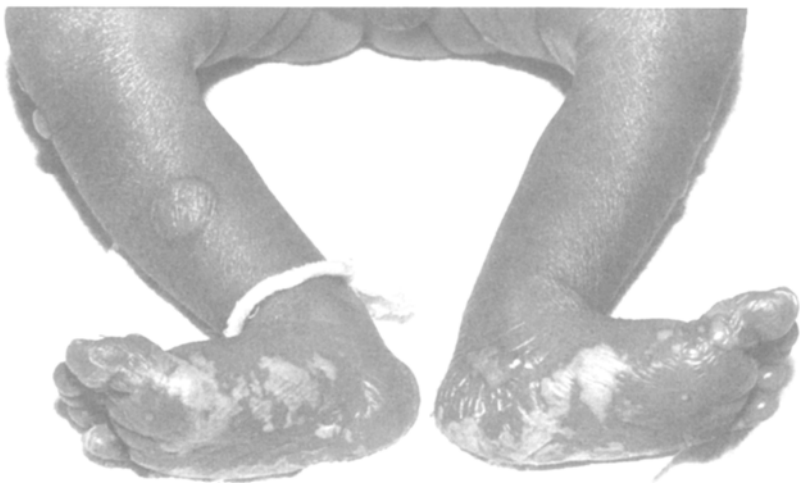

Figure 4. Bullous lesions of the legs and feet present at birth in a neonate with congenital syphilis. Similar lesions were present on the hands, genitalia and round the mouth together with prominent hepatosplenomegaly.

Neurosyphilis

Recent work has shown that *Treponema pallidum* can reach the nervous system very early in the course of infection. CSF abnormalities and isolation of *Treponema pallidum* from CSF is possible in up to 25% of patients with primary syphilis (Lukehart et al., 1988) but it is only during secondary syphilis that the first neuro-ophthalmological complications of syphilis may be observed such as aseptic meningitis, isolated cranial nerve palsies, uveitis, and optic neuritis. All of these are rare. It is estimated that 25% of patients who pass untreated through the secondary stage of syphilis will have demonstrable CSF abnormalities 3 to 18 months after infection. These include a CSF lymphocytosis, raised protein, low glucose, and positive CSF VDRL and CSF FTA-ABS tests. The neuropsychiatric manifestations of late neurosyphilis show such enormous diversity that it has become standard practice to perform serological tests for syphilis in any undiagnosed patient with neurological or psychiatric symptoms.

Late Benign Syphilis

The **gumma** is a chronic granulomatous lesion most common in skin or bone but also described in a range of other sites. The skin **gumma** presents as a chronic painless, indurated, punched-out ulcer which may heal centrally as it advances peripherally. Although treponemes are scanty in such lesions the response to penicillin is often remarkable.

Congenital Syphilis

When a woman with either active or latent syphilis becomes pregnant, the infection readily crosses the placenta. In developing countries where syphilis is prevalent, congenital syphilis accounts for a substantial proportion of stillbirths and pediatric morbidity and mortality in the first year of life. Lesions may be present at birth or appear months or years later.

Syphilis in Patients with HIV Infection

A number of reports have suggested that neuro-ophthalmologic complications of syphilis may occur earlier and more frequently in patients with HIV infection (Musher et al., 1990), though there is some controversy about this. Of clinical importance are reports that serological tests for syphilis may be unreliable in patients with HIV infection and that standard penicillin-based treatment schedules may fail to eradicate *Treponema pallidum* from the central nervous system of patients with syphilis and HIV. HIV testing should be considered in patients with syphilis because of shared risk factors and more specifically because genital ulceration facilitates the acquisition and transmission of HIV.

DIAGNOSIS

Material obtained directly from a primary or secondary lesion or by gland puncture will often enable the clinician to identify *Treponema pallidum* rapidly. Characteristic organisms can be visualized with a dark ground microscope and direct fluorescent anti-treponemal antibody can be used to confirm the identification and differentiate *Treponema pallidum* from saprophytic spirochetes that occur in the mouth and in association with erosive balanitis. For further confirmation serological tests are carried out. For the diagnosis of other forms of syphilis these tests are crucial. The serological tests for syphilis fall into two broad categories. The less specific reagin tests become positive in patients with active syphilis and show a declining titer following adequate treatment. The more specific anti-treponemal tests, which often remain positive for many years, help to differentiate patients whose reagin tests are positive from syphilis from those that have the positive tests associated with a wide variety of other conditions in which similar antibodies develop; these include certain acute infections such as malaria and infectious mononucleosis, and autoimmune diseases, particularly systemic lupus erythematosus. The antibody that is assayed in reagin tests is directed against a complex antigen comprising cardiolipin, lecithin and cholesterol and which is believed to be the product of the interaction of *Treponema pallidum* with host tissues. Such antibodies are measured with either the VDRL (Venereal Diseases Research Laboratory) or RPR (rapid plasma reagin) test. Antibodies are produced 1 to 3 weeks following the appearance of the primary chancre which results in a significant proportion of patients testing negative at presentation. Negative serology may also be reported when high titers of antibody are present in undiluted specimens (prozone phenomenon) and in about 30% of patients with late syphilis. Specific antibodies to *T. pallidum* may be detected by fluorescent antibody methods (FTA-ABS), microhemagglutination (MHA-TP) or by the specialized and rarely used *Treponema pallidum* immobilization (TPI) test. These tests give identical results in patients with non-venereal treponematosis. The FTA-ABS detects antibodies to *Treponema pallidum* after absorption of antibodies that react with non-pathogenic treponemes. It is generally the first of the serological tests to become positive. For the diagnosis of congenital syphilis it is important to differentiate maternal antibody that has crossed the placenta (IgG) from IgM produced by the fetus in response to recently acquired infection. The preferred test is an IgM FTA-ABS.

TREATMENT

Parenteral penicillin G remains the treatment of choice for syphilis and resistance to penicillin has not been reported. The principal drawbacks to the use of penicillin are the possibility of serious adverse reactions in allergic subjects and the difficulty in achieving adequate CSF penetration. Uncomplicated cases of primary and secondary syphilis respond adequately to a single injection of benzathine penicillin.

Table 3. Treatment of Syphilis*

Non-allergic patients with primary, secondary or early latent (<1 year duration) syphilis:
Benzathine penicillin G, 2.4 million units IM in a single** dose
Late latent (>1 year duration) syphilis:
As above, repeated twice at weekly intervals
Neurosyphilis:
Crystalline penicillin G 2-4 × 10^6 units 4 hourly IV for 10–14 days or procaine penicillin 2.4 × 10^6 units daily IM + probenecid 2g daily for 10–14 days

Notes: *Treatments as recommended by Centers for Disease Control 1993 Sexually Transmitted Diseases Treatment Guidelines which should be consulted for the treatment of congenital syphilis, syphilis in the immunocompromised and allergic subjects.

**A second dose may be given to pregnant women a week later.

For patients with congenital syphilis, neurosyphilis and for the immunocompromised, more extended courses of penicillin are preferred combined with the use of probenecid. When patients report allergy to penicillin the options are to resort to a two week course of second line drugs such as a tetracycline or erythromycin or to attempt desensitization. Allergy to penicillin is often transient and it is frequently possible to administer penicillin to patients with a history of penicillin allergy after carrying out skin tests, and, if necessary, a course of desensitization, according to carefully designed protocols (Wendel et al., 1985). Ceftriaxone has recently been introduced in the treatment of syphilis but does not show any clear advantages over penicillin. When treatment is commenced patients should be warned of the 12 to 24 hours of flu-like symptoms that are likely to develop some hours after the start of treatment. This is the Jarisch-Herxheimer reaction and results from the release of heat stable pyrogen from dead treponemes. Where procaine penicillin is used for treatment some patients may develop transient hallucinations following injection (Hoigné syndrome). Follow-up should comprise further reagin tests conducted 3, 6, and 12 months after infection to document a fall in titer to near zero by 1 year (Table 3).

SCREENING AND PREVENTION

Serologic tests provide a useful tool for detecting latent and untreated cases of syphilis amongst groups such as attenders at clinics for sexually transmitted diseases and their contacts, antenatal mothers (ideally screened in the first and third trimesters and at delivery), women who have had stillbirths, sex workers and patients with unexplained neuropsychiatric disorders. Transmission of *T. pallidum* through blood transfusion can be prevented by serologic testing or storage of blood for 48 hours at 4°C. Treatment of all partners of the last 3 months of a confirmed case of primary syphilis is generally advocated. This period is extended to 6 months

and 1 year for secondary syphilis and early latent syphilis. These measures need to be combined with general measures for the prevention of sexually transmitted diseases such as health education and the use of condoms. In settings where laboratory facilities are limited or absent and the prevalence of syphilis is high, a policy of treating for syphilis all patients with genital ulceration can be justified.

SUMMARY

The genus *Treponema* is responsible for a group of closely related skin infections, of which the most important is the sexually transmitted infection caused by *Treponema pallidum* and known as syphilis. Infection with *T. pallidum* does not elicit a typical acute inflammatory response and the organism possesses special properties that enable it to persist within its host for many years. Clinical syphilis is primarily a disease of the skin, where its earliest manifestation, the primary chancre is seen. In untreated patients, secondary and tertiary lesions which can involve internal organs (notably the brain and thoracic aorta) are seen following periods of latency. Syphilis in pregnancy can be transmitted to the unborn child. The laboratory diagnosis of syphilis depends on the direct demonstration of treponemes and on serological tests for infection. *T. pallidum* is fully sensitive to penicillin which is the treatment of choice. Dosage schedules depend on the type of syphilis being treated. Serological screening of pregnant mothers and individuals at risk, identification and epidemiologic treatment of contacts and sexual health promotion are all employed in the control of syphilis.

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Chapter 16

Brucellosis

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INTRODUCTION

Just over one hundred years ago the microbial etiology of "Malta Fever" was demonstrated by Bruce and as a result of subsequent detailed epidemiological studies by the Malta Fever Commission, set up by the Royal Society of London and the Ministry of Defence, the animal reservoir of Bruce's *Micrococcus melitensis* was found in goats by Themistocles Zammit. The consumption of milk and milk derived products by military personnel was forbidden and the incidence of the disease declined dramatically among soldiers and their dependents. Thus the zoonotic nature of this infection was clearly defined. The *Bacillus abortus* infection of cattle was identified by Bang and the American microbiologist, Alice Evans, herself a longtime sufferer from brucellosis, concluded from the similarities between these two organisms that they belonged to the same genus and predicted that *B. abortus* would cause infection in man. A few years later her prediction was fulfilled.

The genus came to be called *Brucella* and we presently recognize six species in the genus. *Brucella melitensis*, *B. abortus* and *B. suis* causing disease most often in man while *B. canis* occasionally does so. *Brucella ovis* and *B. neotomae* do not cause human infections. The first three species infect man's domesticated animals and so this zoonosis occurs most commonly among pastoralist and herding peoples, people who live in close proximity to their animals and in those whose occupations exposes them to close contact with animals and animal products. Brucellosis has a worldwide distribution. Eradication has been achieved in relatively few areas and then only by considerable expenditure of time, effort and money.

BACTERIOLOGY

The brucellae are aerobic, non-motile, non-spore forming Gram-negative cocco-bacilli. There is some variation in the Gram-stained appearances of the three species infecting man: *B. abortus* is obviously bacilliform while the small oval bacteria seen by Bruce were described as cocco-bacilli (*B. melitensis*). These organisms grow slowly even in enriched culture media at 37°C, *B. abortus* and *B. ovis* requiring an atmosphere with added CO_2. This slow growth means that cultures must be maintained for up to six weeks before being discarded and blind sub-culture is routinely carried out. Use of the Bactec or similar system may lead to earlier identification but even with this method cultures usually take 12 days to yield a detectable growth. Fresh isolates from clinical samples have a smooth colonial appearance which becomes rough after a time in culture. Organisms from this genus are catalase positive with oxidase production commonly seen. An interesting and, clinically very significant, observation was the identification of *B. melitensis* as *Moraxella phenylpyruvica* using API20NE test strips. This is important because brucellosis can readily be transmitted in microbiology laboratories.

Three biovars are recognized for *B. melitensis*, nine for *abortus* and five for *suis*. Studies of DNA have shown greater than 80% homology among the presently defined species. No change in nomenclature is presently proposed.

The antigenic structure of the brucellae is, like that of any bacterium, very complex. Most attention has focused on the surface lipopolysaccharide. Two are identified: A and M. The A antigen predominates in *abortus* and *suis* with M as the major component in *melitensis*. Outer membrane proteins and cytosolic proteins have also been defined in recent work with evidence that protection can be conferred by passive transfer of antibodies directed to some of these proteins.

EPIDEMIOLOGY

Animal Infection

Brucella melitensis most often infects goats but sheep and camels can also be infected; *B. abortus* infects cattle and *B. suis* pigs with prolonged infection usually resulting. *Melitensis* can get into cattle as can *suis* but short lived infection is usual in these circumstances. Infection *in utero* is common and erythritol, a ribose-alcohol found in amniotic fluid of animals but not man, is a specific growth factor for the organisms. The udders of most animal species become infected and so even if intrauterine infection has not occurred, the young are likely to become infected by taking milk. Infection in animals has significant consequences with reduced fecundity, abortion and reduced milk yields though reduced weight gain among beef cattle is not a problem. Arthritis among pigs is a problem.

Transmission

Milk from infected cattle or goats will contain brucellae and be ingested by anyone drinking the fresh milk. *Brucellae* are very acid sensitive and so gastric acid will readily kill these organisms as will the production of acid when milk becomes sour but fresh milk will buffer gastric acid allowing organisms to avoid the gastric barrier. The presence of protein and fat in milk may also serve to protect the organisms. Persons taking antacids and drugs that reduce gastric acid secretion, e.g., H_2 receptor antagonists, may also be at increased risk of brucellosis. Milk products such as soft cheeses or buttermilk, called laban in a number of Middle Eastern countries, made with contaminated milk can be vehicles of infection as the pH does not fall sufficiently to kill the bacteria.

There is evidence to show that brucellae can be resistant to cold and desiccation with the possibility that they can survive in dust, in dead animals, and in placenta so that infection from contaminated earth and dust is possible.

Laboratory transmission was mentioned briefly above but it represents a very real risk. Two situations are particularly relevant. The first in endemic areas when there are poor and careless laboratory practices and the second in a non-endemic area

where very occasional imported cases are seen. The biochemical similarities with a *Moraxella* species were noted earlier. A broken culture flask creates an aerosol which infects by contact with the conjunctivae and the mucosa of the naso-pharynx and respiratory tract.

Cuts and abrasions on the hands of persons having contact with infected carcases may also be a site for entry of infection. This was clearly shown in an epidemiological investigation of brucellosis in an abattoir where pigs were slaughtered. Workers who used knives to cut up carcases had an increased risk of infection with a much lower risk to those packing meat. The importance of the respiratory route of infection was shown by the occurrence of cases in workers whose office windows were close to the opening of an extractor fan venting the slaughtering area. Infection has probably been transmitted by semen, breast milk, blood transfusion and organ donation though these are likely to be numerically of minor significance.

Veterinarians and their assistants may inadvertently inoculate themselves with the vaccine strains of *B. abortus* (S19) and *B. melitensis* (Rev 1) and develop disease caused by these strains. These strains are attenuated for animals but not man.

Distribution and Incidence

Brucellosis has a worldwide distribution and while it is more common in rural communities it does occur in towns and even cities because animals, particularly goats, are kept in and around houses. The 1980s saw a great increase in brucellosis in the countries of the Arabian peninsula. This was due initially to an increase in the number of livestock and, in part, to increased awareness of the disease among physicians as the clinical syndrome became familiar. Human infections in Kuwait increased from 1.15 to 42.8 per 100,000 of the adult population over a ten year period up to 1984. *Brucella abortus* is fairly common among the countries of south America where cattle are kept. *Melitensis* infections are also common especially in Peru and Chile. This infection is also endemic in southern Europe with incidence in France varying between 1.07 and 18.4 per 100,000 depending on the area.

The disease has an increased incidence in microbiology workers, both human and veterinary laboratories, abattoir workers, veterinarians and their assistants, shepherds, cowherders, and farm workers. Occupational exposure needs to be borne in mind.

In a given population there seems to be a preponderance of infection among males, though both sexes may become infected, most commonly in the second, third, and fourth decades. Children are less often infected. The peak incidence of human infection occurs in the months after animals have produced their young. Outbreaks among families exposed to the products of one or more infected animals are seen. Seropositivity among apparently healthy blood donors varies between 0.2% and 4% in endemic areas.

PATHOLOGY AND PATHOGENESIS

Phagocytic cells of the reticuloendothelial system ingest and kill microorganisms. The paradox of brucellosis and other infections which are characterized by intracellular parasitism is why this line of defence fails in those persons who develop disease.

There is evidence to suggest that the virulence of the infecting strain is a determinant of the ability of organisms to survive within macrophages and that genetically determined host factors determine the ability of macrophages to inhibit bacterial growth within them. The organisms can be arranged as follows with regard to virulence, *B. melitensis* (most virulent), *B. abortus*, and *B. suis* (least virulent).

When mice are infected with brucellae numbers of organisms in the organs with the largest numbers of macrophages, the liver and spleen, rise over ten days and then decline markedly. This decline is concurrent with the appearance of sensitized T-cells of CD-8 phenotype, which interact with macrophages to stimulate killing. Gamma-interferon is likely to mediate this interaction and the T_h-1 phenotype is likely to be involved. Numbers of circulating gamma-delta T-lymphocytes are markedly increased in human brucellosis but cellular reactivity to brucella antigens in the blood compartment is found in cells bearing the alpha-beta phenotype.

Despite the decline in numbers of organisms in mice at this stage of the infection, a few bacteria persist in liver macrophages. If the pattern of persistence of organisms in macrophages has a counterpart in man, then this may be the basis of relapse, a prominent feature in brucellosis.

The main emphasis in efforts to try to understand the pathogenesis of this infection has focussed on cell mediated immune responses that are associated with granulomatous pathology which characterizes this and other infections with intracellular pathogens. However, it is well recognized that passive immunization with antibodies directed to a range of antigenic determinants including peptidoglycan (cell wall) and lipopolysaccharide can prevent infection so that when macrophages burst and release organisms that have replicated within them, antibodies may bind to their appropriate antigens and perhaps contribute to the control of the infection.

The pathology of brucellosis is characterized by granulomatous histology in infected organs and by bacteremic spread to all organs of the body, though liver, spleen, lymph glands and osteo-articular structures are most commonly involved. Epithelioid cells are seen with a surrounding layer of lymphocytes, monocytes and fibroblasts. Giant cells may be present. Pus formation and necrosis may occur. Caseation is not a feature of this infection which helps to distinguish it histologically from tuberculosis.

CLINICAL FEATURES

The incubation period can best be judged from laboratory accidents when a flask has been broken. The usual period is two to four weeks before the onset of

symptoms. Fever, lethargy, and night sweats are usual initial symptoms. These are prominent but not so severe as to take patients in endemic areas to a physician immediately. The persistence of symptoms for several weeks or the development of localizing features brings the patient to medical attention. In the pre-antibiotic era it was recognized that brucellosis could go on for months continuously, or show undulation of fever or show spontaneous regression. Almroth Wright "vaccinated" himself with killed brucellae and then inoculated himself with living brucellae. He developed a febrile illness with an undulant pattern which resolved spontaneously after several weeks. Spontaneous resolution of infection occurs in a proportion of those infected. There was a mortality of up to 7% with brucellosis in the pre-antibiotic era.

Enlargement of liver and spleen are usually found in this infection. Forty percent of patients will have hepatosplenomegaly or splenomegaly. Lymph gland enlargement is found in about 10% of cases.

Skeletal Disease

Joint pains are common in this infection but true arthritis occurs in about 30% of cases. While any joint or joints can be involved the large weight bearing joints, hip, knee and ankle, are commonly affected. Up to 35% of patients with *B. melitensis* may have joint problems. The joint is swollen, tender and hot. The effusion in the knee can be so large as to produce a Baker's cyst and rupture of a Baker's cyst leaking joint fluid into the calf to simulate a deep vein thrombosis or cellulitis can occur.

Joint problems in the spine are common with any part of the axial skeleton involved. The lumbar spine is most often involved. It is likely that there is initial inflammation of the intervertebral disc. The swollen disc can compress a nerve root in an intervertebral foramen to produce the symptoms and signs of sciatica. Inflammation may continue over a longer period to result in erosion of the margins of the vertebral body and destruction of the affected disc. Osteophyte and syndesmophyte formation occur with time. In the early stages pus is not present but with time the inflammation may progress to pus formation and abscesses, paravertebral or even a psoas abscess, may form. The clinician has to bear in mind the similarity between brucellosis and tuberculosis in this respect. Sacro-iliitis also occurs.

Localization at other Sites

Epididymo-orchitis is a localizing feature in up to 9% of males. Renal infection with pyelonephritis is reported though not commonly and an IgA mesangial nephropathy has been seen. There is a large population of macrophages in the alveoli of the lungs and by inhalation of an aerosol pulmonary disease may be produced. One of the most serious complications of brucellosis is endocarditis affecting either a normal or abnormal valve, particularly the aortic. Pericarditis is also recognized. A number of authors have stressed the wide range of manifestations

of neurologic involvement including meningitis with CSF features similar to those of tuberculous meningitis, an encephalopathy, papilledema, cerebral vasculitis, and cerebellar features. Neurologic features occur in about 2% of cases and can occur early or late in the course of infection. Skin rashes and ocular inflammation have been documented.

Chronic Infection

Before effective treatment was available prolonged infection continuing for months and even years was well recognized. Alice Evans described her own illness which was on more than one occasion diagnosed as neurasthenia before brucella were cultured from lesions found at surgery. The major series of cases reported in recent years have come from the Arabian peninsula and 10% in a Kuwaiti series had symptoms for more than a year.

DIAGNOSIS

Clinical suspicion is essential for the diagnosis to be considered. Blood culture is most often used for isolating the organism but for the reasons noted above isolation rates are frequently not very high. In addition to the rather fastidious nature of the organism *in vitro*, treatment with antibiotics prior to taking samples will rapidly sterilize the peripheral blood, further reducing the chances of success. Bone marrow culture increases the chances of isolation even when antibiotics have been taken and an isolation rate of 90% from bone marrow has been reported. Tissue samples or pus may yield organisms. Prolonged culture and blind sub-culture should be carried out. Clot culture can give a higher yield of organisms than whole blood but this has to be balanced against the risk of laboratory infection as a result of manipulating the clots. Brucellosis is an ideal organism for diagnosis using the polymerase chain reaction (PCR) as it is present in blood in small numbers, and difficult and hazardous to isolate using standard techniques. Primers for diagnosis by PCR have been described and used with success but for endemic areas this technology is perhaps not yet appropriate for routine use.

Serological testing has been the mainstay of diagnosis for many years. The basic test of tube agglutination (SAT) to which may be added the step of treating serum with 2 mercaptoethanol (2-me), to destroy IgM antibody, prior to carrying out SAT helps distinguish between acute and more chronic infection. Non-agglutinating anti-brucella antibodies are well-recognized to cause a prozone phenomenon at low titer and so screening in the SAT should be carried out at low and high dilutions. The anti-globulin test is useful in detecting significant antibody responses in chronic infections when SAT titers may be insignificant. Over the last decade there has been great interest in applying the enzyme linked immunosorbent assay (ELISA) technique to brucellosis. Whole cell and sub-cellular antigens have been used with good results and commercial kits are starting to become available.

However, many of the areas of the world where brucellosis is most common are relatively poor and the SAT is perhaps most relevant to their practice because of its simplicity and requirement for simple antigens which could be produced locally. It is essential in interpreting serological test results that they should always be considered in association with the clinical information about the patient, a single titer in isolation may not always be significant.

Radiological examinations in the presence of skeletal or articular involvement may be normal in the early stages though technetium bone scans often show evidence of disease. The bone changes occur later and appear as destruction, osteophyte formation and syndesmophyte formation. Soft tissue shadows from abscesses or inflammation occur. CT scanning gives more detail of bone changes and associated inflammatory reaction.

Laboratory investigations give non-specific results. Anemia is usual but moderate in degree. The white cell count is at the lower end of the normal range with a lymphocytosis of 40 to 50%. Platelet counts may be reduced significantly but bleeding due to this or coagulopathy is very uncommon. Liver function tests show an elevated alkaline phosphatase which may relate to hepatic granulomata or to bone infection. Transaminases may also be mildly elevated.

DIFFERENTIAL DIAGNOSIS

There is a wide range of potential causes for prolonged fever and include tuberculosis, Q fever, typhoid and visceral leishmaniasis. The patient is usually more ill more quickly in typhoid. Q fever can be difficult to distinguish clinically as it can occur in persons who have had contact with sheep and it causes hepatosplenomegaly and epididymo-orchitis in males. Blood cultures are sterile and serological testing is required for diagnosis. Clinical similarities with tuberculosis have been apparent in clinical descriptions of bone and joint disease and CNS involvement. In leishmaniasis the causative organisms are usually found in bone marrow aspirate and this is therefore another indication for obtaining marrow aspirate early in the investigation of a patient with fever and hepatosplenomegaly. Anemia and pancytopenia are usual in leishmaniasis.

TREATMENT

Antibiotic treatment is effective and acceptable but needs to be carried out for at least six weeks and often for 12 weeks. Because of the long duration everything possible must be done to educate the patient regarding the necessity of taking the full course. The use of doxycycline makes the regimen easier for patients than four times daily regimens with tetracycline. In children below eight years tetracycline is contraindicated and trimethoprim-sulphamethoxazole (TMP-SMZ) can be used instead. The latter must not be used alone as it is associated with an unacceptably high relapse rate. Doxycycline cannot be used in pregnancy according to manufac-

turer's recommendations though there have been claims for its safety in pregnancy in the literature (see treatment review cited in the reference list). Rifampicin can also be used with doxycycline in adults and children over eight years or with TMP-SMZ in non-pregnant persons of any age. Other effective drugs include chloramphenicol, ciprofloxacin, cefotaxime, and ceftriaxone.

The duration of treatment must be considered when the regimen to be used has been decided upon. Acute disease will respond well to six weeks' treatment. When localized disease affecting the joints, skeleton, and nervous system is present, up to 12 weeks' medication is needed. Some authors recommend that three drugs be used when there is neurological involvement. It may be necessary to maintain treatment beyond these rather arbitrary limits depending on the adequacy of response. It is likely that prolonged therapy is needed in managing endocarditis. Surgical intervention may be needed to drain pus or excise infected heart valves.

Relapse is a particular problem in brucellosis, probably the result of survival of organisms within macrophages. Relapse rates of 4.5% are reported for doxycycline plus streptomycin and 8.4% for rifampicin plus doxycycline.

CONTROL

Boiling milk before it is drunk or used to make other products will prevent this route of transmission of infection as will pasteurization in commercial dairy practice. Control of infection in herded animals requires vaccination with S19 strain for *B. abortus*, Rev 1 for *B. melitensis* and attenuated *B. suis* strains. In order to eradicate brucellosis from domestic livestock it is necessary to institute a program of testing of animals, slaughter and safe disposal of infected animals and vaccination of uninfected animals. Compensation for animals destroyed is usual. Such measures are expensive in time, money, and effort and once eradication is achieved, continued surveillance is required.

SUMMARY

Brucellosis is an important zoonotic infection acquired by man from herded animals. Several species of the genus Brucella cause the infection with melitensis, abortus and suis in declining order of virulence. They are small, Gram negative bacilli or cocco-bacilli found intracellularly and evoke granulomatous changes in infected tissues.

The geographic distribution of these infections is widespread in temperate and tropical regions. In animals, infection causes abortion, reduced milk yields and reduced fecundity and so the economic losses are considerable. There are very few countries from which brucellosis has been eradicated and where this has been achieved or there have been substantial reductions in infection rates by serological testing, destruction of positive reactors, vaccination of non-reactors and then continued surveillance, the cost has been considerable. The vaccines used for

animals are live, attenuated strains but attenuation relates to animals and not to man in whom they can cause disease.

Human infection occurs by various routes but ingestion of milk, milk products from an infected animal is among the most common. The illness is characterized by prolonged infection lasting weeks or months rather than days. Persisting fever with weight loss, night sweats and lethargy are typical but non-specific symptoms. These symptoms will often not bring a patient to medical attention until localized disease develops. Commonly this is an arthritis, frequently affecting a large weight bearing joint or the axial skeleton, but any system of the body can be involved including genito-urinary system, lymph glands, skin, eye, heart, and nervous system. Abscess formation can occur at a range of sites.

Diagnosis should depend on demonstrating the organism but Brucellae can be difficult to grow in blood culture and prior use of antibiotics can reduce further the chances of obtaining a positive culture. Culture of bone marrow aspirate increases the yield of culture, particularly when antibiotics have been used, and culture of lymph gland, liver biopsy, other tissue samples and pus may yield organisms. Because of these difficulties serological diagnosis is of major importance with the agglutination test giving satisfactory results for laboratories with basic facilities. Pre-treatment of test sera with 2-mercapto-ethanol (2me), which destroys IgM, allows differentiation of recent infection (2me labile titer) from a more longstanding infection with a predominantly IgG response.

Treatment with antibiotics is effective, usually requiring combination therapy, for example rifampicin plus doxycycline, for 6 weeks in cases without major localization and for 12 weeks when there is joint disease or other major localizing manifestation. Endocarditis though rare, can be very difficult to treat and may require valve replacement in addition to prolonged antibiotic therapy.

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Chapter 17

Fungal Diseases

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INTRODUCTION

Fungal diseases are generally divided into categories representing the site at which the organism first enters the tissue to initiate disease. There are four such categories: the systemic fungal diseases, wherein the organism is inhaled and the primary lesion(s) develops in the lung; the subcutaneous mycoses, characterized by the traumatic implantation of the fungus into the subcutaneous tissue; the cutaneous mycoses which involve attack of the keratinized layers of the skin by fungi; and the

Table 1. The Most Common Mycotic Diseases and their Etiologic Agents

Primary Mycoses	
blastomycosis	*Blastomyces dermatitidis*
coccidioidomycosis	*Coccidioides immitis*
histoplasmosis	*Histoplasma capsulatum*
paracoccidioidomycosis	*Paracoccidioides brasiliensis*
Opportunistic Mycoses:	
candidiasis	*Candida albicans, C. tropicalis*
cryptococcosis	*Cryptococcus neoformans**
aspergillosis	*Aspergillus fumigatus, A. flavus*
zygomycosis	*Rhizopus* sp.
Subcutaneous Mycoses:	
sporotrichosis	*Sporothrix schenckii*
chromoblastomycosis	*Fonsecaea pedrosoi*
phaeohyphomycosis	multiple darkly-pigmented fungi
mycotic mycetoma	*Pseudoallescheria boydii*
Cutaneous Mycoses:	
dermatophytosis	*Trichophyton rubrum>T. tonsurans>T. mentagrophytes>Microsporum canis>Epidermophyton floccosum*
Superficial Mycoses:	
pityriasis versicolor	*Malazzesia furfur* (presumed etiologic agent)

Note: *may be primary in some cases and opportunistic in others

superficial mycoses which involve minimal, if any, pathology, and the mildest of associations between host and parasite on the skin or hair.

A list of the four groups of mycoses and the most common diseases and etiologic agents found in the United States is presented in Table 1. Cryptococcosis and candidiasis are diseases for which the classification scheme does not work well, in that cryptococcal meningitis is largely an opportunistic disease whereas pulmonary cryptococcosis occurs with some frequency in individuals with no known underlying abnormalities. Moreover, candidiasis is more often seen as a cutaneous or mucocutaneous disease than a truly systemic disease, and the *Candida* sp. most often causing disease are normal flora of humans, usually residing in the gastrointestinal tract; thus, disease results from endogenous spread.

SYSTEMIC MYCOSES: PRIMARY PATHOGENS

The term primary pathogen refers to the property of the fungus to infect and initiate disease in immunologically competent and otherwise healthy individuals, the only provision being that a sufficient number of infectious particles must be inhaled to circumvent the normal innate defenses and establish some level of disease. Having evaded the initial defenses, however, previously well patients who do not encounter an overwhelming number of infectious particles, often recover from the initial infection without treatment and with a long-standing immunity to reinfection.

There are four primary fungal pathogens, namely, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*. Each of these organisms is a dimorphic fungus. *B. dermatitidis*, *H. capsulatum* and *P. brasiliensis* are dimorphic by virtue of the fact that they may exist as either yeast or mold forms depending upon the environment in which they are growing. *C. immitis* is termed dimorphic because it exists in the soil and on routine laboratory media as a mold producing arthroconidia, but when invading tissue the arthroconidia convert to large, round, thick-walled structures called spherules which, when mature, divide into many endospores. The endospores are released and subsequently mature into endospore-producing spherules. It is difficult, but not impossible, to produce endosporulating spherules *in vitro*. Two additional terms, saprophytic and parasitic, may be added to the description of the dimorphic fungi as well. The mold forms are saprophytic in that they occur in the environment and maintain a normal growth pattern in the presence of dead or decaying organic matter and the yeast or spherule forms are parasitic, in that those forms are found almost exclusively when the fungus is present in host tissue or when the organism is incubated *in vitro* at 35 to 37°C on appropriate media.

Epidemiology and Ecology

Summarized in Table 2 are the epidemiological and ecological factors associated with the primary mycoses. The epidemiology of a disease can be defined by using

Table 2. Epidemiologic, Ecologic and Clinical Aspects of the Primary Mycoses

Disease	*Blastomycosis*	*Histoplasmosis*	*Coccidioidomycosis*	*Paracoccidioidomycosis*
Organism	*Blastomyces dermatitidis*	*Histoplasma capsulatum var. capsulatum*	*Coccidioides immitis*	*Paracoccidioides brasiliensis*
Epidemiology	predominantly North America, i.e., north, central and southern United States bordering the Missouri, Mississippi, Ohio River Valleys, and southern Canada bordering the mid-western states and extending up the St. Lawrence seaway	predominantly North America, i.e., north, central and southern United States bordering the Missouri, Mississippi, Ohio River Valleys; extending into northern Florida	in the Lower Sonoran Life Zones of North, Central and South America; in United States, southern and central California, Arizona, Utah, Nevada, New Mexico and western Texas	restricted to selected areas of Central and South America; not found in the United States
Ecology	uncertain: may be rotting wood, may involve high humidity and soil along river banks; very difficult to cultivate from the environment	grows well in environments containing nitrogenous compounds. eg., soils contaminated with bird feces or bat guano; readily cultured from soil	survives well in soil with a high alkaline content, as found in Lower Sonoran Life Zone; readily cultured from soil	presumed to be soil but ecologic niche has not been definitively identified; difficult to cultivate from soil

Clinical	clinical conditions variable; acute or chronic pulmonary disease, with or without apparent systemic spread; degree of self-limited disease unknown; patients often seen first by dermatologist because of skin lesions skin, bone, and genitourinary tract most common sites for dissemination	clinical conditions quite variable: 1. acute, self-limited, or chronic pulmonary disease; majority of acute never specifically diagnosed 2. acute fulminant or chronic disseminated progressive disease; multisystem involvement but especially organs of reticuloendothelial system	clinical conditions quite variable: 1. acute or chronic pulmonary disease: 60% clinically inapparent 38% clinically apparent but resolve spontaneously 2% develop chronic cavitary disease 2. 1–2% develop disseminated disease, which may include meningitis; skin, bone, and meninges most common sites for dissemination	clinical conditions quite variable: 1. acute or subacute juvenile form 2. chronic adult form (most common) primary infection believed to be pulmonary, but disease usually diagnosed on the basis of extrapulmonary lesions, e.g., cutaneous or mucocutaneous
Risk Factors for Serious Disease		AIDS; other forms of immunosuppression	Dark-skinned races more susceptible to disseminated disease; AIDS; other forms of immunosuppression	adult males more likely to develop clinical disease than females or children

criteria such as cultivation of the organism from the environment, correlation of positive environmental cultures with the geographic distribution of case reports, and detection of delayed hypersensitivity specific to a particular fungus in the inhabitants of various geographic areas. It is possible to culture only two of the four primary fungal pathogens from soil on a regular basis, namely, *H. capsulatum* and *C. immitis*. Thus the ecologic niche for these two fungi is well established. *C. immitis* has been described as a poor competitor and good survivor. The arthroconidia of the mold phase survive well in the top twelve inches of sandy soil during the hot dry seasons in desert-like areas, conditions not suited to other organisms that might have a competitive advantage in more temperate environments. During the rainy season arthroconidia germinate, new hyphae are formed that subsequently break into new arthroconidial units, and survival is ensured through the next dry season. These environmental conditions are found only in the Lower Sonoran Life Zone in the New World, i.e., the southwestern areas of the United States and selected areas of Central and South America.

The mold form of *H. capsulatum*, on the other hand, appears to need considerably more moisture to maintain its viability and grows particularly well in an environment high in nitrogenous compounds. Bat guano and bird feces, especially those of blackbirds and chickens, provide suitable enrichments for soil for the maintenance and growth of the organism. The endemic areas for *H. capsulatum* and *C. immitis* do not overlap.

There are two skin-test antigens available for the detection of delayed hypersensitivity to *C. immitis*, and one for the detection of hypersensitivity to *H. capsulatum*. Skin-test surveys have confirmed the data on geographic distribution acquired by analysis of case reports and isolations of the fungus from soil. The skin-test material for *H. capsulatum*, as well as of one of the *C. immitis* preparations, is a mixture of antigens found in the culture filtrate of long-term cultures of the mold phases of each of these fungi. The second extract available for *C. immitis* is a toluene extract of the spherule form. Positive skin tests cannot be used diagnostically, as they are indicative only of previous exposure to the organism.

B. dermatitidis and *P. brasiliensis* have been elusive to scientists attempting to culture them from the environment and define their ecologic niche. *P. brasiliensis* has been isolated from soil only twice, and *B. dermatitidis* only a few more times. There is some evidence to suggest that both of these fungi require a humid environment such as that found associated with the banks of rivers and streams, but the specific ecologic niche for each remains a mystery. Moreover, there is no skin-testing material available for the detection of delayed hypersensitivity to *B. dermatitidis*, and despite the fact that a number of skin-test surveys have been performed with antigens of *P. brasiliensis*, the surveys have not been particularly useful in identifying the endemic areas.

Risk Factors and Clinical Presentations

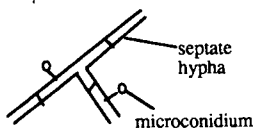
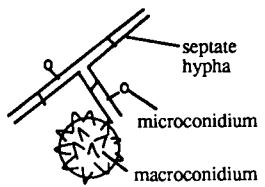
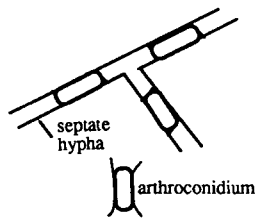
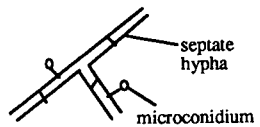
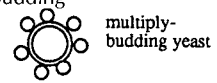
Clinical conditions and the risk factors associated with the development of clinical and/or disseminated disease with the primary pathogens are also summarized in Table 2. What they all have in common is that, since the fungus is inhaled, initiation of the disease process is in the lung. Beyond that, however, each disease has unique features which distinguishes it from the others. For example, the pathologic response to both *C. immitis* and *B. dermatitidis* is similar, in that polymorphonuclear leukocytes figure prominently in lesions caused by both fungi. Moreover, when disseminated disease occurs, skin and bone are likely to be involved. *B. dermatitidis*, however, rarely attacks the meninges, whereas coccidioidal meningitis occurs in a large percentage of individuals with disseminated disease. *H. capsulatum* is unique in that the pathologic response to the fungus is almost purely granulomatous and the fungus is a facultative intracellular parasite within cells of the monocyte lineage. Thus it is a disease of the reticuloendothelial system and hepatosplenomegaly is a common finding during disseminated disease. Since recovery from histoplasmosis is a T-lymphocyte mediated event, histoplasmosis has become an AIDS-defining disease in highly endemic areas of the United States, e.g., Indianapolis, Indiana.

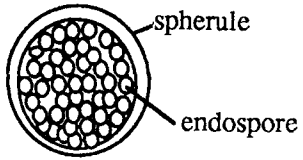
Coccidioidomycosis is the best defined systemic mycotic disease with regard to the clinical conditions which result following exposure and with respect to the risk factors which influence the disease process negatively. There are several reasons for our wealth of knowledge. First, the fungus is highly infectious and only a few arthroconidia will initiate disease. This insures that virtually all who come in contact with the arthroconidia in nature become infected. Secondly, during World War II, a large number of military personnel were moved into the endemic area from nonendemic areas. These individuals had no underlying immunity and were a captive population to study as they became exposed to the fungus. Therefore, it is known that about 40% of the individuals exposed to arthroconidia develop clinically apparent disease. One to two percent of these individuals will develop severe disseminated forms of the disease, which may include meningitis. Skin is the most common site for the appearance of dissemination, followed by bone and the meninges. Dark-skinned races in general are more susceptible to the development of severe disease, with persons of Filipino ancestry being at highest risk. With the advent of AIDS, it, too, is becoming a serious risk factor for the development of disseminated disease.

Laboratory Considerations

Laboratory considerations for the primary pathogens and the diseases they cause are presented in Table 3. Patients with primary mycoses usually have pulmonary involvement, with the exception of those with paracoccidioidomycosis where the pulmonary involvement may be transient and mild. Diagnosis of the primary

Table 3. Selected Laboratory Considerations for the Primary Mycoses

Disease	*Blastomycosis*	*Histoplasmosis*	*Coccidioidomycosis*	*Paracoccidioidomycosis*
Organism	*Blastomyces dermatitidis*	*Histoplasma capsulatum var. capsulatum*	*Coccidioides immitis*	*Paracoccidioides brasiliensis*
Specimens	sputum, bronchial washings pus or skin biopsy, prostatic secretions	sputum, bronchial washings blood, bone marrow, biopsy of lymph node, spleen, liver	sputum, bronchial washings pus or skin-biopsy, spinal fluid	cutaneous or mucocutaneous biopsy
In vitro Culture	≤ 30 C: white to tan mold producing microconidia, 2–10 μ 	≤ 30 C: white to tan mold producing microconidia, 2–4 μ, and macroconidia, 8–15 μ, the latter of which have elongated projections from the cell wall; cultures without macroconidia indistinguishable from those of *B. dermatitidis* 	≤ 30 C: white to gray mold producing masses of arthroconidia 	≤ 30 C: colonial morphology indistinguishable from *B. dermatitis* or *H. capsulatum* when the latter is producing microconidia only 
	35–37 C: large, thick-walled, often multinucleate, budding yeast, ≥ 8–15 μ, with broadly-based connnected between mother and daughter cells 	35–37 C: small, oval, thin-walled yeasts, 2–4 μ 	35–37 C: on ordinary laboratory media, white to gray mold producing masses of arthroconidia	35–37 C: large, multiply-budding yeast cell. 4–30 μ; gives a pilot's wheel appearance because of the budding 

In vivo morphology	yeast, same as *in vitro* appearance	small, oval, thin-walled yeasts found routinely inside the cytoplasm of macrophages 	large round structures, spherules, which when mature divide into small endospores, the latter of which are released into the tissue: spherules, 10–60 μ endospores, 2.5 μ 	yeast, same as *in vitro* appearance
Laboratory	Direct: KOH, yeast can be visualized easily in specimen	Direct: KOH of no value Special Stain: Giemsa or Wrights stain of blood, bone marrow, or tissue impression, look for small intracellular yeasts	Direct: KOH, spherule can be visualized easily on direct	Direct: KOH, yeast can be visualized easily on direct
	Periodic Acid-Schiff's or Gomori stains for histopathology	Periodic Acid-Schiff's or Gomori stains for histopathology	Periodic acid-schiff's or gomori stains for histopathology	Periodic Acid-Schiff's or Gomori stains for histopathology

mycoses, exclusive of paracoccidioidomycoses, may result from culture of sputum specimens. Presumptive diagnoses of blastomycosis, coccidioidomycosis and paracoccidioidomycosis are sometimes made on the basis of KOH preparations of sputum or the drainage from lesions. Presumptive diagnosis is based on the demonstration of the typical *in vivo* form of the fungus, i.e., large budding yeasts with a doubly-refractile cell wall in the case of blastomycosis, intact spherules containing endospores in the case of coccidioidomycosis, and large, multiply-budding yeast cells in the case of paracoccidioidomycosis. *H. capsulatum* can be difficult to culture from sputum and it cannot be visualized in a KOH preparation of sputum or any other specimen. Since it is an intracellular parasite in macrophages, however, it can be visualized in such cells by performing a Giemsa or Wrights stain of the buffy coat of blood or bone marrow during the early stages of disease when the fungus is being transported from the lung to other organs involved in the reticuloendothelial system.

All of the primary pathogens grow from clinical specimens at ≤30°C as molds, and all, with the exception of *C. immitis*, are slow growers. They may take 4 to 6 weeks to grow from a clinical specimen. To determine that the mold growing from the specimen is actually a form of one of the primary pathogens, additional manipulations must be performed. There are many nonpathogenic saprophytes in nature, all of which are molds incapable of converting to yeasts or spherules, which may contaminate clinical specimens. *B. dermatitidis*, *H. capsulatum* and *P. brasiliensis* may be converted to the yeast forms to confirm the identification. *C. immitis* must be cultured under highly controlled *in vitro* conditions to acquire the spherule form and it is not common practice to do so in routine diagnostic laboratories. The identity of all four fungi, however, can be confirmed by extracting antigens from the medium in which they are grown and demonstrating, using the technique, immunodiffusion in agar, that the extracts contain antigens which are specific for the fungus.in question. Commercial kits are available for this purpose. The test is called an exoantigen test. DNA probes are also available commercially for the detection of specific fungal nuclear material. Occasionally mice are inoculated with the suspected pathogen in an attempt to demonstrate the typical parasitic form of the fungus and confirm the suspected identity of the isolate.

Immune Response

Antibody Response

All of the primary pathogens stimulate vigorous antibody responses. In fact, the same antigens that are used to identify the fungi in the exoantigen test also stimulate specific responses in patients. The antigens have been given various letter designations. While serology may be of value diagnostically and prognostically, especially in coccidioidomycosis and histoplasmosis where the responses in specific clinical

conditions are reasonably well defined, it must be kept in mind that antibody is not protective. In fact, in general, the more antibody produced, the worse the prognosis.

Serology in blastomycosis is problematic, because, as mentioned above, *B. dermatitidis* contains antigens which cross-react with those of other fungi. *B. dermatitidis* has one antigen which is not shared with other fungi, designated A, but it has not yet been obtained in sufficiently pure form, i.e., without cross-reacting components, to be used for skin-testing or for sensitive serologic tests such as ELISA. However, if antibody to the A antigen can be demonstrated by immunodiffusion, recent or current infection with *B. dermatitidis* is presumed. However, only 50 to 80% of patients with culturally-proven blastomycosis, develop demonstrable antibody to the A antigen. Table 4 provides a summary of skin and serodiagnostic tests for the primary mycoses.

Cell-mediated immunity

As mentioned above, skin-test surveys with *Histoplasma*-specific and *Coccidioides*-specific antigen preparations have provided evidence for delayed hypersensitivity, one manifestation of cellular immunity, to these two fungi. Interpretation of the skin-test is the same as that for any delayed hypersensitivity skin-test, a positive reaction simply implies exposure to the fungus at some time prior to skin-testing. Although the patient may have active disease, a positive skin-test does not confirm that. *In vitro* assays can be performed as well to detect proliferative responses of peripheral blood lymphocytes in the presence of fungus-specific antigens.

Negative skin-test reactions may be interpreted in one of three ways. First, individuals who have never been exposed to the fungus in question will be negative due to the absence of stimulation. Secondly, insufficient time between exposure and testing may have occurred for sensitization to reach a demonstrable level. Thirdly, a previously positive patient may become anergic, i.e., specifically unreactive to the antigen. In the latter situation, the patient usually expresses normal delayed hypersensitivity early in the course of the disease but as the disease worsens, the capacity to mount a delayed hypersensitivity reaction when challenged with specific antigen is diminished or lost.

Suitable commercial antigens for skin-testing and *in vitro* testing are not generally available for blastomycosis or paracoccidioidomycosis, although delayed hypersensitivity to *P. brasiliensis* has been demonstrated with experimental antigen extracts. It is presumed that delayed hypersensitivity in blastomycosis will be demonstrable when suitable non-cross-reactive antigens become available.

Mechanisms of Resistance

Resistance to reinfection with the etiologic agents of all the primary mycoses is thought to be T-lymphocyte-mediated cellular immunity. The major phagocytic cells in humans, polymorphonuclear leukocytes (PMNL) and macrophages, are

Table 4. Skin and Serodiagnostic Tests for the Primary Mycoses

Disease	*Blastomycosis*	*Histoplasmosis*	*Coccidioidomycosis*	*Paracoccidiomycosis*
Organism	*Blastomyces dermatitidis*	*Histoplasma capsulatum var. capsulatum*	*Coccidioides immilis*	*Paracoccidioides brasiliensis*
Organism-Specific Antigens	A	H, M	TP, F	several different antigens suggested; no standardized antigens available
Serodiagnostic Tests of Value	Immunodiffusion: detection of antibody in patient sera to the A antigen	Immunodiffusion: detection of antibody in patient sera to the M and/or H antigens	Immunodiffusion: detection of antibody to the F antigen	several different tests are used; no standardized approach
			Tube Precipitin Test: detection of IgM antibody using heated coccidioidin as antigen	
	Complement Fixation: detection of antibody in patient sera using whole yeast cells	Complement Fixation: detection of antibody in patient sera using histoplasmin or whole yeasts as antigen	Complement Fixation: detection of antibody to CF antigens in patient sera using unheated coccidioidin	
Interpretation of Serodiagnostic Tests	Immunodiffusion: antibody to A antigen denotes current or recent infection; test only positive in 50–80% culturally-positive cases	Immunodiffusion: antibody to H or M antigens in patient who has not been skin-tested recently suggests current or recent disease, but test is insensitive	Immunodiffusion: antibody to F antigen evidence of active disease but test insensitive	complement fixing antibodies appear late and linger long after cure; titers high in serious forms of disease
			Tube Precipitin Test: IgM antibodies detected early in disease, denotes recent infection; disappear as disease progresses	

		Complement Fixation: extremely difficult to interpret because *B. dermatitidis* shares so many antigens with other fungi	Complement Fixation: presence of CF antibodies at a titer of ≥ 1:8 denotes recent or current disease and 4-fold increases or decreases in titer denote worsening or improvement, respectively	Complement Fixation: presence of CF antibodies of any titer presumptive evidence of disease; high titers indicate progression to disseminated disease; low titers may linger for long periods	
		serology of limited diagnostic value	serology of more diagnostic than prognostic value	serology of both diagnostic and prognostic value	serology of both diagnostic and prognostic value
	Skin Tests	none available	culture filtrate antigen: histoplasmin	1. culture filtrate antigen: coccidioidin 2. spherule extract: spherulin	culture filtrate antigen; not available commercially
			positive reaction indicates exposure only at some time prior to skin testing; not diagnostic of current disease	positive reaction indicates exposure only at some time prior to skin testing; not diagnostic of current disease	positive reaction indicates exposure only at some time prior to skin testing; not diagnostic of current disease

Table 5. Innate and Acquired Immunity in the Primary Mycoses

Disease	*Blastomycosis*	*Histoplasmosis*	*Coccidioidomycosis*	*Paracoccidioidomycosis*
Innate Immunity: Polymorphonuclear Leukocyte (PMN)	PMN relatively ineffective against both parasitic and saprophytic forms of *B. dermatitidis*, despite the fact that blastomycotic lesions filled with PMN	PMN relatively ineffective against both parasitic and saprophytic forms of *H. capsulatum*	PMN relatively ineffective against both parasitic and saprophytic forms of *C. immitis*, despite the fact that coccidioidal lesions contain many PMN	PMN relatively ineffective against both parasitic and saprophytic forms of *P. brasiliensis*
Innate Immunity: Macrophage in Nonimmune Host	macrophage relatively ineffective against both parasitic and saprophytic forms	macrophage relatively ineffective against both parasitic and saprophytic forms; yeast is facultative intracellular parasite within macrophage	macrophage relatively ineffective against both parasitic and saprophytic forms	macrophage relatively ineffective against both parasitic and saprophytic forms
Acquired Immunity	antibody: produced, but not protective	antibody: produced but not protective	antibody: produced but not protective	antibody: produced but not protective
	cell-mediated immunity: probably the mechanism of protection but poorly understood	cell-mediated immunity: protective, macrophages activated by lymphokines, e.g., interferon-gamma, can kill	cell-mediated immunity: protective, macrophages activated by lymphokines, e.g., interferon-gamma, can kill	cell-mediated immunity: poorly understood but since macrophages activated by interferon-gamma can kill yeasts, CMI believed to be important

relatively ineffective at killing either the saprophytic or parasitic forms of the primary pathogens unless activated by soluble mediators (lymphokines) produced by T lymphocytes following exposure to fungal antigen. Table 5 provides a summary of innate and acquired immunity in the primary mycoses.

SYSTEMIC MYCOSES: OPPORTUNISTIC PATHOGENS

The opportunistic mycoses are those whose etiologic agents are not inherently highly invasive but which rely upon some underlying condition of the host to increase susceptibility to the development of disease. Underlying conditions are too numerous to list exhaustively but include such factors as immunosuppression, either drug-induced or by virtue of disease involving the lymphoid system, long-term antibacterial therapy, surgery, and indwelling catheters. Aside from a growing number of anecdotal cases of nonpathogenic saprophytic fungi causing disease in highly immunosuppressed patients, including those with AIDS, there are four groups of fungi which contain the most common opportunistic pathogens, namely, organisms within the genus *Candida*, two cryptococcal organisms, *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gatti*, several species in the genus *Aspergillus*, in particular *A. fumigatus* and *A. flavus*, and several species within the class, zygomycetes.

Diseases caused by *Candida* sp. and the cryptococci are referred to as opportunistic yeast infections, whereas those due to the aspergilli and zygomycetes are called opportunistic mold infections. Cryptococcosis is actually a disease which can be categorized as either primary or opportunistic. Approximately 50% of the patients with pulmonary cryptococcosis have no known underlying condition predisposing to the development of the disease. Virtually all patients with cryptococcal meningitis, however, have some underlying deficiency which increases their susceptibility to the development of the most serious form of cryptococcosis. Approximately 10% of AIDS patients, for example, succumb to cryptococcal meningitis. Those that survive must be maintained on suppressive antifungal therapy for life.

C. neoformans is the only encapsulated yeast of medical importance. Although there have been a few reports of the observation of hyphae associated with tissue infected with *C. neoformans*, it is not a dimorphic fungus in the traditional sense. *Candida* sp. are dimorphic fungi, however. Poorly defined nutritional factors appear to be associated with the transition from one form to another, but both forms can be demonstrated coexisting in the same tissue section. *In vitro*, the mycelial form grows better at 37°C than 25°C in appropriate media; this pattern of growth is opposite to that seen with the dimorphic primary pathogens capable of yeast-mold conversion.

The aspergilli and the zygomycetes are both molds; they are not dimorphic. Sporulating structures are produced at the tips of specialized hyphal elements. In the aspergilli the conidia are produced in chains over the surface of a vesicle

produced at the hyphal tip. The chains of conidia are susceptible to breaking apart in air currents and individual conidia are inhaled to initiate disease. The zygomycetes most frequently involved in disease produce spores within a sac-like structure called a sporangium and the sporangiospores are released into the air following rupture of the sporangial wall. When these organisms invade tissue, they do so by producing hyphae, and they have a predilection for invasion of blood vessels.

Epidemiology and Ecology

C. neoformans, the aspergilli, and the zygomycetes are all acquired from environmental sources. The latter two are ubiquitous whereas *C. neoformans* is found in specific niches in nature. *C. neoformans* var. *neoformans* is found worldwide associated with soils contaminated with pigeon excreta or, more rarely, with the excreta of chickens. *C. neoformans* var. *gatti*, on the other hand, is found associated with detritus around red gum trees, *Eucalyptus calmaldulensis*. Isolation of, as well as infection with, *C. neoformans* var. *gatti* is much less common than *C. neoformans* var. *neoformans*. Most reports of cryptococcal disease being caused by *C. neoformans* var. *gatti* come from Australia, Southern California, and Brazil. The etiologic agent of cryptococcosis in AIDS patients, regardless of geographic location, has been almost exclusively *C. neoformans* var. *neoformans*.

Candida albicans and *C. tropicalis*, the two most common species of *Candida* involved in human disease, are normal flora associated most often with the gastrointestinal tract. When disease occurs, therefore, it is endogenous in origin. To establish systemic disease, the organism appears to traverse the lining of the gut, gain access to the circulation, and seed various internal organs.

Risk Factors and Clinical Presentations

As these are opportunistic diseases, by definition there must be underlying conditions which predispose to the development of disease. In cryptococcosis, the most prominent factor which predisposes to disease is some alteration in T-cell function. For example, approximately 10% of AIDS patients acquire cryptococcosis. Each of the other diseases described in this section occurs in a particular clinical form depending upon the specific underlying condition. These conditions and the type of disease expressed are summarized in Table 6. For example, cutaneous candidiasis may occur in skin folds in obese individuals because the warm, moist environment, in the skin folds provides a suitable medium in which the fungus may grow, or it may occur on the hands of individuals who wear protective gloves regularly, the gloves preventing evaporation of water from the skin and occluding the skin surface. Life-threatening systemic diseases with *Candida* sp. and the opportunistic molds usually occur in neutropenic individuals and in individuals with abnormal macrophage function. The polymorphonuclear leukocyte (PMNL) and the macrophage are critical as a first line of defense against the opportunistic molds and *Candida* sp.

Table 6. Epidemiologic, Ecologic, and Clinical Aspects of the Opportunistic Mycoses

Disease	*Cryptococcosis*	*Candidiasis*	*Aspergillosis*	*Zygomycosis (mucormycosis, phycomycosis)*
Organism	*Cryptococcus neoformans C. neoformans var neoformans C. neoformans var gatti*	*Candida albicans, C. tropicalis*	*Aspergillus fumigatus, A. flavus*	*Rhizopus sp, Absidia sp.*
Epidemiology	world-wide but varieties are ecologically-restricted	endogenous, normal flora	ubiquitous	ubiquitous
Ecology	*C. neoformans* var neoformans associated content; *C. neoformans var gatti* associated with *Eucalyptus detritus*	primarily mucous membranes, especially gastrointestinal	lives on dead or decaying organic matter	lives on dead or decaying organic matter
Clinical	multiple classifications, but in general	wide variety of clinical conditions, but in general	wide variety of clinical conditions, but in general	wide variety of clinical conditions, but in general
	1. pulmonary	1. cutaneous disease	1. intoxications due to preformed mycotoxins	1. rhinocerebral—±50%
	2. meningeal	2. mucocutaneous disease	2. hypersensitivity disease	2. pulmonary and/or disseminated—±20%
	3. disseminated disease with multi-system involvement	3. systemic disease—multisystem involvement	3. colonizing disease (aspergilloma)	3. abdominal, pelvic or gastric—±11%
		4. focal disease, e.g. peritoneum, heart valves	4. invasive disease	4. cutaneous—±15

continued

Table 6. Continued

Disease	*Cryptococcosis*	*Candidiasis*	*Aspergillosis*	*Zygomycosis (mucormycosis, phycomycosis)*
Organism	*Cryptococcus neoformans C. neoformans var neoformans C. neoformans var gatti*	*Candida albicans, C. tropicalis*	*Aspergillus fumigatus, A. flavus*	*Rhizopus sp, Absidia sp.*
Risk Factors	AIDS; other forms of immunosuppression; however, about 50% of patients with pulmonary disease only, have no known underlying factor contributing to disease	predisposing conditions too numerous to list; the following examples, however, are typical: cutaneous disease occurs on skin folds of obese patients; mucocutaneous disease occurs in AIDS patients and other patients with T lymphocyte disorders, systemic disease occurs in neutropenic patients; localized disease may follow surgery	type of disease depends on underlying factors: e.g., patients appear to have a genetic predisposition to hypersensitivity disease, which includes atopy; colonizing disease occurs in patients with preexisitng pulmonary cavities such as seen with tuberculosis; invasive disease occurs in immunosuppressed neutropenia	type of disease depends on underlying condition, e.g., rhinocerebral in uncontrolled diabetic; disseminated disease most often in patients with leukemia/lymphoma; gastric under conditions of malnutrition; cutaneous in burn patients

Laboratory Considerations

Cryptococcosis is usually diagnosed on the basis of culture of the organism from sputum or spinal fluid, or demonstration of capsular polysaccharide in serum or spinal fluid. Presumptive diagnosis of cryptococcosis can also be made on the basis of the demonstration of encapsulated, budding yeasts in the centrifugate of spinal fluid. When present, however, *C. neoformans* is relatively easy to culture and identify. Unencapsulated or poorly-encapsulated cryptococci can be found in some patients, so that characteristics other than capsule formation, are used to identify and speciate cryptococcal organisms. For example, pathogenic cryptococci produce melanin (brown pigment) when cultured in the presence of caffeic acid or other substrates which can be hydrolyzed by the phenoloxidase of the fungus, all cryptococci synthesize urease and have the ability to utilize inositol as a carbon source. Cryptococci will grow from a specimen relatively rapidly, e.g., in 3 days, and they can then be identified in 1 to 6 days depending upon the yeast identification system used in the laboratory.

The laboratory test for the detection of cryptococcal polysaccharide is called the cryptococcal antigenemia test. There are four serotypes of cryptococci based on the antigenic composition of the capsular polysaccharide. Serotypes A and D are found to be *C. neoformans* var. *neoformans* species and serotypes B and C are *C. neoformans* var. *gatti*. Rabbits immunized with a mixture of capsular polysaccharides consisting of types A, B, and C, produce antibodies capable of recognizing all four serotypes. Therefore, rabbit IgG specific for capsular polysaccharide is used to detect cryptococcal polysaccharides in body fluids.

Diagnosis of candidiasis, especially the systemic forms, is much more problematic. The isolation of a *Candida* sp. from blood, for example, may indicate only that the plastic surface of a central or peripheral line is colonized with the organism. Likewise, the isolation of *Candida* sp. from sputum usually indicates only that some abnormal pathologic condition is present in the lung, permitting the mucocutaneous overgrowth of the organism. Pulmonary disease, or other systemic forms of candidiasis, in fact, can only be established unequivocally if hyphal and yeast forms can be demonstrated histopathologically in biopsy specimens. On the other hand, the demonstration of typical hyphae/pseudohyphae and yeasts in 10 to 20% potassium hydroxide (KOH) preparations of cutaneous and mucocutaneous lesions is strong presumptive evidence of candidal disease at those sites. *Candida* sp. grow rapidly from specimens in the laboratory, 2 to 3 days, and are easily identified and speciated on the basis of their ability to produce hyphae under the appropriate incubation conditions, their ability to assimilate various sugars when the sugars are provided as the sole carbon sources, and their ability to reduce nitrate to nitrite. *C. albicans* is the only candidal species which regularly produces germ tubes in fetal calf serum or in special media designed to induce germ tube production, so that *C. albicans* can be identified the same day colonies appear on primary isolation media.

Speciation of *Candida* (not *albicans*) sp., however, as with cryptococcal species, requires additional time.

Diagnosis of aspergillosis and zygomycosis are also problematic as spores of these organisms are often encountered in air and may contaminate specimens being cultured. Furthermore, the zygomycetes are often difficult to recover from clinical specimens, especially biopsies, and diagnosis may depend upon the demonstration of the organism in the biopsy specimen by histopathologic techniques. Diagnoses of opportunistic mold infections, in general, often depend upon the repeated isolation of the same organism from sequential samples, and the demonstration of the organism in tissue by histopathology. When these organisms actually invade tissue, one sees only hyphae, no sporulating structures are produced. In fact, the only occasion in which a sporulating structure may be seen associated with tissue in aspergillosis or zygomycosis is in the case of colonization of a preexisting cavity in the lung with *Aspergillus* sp. In this instance, tissue invasion *per se* does not occur and the organism is simply growing on the surface of the cavity exposed to the air.

The histopathologic appearance of the hyphae from these two groups of opportunistic molds is very different. The hyphae of *Aspergillus* sp. are septate with parallel walls and they often divide at acute angles to produce brush-like structures observable by staining with special stains such as Gomori methanamine silver (GMS) or Periodic acid Schiff's (PAS). GMS results in the deposition of silver in the cell wall and fungi become black, and PAS results in the deposition of red pigment in the fungal cell wall. The agents of zygomycosis produce broad hyphae with no crosswalls and their appearance in tissue is difficult to detect because the hyphae may look like irregularly shaped tears of the tissue.

Identification of the opportunistic molds is based almost solely on the macroscopic and microscopic appearance of sporulating organisms growing on solid media. Table 7 gives a summary of important laboratory considerations for the opportunistic mycoses.

Immune Response

Most individuals with intact immune systems produce antibody to the opportunistic yeasts and molds during the course of disease. In the case of cryptococcal disease, however, the antibody appears to have specificity for capsular polysaccharide and combines readily with it, so that free antibody is difficult to detect in individuals with active disease. Antibody detection, therefore, is of no diagnostic value in cryptococcosis. Antibody detection in candidiasis is of little value at the present time as well. Since a large proportion of healthy individuals is colonized with *Candida* sp., many individuals have antibody to cell wall antigens of the fungus and some may also have antibody to cytoplasmic antigens. It is, therefore, very difficult to use serology to separate patients who are only colonized with the fungus from those in whom the fungus is causing disease.

Table 7. Selected Laboratory Considerations for the Opportunistic Mycoses

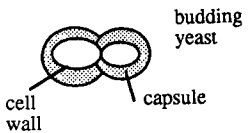
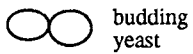
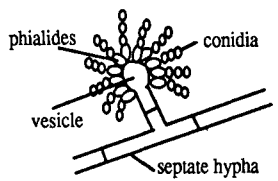
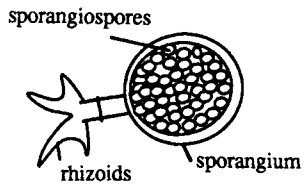
Disease	*Cryptococcosis*	*Candidiasis*	*Aspergillosis*	*Zygomycosis (mucormycosis, phycomycosis)*
Organism	*Cryptococcus neoformans C. neoformans var neoformans C. neoformans var gatti*	*Candida albicans, C. tropicalis*	*Aspergillus fumigatus, A. flavus*	*Rhizopus sp., Absidia sp.*
Typical Specimens	sputum, spinal fluid	most known specimen types, e.g., blood, urine spinal fluid, sputum, biopsies, etc.	sputum and biopsy most common	sputum and biopsy most common
In vitro Culture	encapsulated budding yeast at any temperature 	unencapsulated budding yeast on standard laboratory media such as Sabourauds agar 	rapidly growing, sporulating mold; two most common species causing disease produce conidia which contain various shades of green pigment in the conidial wall and which are produced in chains from the surface of a specialized structure 	rapidly growing mold producing sac-like structures, sporangia, which contain hundreds of small spores, called sporangiospores; colonies become gray to black in color 
	easy to culture from specimen; difficult to determine significance if from a normally contaminated site			often difficult to recover by culture from specimen

Table 7. Continued

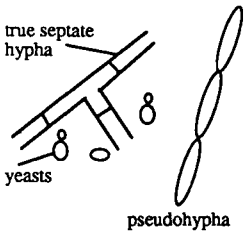
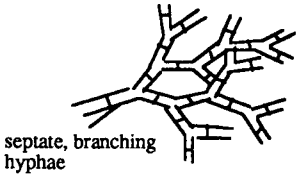
Disease	*Cryptococcosis*	*Candidiasis*	*Aspergillosis*	*Zygomycosis (mucormycosis, phycomycosis)*
Organism	*Cryptococcus neoformans C. neoformans var neoformans C. neoformans var gatti*	*Candida albicans, C. tropicalis*	*Aspergillus fumigatus, A. flavus*	*Rhizopus sp., Absidia sp.*
In vivo morphology	same as *in vitro* appearance	lesions or specimens usually contain a mixture of yeasts and hyphal forms; some specimens also contain pseudohyphae 	lesions contain hyphal elements with cross-walls (septa) dividing the hyphal elements into individual cells; the hypha often have the appearance of a fan due to the manner in which the hyphal tips divide and grow 	hyphae in the tissues have very irregular shape and may simply resemble elongated holes with unparallel walls; septa are seldom formed
Laboratory	Direct: India Ink preparation of spinal fluid allows visualization of encapsulated yeasts; KOH of no value	Direct: KOH of some value; Gram stain may be helpful for vaginal or buccal lesions	Direct: KOH occasionally helpful for aspergilloma but not helpful for other forms of disease	Direct: KOH may be helpful
	Periodic Acid-Schiff's or Gomori stains for histopathology; mucicarmine stains capsule	Periodic Acid-Schiff's or Gomori stains for histopathology	Periodic Acid-Schiff's or Gomori stains for histopathology	Periodic Acid-Schiff's or Gomori stains for histopathology

Table 8. Serology as Diagnostic or Prognostic Aid for Opportunistic Fungal Diseases

Disease	*Cryptococcosis*	*Candidiasis*	*Aspergillosis*	*Zygomycosis*
Organism-Specific Antigens	capsular serotypes A, B, C, D	none are diagnostically useful in the routine laboratory at the present time	none that are routinely diagnostically useful at the present time	none that are routinely used at the present time
Serodiagnostic Tests Available	cryptococcal antigenemia test: latex beads coated with anticapsular antibody; test may also be configured as an enzyme-linked immunoassay	Immunodiffusion: detection of antibody to multiple cytoplasmic antigens in patient sera	Immunodiffusion: detection of antibody to cytoplasmic antigens	none that are routinely used at the present time
		commercial kits for detection of antigenemia available	Complement Fixation: detection of antibody to cellular antigens in patient sera	
Interpretation of Serodiagnostic Tests	detection of cryptococcal antigen at almost any titer in serum or CSF is indicative of active disease; CSF is positive in essentially all cases of cryptococcal meningitis, serum is positive in approximately 50% of culturally-proven pulmonary disease	Immunodiffusion: immunodiffusion assays are performed in many laboratories but since some normal individuals, as well as patients with nonfungal diseases, have precipitating antibodies, the test is of little value	Immunodiffusion: immunodiffusion tests have been used for some time as an aid in diagnosis of hypersensitivity disease and aspergilloma; many patients with invasive disease are too ill to produce antibody	
	detection of antibody is of no value routinely	Tests for antigenemia: the reagents in the commercial kits are poorly defined and the tests are very insensitive	Complement Fixation: CF assays for Aspergillus antigens are frequently done in parallel with those for the organisms causing the primary systemic mycoses but meaningful interpretation of the results is difficult	

Table 9. Innate and Acquired Immune Responses to the Opportunisitic Fungal Pathogens

Disease	*Cryptococcosis*	*Candidiasis*	*Aspergillosis*	*Zygomycosis (phycomycosis, mucormycosis)*
Innate Immunity: Polymorphonucelar Leukocyte (PMN)	effective against poorly-encapsulated *C. neoformans*; as capsule size increases phagocytosis and killing decrease	can damage both yeasts and hyphae of pathogenic *Candida* sp	can damage and kill hyphae of *A. fumigatus* but ineffective against conidia	can damage and kill hyphae but PMN relatively ineffective against conidia and spores;
Innate Immunity: Macrophage in Nonimmune Host	relationship between macrophage and *C. neoformans* poorly understood	can damage *C. albicans* but its role *in vivo* poorly understood	first line of defense against conidia, prevents germination; can kill hyphae without ingestion but PMN appears more important	first line of defense against spores; inhibits germination, but difficult to kill
Acquired Immunity	protective immunity may be mediated by a combination of antibody- and cell-mediated immunity	controversial: protection may be mediated by a combination of antibody- and cell-mediated immunity	cell-mediated immunity probably protective but patient either recovers or dies before CMI has time to influence the outcome	cell-mediated immunity probably protective but patient either recovers or dies before CMI has time to influence the outcome
			antibody produced but it has no known role in protection	little known about antibody response

Antibody detection may be of some diagnostic aid in aspergillosis, specifically in those individuals with allergic or colonizing disease. Those with colonizing disease especially, frequently produce large numbers of specific antibodies to many *Aspergillus* sp. antigens. A summary of serodiagnotic tests for opportunistic fungal diseases is given in Table 8.

Mechanisms of Resistance

Two types of resistance must be considered when discussing the opportunistic fungi, viz., innate versus acquired (see Table 9). Intact innate immunity, i.e., properly functioning PMNL and macrophages, are critical for normal defense against *Candida* sp. and the opportunistic molds. Moreover, intact T-cell function appears to be critical for the normal defense of mucocutaneous tissue against *Candida* sp. The basis of acquired resistance, defined here as resistance to reinfection, against *Aspergillus* sp. or zygomycetes is unknown, and in candidiasis it is controversial. It is likely that both antibody and cellular immunity play a role in resistance to reinfection to *Candida* sp.

Innate and acquired responses to *C. neoformans* are controversial as well. The capsule is clearly antiphagocytic, but poorly encapsulated forms can be phagocytized and killed. It has been suggested that cryptococcal disease is not rampant among healthy individuals living in urban environments where they are probably exposed fairly often, because poorly-encapsulated organisms are encountered in nature and are disposed of in the healthy individual by normal alveolar defenses before they have had sufficient time to multiply and produce large amounts of capsule. It is clear from experimental studies, and from observations of disease in humans, that intact cellular immunity is critical to successful defense against the organism as well. It also appears that capsule-specific antibody plays a role in protection.

SUBCUTANEOUS MYCOSES

The subcutaneous mycoses are, by definition, those fungal diseases which result in a subcutaneous lesion following the traumatic implantation of a fungus into the subcutaneous tissue. All subcutaneous mycoses have in common the fact that the etiologic agents, normal inhabitants of soil, are traumatically implanted into the subcutaneous tissue to initiate disease. There are, however, only four forms of subcutaneous disease which are seen in the United States, namely, sporotrichosis, phaeohyphomycosis, chromoblastomycosis, and mycetoma.

Sporotrichosis

Sporotrichosis is caused by the fungus *Sporothrix schenckii*, a dimorphic organism which grows as a small yeast in tissue or on artificial media at 37°C and as a sporulating mold at temperatures ≤30°C. The fungus is found world-wide in

temperate, subtropical and tropical climates. Sporotrichosis is predominantly a disease of individuals at risk for puncture wounds with sharp objects such as rose thorns, wood splinters, or pine needles. Sphagnum moss is frequently contaminated with the fungus and numerous outbreaks have occurred in individuals handling objects packed in sphagnum moss.

The most common clinical form of sporotrichosis is lymphocutaneous disease wherein the primary lesion, commonly on an extremity, develops at the site of deposition of the fungus, erodes to the surface at that point, but also from which organisms spread via the lymphatics. Where a sufficient number of organisms gather at a particular site in the lymphatic tract a new lesion develops. Thus, for example, it is not uncommon to see a patient with a lesion on the hand and several additional lesions on the forearm associated with the lymphatic tract. In addition to the lymphocutaneous form of the disease, however, a form which usually occurs in previously healthy individuals, pulmonary sporotrichosis may be seen in chronic alcoholics where it mimics histoplasmosis, and disseminated sporotrichosis with multisystem involvement may be seen in immunocompromised individuals.

Presumptive diagnosis of sporotrichosis is made on the basis of the patient's history and clinical picture. Definitive diagnosis is based on culture of the organism from the lesion. It is necessary to convert the mold phase of the organism growing at ≤30°C to the yeast phase at 35°–37°C to confirm that the organism isolated is *S. schenckii*. The mold form frequently produces a brown-black pigment when growing *in vitro*. KOH preparations of tissue are of no value as diagnostic aids. An acid-fast stain, however, can sometimes be helpful, because *Nocardia* sp., which are filamentous bacteria and partially acid-fast, can produce a clinical picture identical to that of sporotrichosis. *Nocardia* sp. will grow on Sabourauds agar without antibacterial agents. Serology and skin-testing are not done in the United States but both are employed in selected medical centers in Latin America.

Chromomycosis and Phaeohyphomycosis

Chromoblastomycosis and phaeohyphomycosis are both diseases which have dematiaceous, i.e., brown or black pigmented, fungi as etiologic agents. Some mycologists consider the terms chromoblastomycosis and phaeohyphomycosis to be synonyms. However, chromoblastomycosis is traditionally described as a chronic disease involving both cutaneous and subcutaneous disease in which the fungal form observed in tissue is a round cell, 4 to 12 μ in diameter, with a thick brown wall, divided in several planes with crosswalls. These cells often remain clustered, and are called sclerotic bodies. A common aptly descriptive term applied to them is "copper pennies." Their ontogeny is unknown. In contrast to the tissue form seen in chromoblastomycosis, in phaeohyphomycosis only hyphal elements with brown to black pigments in their walls are seen in tissue. Phaeohyphomycotic lesions are more often localized as "cysts," and treatment, when the lesion is

advantageously placed, is excision. The most common agent of chromomycosis is the dematiaceous fungus, *Fonsecaea pedrosoi.*

Mycetoma

In order to be classified as a mycetoma, a lesion must have three characteristics: tumifaction (swelling), granules (microcolonies of the etiologic agent), and draining sinus tracts. Approximately 50% of the lesions which meet these criteria are produced by fungi, the other 50% being produced by bacteria classified as actinomycetes. The most common fungal cause of mycetoma in the United States is *Pseudoallescheria boydii,* and the most common bacteria isolated as etiologic agents belong to the genus *Nocardia.* Patients with actinomycetomas can be treated successfully with antibacterial agents if the disease is diagnosed early. Treatment of mycotic mycetomas has been much more difficult, however, even when diagnosed early.

CUTANEOUS MYCOSES

The cutaneous mycoses are those where the primary lesions are initiated in the skin. They must be distinguished from diseases where dissemination of the organism from internal sites to the skin has occurred. Since the cutaneous mycoses, exclusive of cutaneous candidiasis, are caused by a group of organisms known collectively as dermatophytes, the cutaneous mycoses are called dermatophytoses. The dermatophytoses are the only contagious mycotic diseases.

Etiologic Agents and Risk Factors

The etiologic agents of the dermatophytoses are collectively termed the dermatophytes. A vernacular term often used to describe these clinical conditions is "ringworm." The Latin word for "worm" is "tinea" and dermatophyte infections are described to this day as tineas. One usually appends a modifier to indicate the anatomical location of the infection, e.g., tinea capitis is ringworm of the head, tinea pedis is ringworm of the feet, and so forth. The names derive from the observations in the 17th century of circular lesions with erythematous borders occurring on the smooth skin or scalp.

The dermatophytes are a collection of keratinophilic fungi whose primary reservoirs are either in soil (geophilic), in lower animals (zoophilic), or in man (anthropophilic). There are three genera of dermatophytes: *Trichophyton, Microsporum,* and *Epidermophyton.* There are a number of species of both *Trichophyton* and *Microsporum* but only a single species of *Epidermophyton, E. floccosum.* The most common etiologic agents of the dermatophytoses are *T. rubrum, T. tonsurans, T. mentagrophytes, M. canis* and *E. floccosum.*

Pathogenesis

The infecting particle depends upon the source of the fungus at the time of infection. For example, an abraded area of skin may become infected by contamination with soil in which the fungus is growing and producing hyphae and spores, or infected skin scales or hairs containing hyphal elements or arthroconidia may be transferred by direct contact with an infected animal or human or by contact with some inanimate object, e.g., a hair brush, a shaver, a bed sheet, and so on. While contact with the fungus is essential for the establishment of disease, contact alone is not sufficient. Other factors which contribute to the outcome of contact with the fungus include, elevated environmental temperatures, relatively high humidity, occlusion of the site of contact, skin abrasions, an appropriate but poorly-understood gene pool, malnutrition, and gender.

All dermatophytes are keratinophilic, and as such they produce keratinases, enzymes capable of hydrolyzing keratin. Dermatophytes attack tissues which contain dead keratin, specifically skin, hair and nails. For some unknown reason, all dermatophytes do not attack the keratin from all three sources. For example, *T. rubrum* attacks skin and nails but is virtually unknown as a pathogen of hair. When attacking skin the fungal hyphae grow in a radial direction. The most physiologically active portion of the fungus is at the growing hyphal tip and the inflammatory response of the host is to that apical area. The centers of the lesions tend to contain dead fungal particles with little remaining inflammation. Thus a typical dermatophyte lesion has an erythematous border with a central area of clearing. The organisms may penetrate to the base of the stratum corneum, but they go no deeper.

Three types of hair infections may occur, namely, ectothrix, endothrix, and favic. They are described on the basis of the form of the fungus and its position in the hair. In an endothrix type of infection, fungal hyphae fill the hair shaft and are converted completely to arthrospores, the latter of which become round. Ectothrix infection is characterized by the production of arthrospores on the outside of the hair. In favic infections one finds only hyphae coarsing through the hair shaft.

Diagnosis

Diagnosis of dermatophyte infections is usually based on the clinical picture in combination with microscopic observations of hair, nails or skin scraping. Tissue samples are mounted in 10 to 20% KOH, which may or may not contain blue ink, heated gently to disrupt the tissue cells, and observed under the microscope. Fungal hyphae, with or without arthrospores, are observed in skin and nail preparations. Hyphae or arthrospores, but not both, are observed in hair preparations. The clinical picture and a positive KOH are sufficient to diagnose and treat dermatophytosis. However, the causative agents can be cultured on routine mycologic media containing antibiotics. When recovered from a specimen they are identified largely on the

basis of their gross and microscopic appearance in culture. They are slow-growing fungi and may require 3 to 4 weeks for identification.

Mechanisms of Resistance

The mechanisms of resistance to dermatophytes appear to include specific immunity as well as nonspecific factors such as a dry, intact epidermis, a high epidermal turnover rate, the presence of transferrin which binds iron and makes it unavailable to the fungus for growth, and selected fatty acids in sebum. Specific immunity appears to be cell-mediated but it has not been proven definitively. An interesting immunologic phenomenon may occur in some individuals called the dermatophytid or "id" reaction. It is presumed that fungal antigens from lesions in one part of the body gain access to the circulation with the subsequent development of sterile lesions resulting from hypersensitivity responses on the skin elsewhere in the body. For example, an individual with tinea pedis may develop "id" reactions on his/her hands.

SUPERFICIAL MYCOSES

There are several superficial mycoses which involve hair and skin. The most common of these is the disease syndrome, pityriasis versicolor. Pityriasis versicolor is a chronic, mild, asymptomatic infection of the stratum corneum, in which slightly elevated, scaly patches appear on the skin, most often on the neck and torso. Dark-skinned individuals tend to develop hypopigmented lesions whereas light-skinned individuals tend to develop lesions which are hyperpigmented. The depigmentation is thought to be due to the toxic effects of dicarboxylic acids produced from sebum by the fungus, *Malessezia furfur*. *M. furfur* is found associated with the disease and is believed to be its causative agent, but the cause-and-effect relationship has not been proven definitively. It is a member of the normal flora of skin, and is believed to initiate disease when proliferating unchecked under poorly-defined environmental conditions. Diagnosis is based on the microscopic observations of yeasts and short hyphal elements in skin scales treated with 10 to 20% KOH, not on the isolation of the organism in culture. The organism can be grown only on media overlaid with olive oil, as it requires components of the olive oil for growth.

ANTIFUNGAL THERAPY

Drugs currently licensed for the treatment of fungal diseases fall into several categories. The first drugs to be developed in the late 1950s, were polyenes. These are large hydrophobic molecules produced by filamentous bacteria known as actinomycetes. One of the polyenes, amphotericin B, remains the gold standard for treatment of systemic fungal infections. It is, however, poorly soluble in water and

must be administered intravenously as a colloid. The polyenes exert their antifungal activity by interacting with the fungal cell membrane sterol, ergosterol, and mediating permeability-associated alterations in the cell. More recently, a series of synthetic compounds with a broader spectrum of activity have been developed which contain one or more azole rings. The azoles exert their antifungal effects by blocking various steps in the synthesis of ergosterol. The earliest of these, miconazole and ketoconazole, are widely used for a variety of non-life threatening fungal diseases. Newer azoles such as fluconazole and itraconazole are also being used with increasing frequency. Since some of the azoles are used for prophylactic and maintenance therapy, a major problem which has evolved is the appearance of azole-resistant organisms. Another antifungal drug, 5-fluorocytosine, is used primarily as an adjunct to therapy with amphotericin B. It is an antimetabolite which inhibits RNA synthesis and interrupts DNA synthesis. It has a narrow spectrum of activity and the appearance of organisms resistant to its action are a problem as well. Finally, griseofulvin, an antibiotic produced by a fungus in the species *Penicillium*, has been an important agent for the treatment of dermatophytoses, the only clinical syndrome in which it is effective. Its mechanism of action involves an interference with microtubule formation; thus, it is only effective against actively growing cells.

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Chapter 18

The Rickettsiae

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INTRODUCTION

Rickettsiae have caused diseases in humans throughout the world since the earliest days of antiquity. It was noted by Zinsser that typhus and other infectious diseases have decided the outcome of many military campaigns. Depending on the outcome for each contending party, either the epidemics were blamed for defeat or the triumphant leaders were credited with victory. With the advent of effective antibiotics, the fear of acquiring a rickettsial infection receded. However, on closer examination, rickettsial diseases are still a global public health problem causing morbidity and in certain instances mortality.

It was and still is difficult to diagnose rickettsial diseases because many other illnesses have similar clinical manifestations. The rickettsias are small Gram-negative bacteria that, with few exceptions, are capable of growth only inside eukaryotic cells. They are associated with arthropods or other invertebrate hosts and often infect vertebrates.

Taxonomy

The order Rickettsiales contains three families: Rickettsiaceae, Bartonellaceae, and Anaplasmataceae. The family *Rickettsiaceae* is divided into three tribes: *Rickettsieae*, *Ehrlichieae*, and *Wolbachieae*. Members of the genus *Rochalimaea* which were previously in the tribe *Rickettsieae* have been moved to the genus

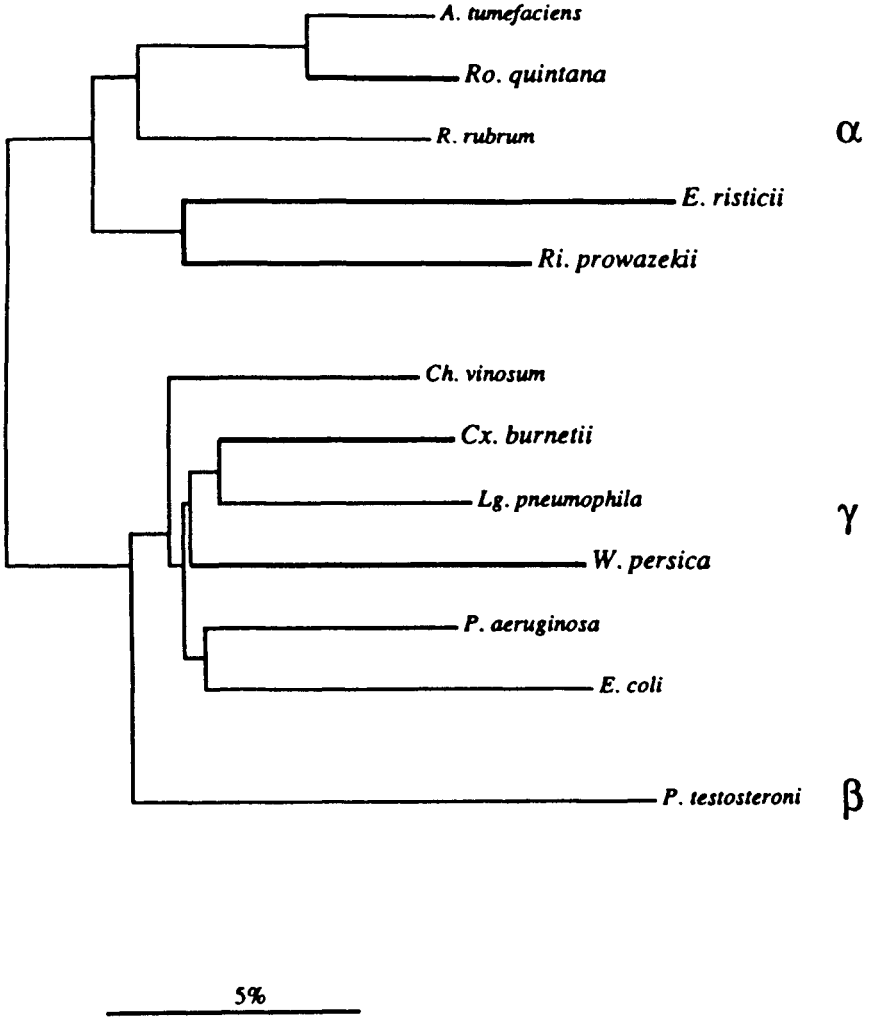

Figure 1. Evolutionary distance tree showing relationships between the organisms in this study and representative members of the α, β, and γ subdivisions of the purple bacteria. Scale bar corresponds to 5 nucleotide substitutions per 100 sequence positions. (Reprinted from Weisberg et al., 1989, with permission).

Table 1. Representative Type of Pathogenic Rickettsiae in Humans

Family Genus	*Biogroup*	*Species*	*Disease*	*Distribution*	*Transmitted by:*	*Target Cell*	*Cellular Location*
Rickettsiaceae Rickettsia	Spotted fever	*R. rickettsii*	RMSF	Western hemisphere	Tick bite	Endothelium	Cytosol
		R. conorii	MSF	Middle East, Africa	Tick bite		
	Typhus fever	*R. typhi*	Endemic typhus	Worldwide	Infected flea feces		
		R. prowazekii	Epidemic typhus	South America, Africa	Infected louse feces	Endothelium	Cytosol
			Recrudescent typhus	Worldwide	Reactivation of latent infection		
			Sylvatic typhus		Contact with flying squirrels		
Orientia	Tsutsugamushi fever	*O. tsutsugamushi*	Scrub typhus	Asia	Chigger bite	?	Cytosol
Coxiella	Coxiella fever	*C. burnetii*	Q fever	Worldwide	Inhalation of infected aerosol	Mononuclear phagocytes	Phagosome
Ehrlichia	Ehrlichia fever	*E. sennetsu*	Sennetsu/Human monocytic ehrlichiosis	Japan	Mite fever	Mononuclear phagocytes	Phagosome

		E. chaffeensis	Spotless fever/Human monocytic ehrlichiosis	United States	Unknown	Mononuclear phagocytes	Phagosome
		E. ?	Human granulocytic ehrlichiosis	United States	Ixodes and/or dermacentor ticks?	Granulocyte	Phagosome
Bartocellaceae *Bartonella (Rochalimaea)*	Trench fever	*B. quintana*	*Trench fever*	*Europe*	louse feces	Erythrocyte (extracellular)	Cytosol & epicellular
			Bacillary angiomatosis	U.S./Europe	?		
		B. henselae	Bacillary angiomatosis	U.S./Europe	?		
			Cat scratch fever	U.S./Europe	Cat flea ?		
		B. elizabethae	Endocarditis ?	U.S.	?		

Bartonella of the family *Bartonellaceae*. This transfer was due to the high degree of DNA and 16S rRNA relatedness between the *Rochalimaea* species and *Bartonella bacilliformis*. While the old genus *Rochalimaea* has been replaced with the genus designation *Bartonella*, the species names remain unchanged. This chapter is an overview of the human rickettsial pathogens in the tribes *Rickettsieae* and *Ehrlichieae* and of the human pathogens in the genus *Bartonella* formerly belonging to the genus *Rochalimaea* (Table 1). The tribe *Rickettsieae* comprise at present three genera: *Rickettsia, Orientia,* and *Coxiella*. The tribe *Ehrlichieae* includes one genus: *Ehrlichia*.

The phylogenetic (Figure 1) diversity of the rickettsiae (Weisburg et al., 1989) showed that the members of the family *Rickettsiaceae* belong to the purple bacterium phylum. However, the family as a whole is not monophyletic. Beyond the level of the genus *Rickettsia*, the relationship within and among the genera seems to disappear resulting in a phylogenetic diversity. For example, the genus *Coxiella*, an obligate intracellular rickettsia, shows a distant relationship to the genus *Legionella*, a bacterium that grows extracellularly in bacteriological media. It was recently suggested that the family *Bartonellaceae* be removed altogether from the order *Rickettsiales*.

GENUS RICKETTSIA

The genus *Rickettsia* is divided into two biogroups based on their antigenic interrelationship: spotted fever and typhus fever. Both the spotted fever and typhus biogroups consist of a number of species.

SPOTTED FEVER GROUP

The spotted fever group includes several species of rickettsias that induce the same or similar pathologic effects with varying degree of pathogenicity. The Rocky Mountain spotted fever (RMSF) agent being, the most pathogenic, will be described as the type species. Other spotted fever agents are those that cause Mediterranean spotted fever (MSF) and rickettsial pox, which induce a relatively milder syndrome.

Rocky Mountain spotted fever, one of the deadliest of all infectious diseases, was first recognized in the late 1800s and early 1900s in the Snake River valley of Idaho and the Bitterroot Valley of western Montana. Between 1906 and 1909, Howard Ricketts demonstrated that RMSF was caused by infectious organisms that were present in the patient's blood. They could be transmitted to guinea pigs and monkeys, and conferred immunity on animals that survived the infection.

Epidemiology

R. rickettsii resides in various species of ticks, which are considered the reservoir or natural hosts. In the Rocky Mountain region and west of the range, *R. rickettsii* is found in the wood tick *Dermacentor andersoni*. To the east of the range, *R.*

rickettsii occurs in *D. variabilis*, the common American dog tick. Transovarian transmission is the major mechanism for the maintenance of *R. rickettsii* in nature. Approximately 4 percent of *D. variabilis* ticks in many locales contain spotted fever-group rickettsiae, nearly all of which are presumably nonpathogenic for humans (e.g., *Rickettsia montana* and *Rickettsia rhipicephali*). Nonpathogenic spotted fever rickettsiae seem to be able to exclude *R. rickettsii* from becoming established in ticks containing these nonpathogenic rickettsiae. Ticks have rarely been found naturally infected with more than one species of spotted fever-group rickettsia. Only the adult stage of the tick feeds on humans. The nymphs feed on small animals.

Cyclic fluctuations in the incidence of RMSF have occurred over a period of decades. Throughout the entire country in 1959, only 199 cases were reported to the Centers for Disease Control. After a steady rise in incidence, the highest number of cases, 1,192, was reported to the CDC in 1981. Subsequently, a decline has occurred, with an average of 600 cases reported. Most cases occur between May and September, but though relatively rare, RMSF cases are reported in the winter months. The disease has a high incidence in children who play out doors and has an affinity for dogs, which are frequently parasitized by *D. variabilis*.

Bacterium

R. rickettsii (Figure 2) is a small 0.2 to 0.5 μm by 0.3 to 2.0 μm obligate intracellular bacterium. Ultrastructurally, these organisms have typical procaryotic cytoplasm containing ribosomes and indistinct strands of DNA in an amorphous cytosol that is surrounded by a plasma membrane. The outer envelope or cell wall resembles that of other Gram-negative bacteria. The rickettsial envelope appears to be the typical Gram-negative structure with a bilayer inner membrane, a peptidoglycan, and a bilayer outer membrane. Rickettsial cell walls contain the constituents of peptidoglycan, namely, sugars, amino acids, muramic acid, and diaminopimelic acid, but not teichoic acid. An indistinct layer, often referred to as the microcapsular layer, is present on the outer surface of the cell wall. Intracellularly the rickettsial organism is not surrounded by a host cell membrane, but rather resides directly in the cytosol or nucleoplasm of the host.

R. rickettsii metabolize glutamate, glutamine, pyruvate, and α-ketoglutarate. *R. rickettsii* synthesizes proteins in the absence of protein synthesis by the host cell. It is also probable that availability of certain amino acids from host cell pools is the limiting factor in rickettsial growth (Austin and Winkler, 1988).

The genome of *R. rickettsii* contains 1.3×10^9 daltons of DNA. The guanine-plus-cytosine content of *R. rickettsii* and other spotted fever group rickettsiae is 32 to 33 mol%.

Three surface proteins, generally designated as 120 (rOmpB), 155 kD, and 190 kD (rOmpA) may be considered as the immunodominant antigens because antibodies to these proteins appear early. In addition, the cell wall contains lipopolysaccharide-like material (LPS). Monoclonal antibodies to heat-labile epitopes

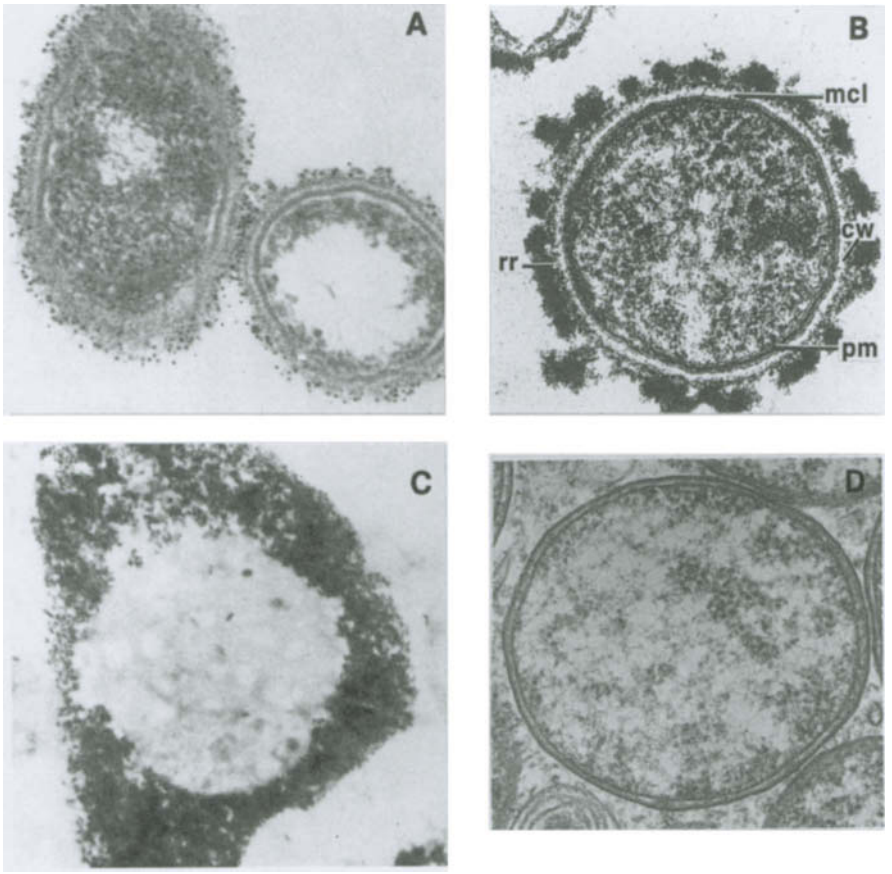

Figure 2. A. Immunolabeling to localize external antigenic sites of *R. rickettsii* Magnification, × 225,000. (Reprinted by permission, Hechemy et al., J. Clin. Microbiol. 27, 377, 1989). B. Ultrathin section of the Breinl strain of *R. prowazekii* from a preparation which was stained with ruthenium red, rr; mcl, microcapsular layer; cw, cell wall; pm, plasma membrane. Bar = 0,2 μm . (Reprinted by permission, Silverman et al., Infect. Immun. 22, 233, 1978). C. Late growth phases of *R. prowazekii* (Breinl) in chicken embryo cells. (Reprinted by permission from Wisseman & Wadell, Inf. Immun., 11, 1391, 1975). D. *R. tsutsugamushi* rickettsiae showing tri-laminar membranes, ribosomes, and DNA filaments. ×1000,000. (Reprinted by permission, Rikihisa, Y., & Ito, S.) In: Rickettsiae and Rickettsial Diseases, Burgdorfer, W., & Anacker, R.L., eds. p. 213, Academic Press, NY, 1981).

neutralize the ability of the rickettsiae to cause febrile disease in guinea pigs. In contrast, monoclonal antibodies to heat-stable epitopes of the 155-kD polypeptides and to LPS do not neutralize toxicity (Anacker et al., 1987).

Pathology

RMSF is a systemic illness with protean manifestation (Walker, 1989). It appears that there is no apparent important immunopathologic component to *R. rickettsii*, e.g., exotoxins. Rickettsemia in human RMSF is usually only on the order of $10^{0.7}$ to $10^{1.2}$ per mL. From the skin, the portal of entry, rickettsiae spread via the lymphatics and bloodstream to all parts of the body. Adsorption of the bacterium and consequently internalization require metabolically competent rickettsiae and active participation of the host cell. *In vitro R. rickettsii* kills parasitized cells directly, thus demonstrating the rickettsial ability to directly exert its pathogenic mechanisms in the absence of the immune, inflammatory, and coagulation mechanisms of the host. The intrinsic and extrinsic pathways of coagulation, platelets, and the fibrinolytic system are activated. Thrombocytopenia occurs in 32% to 52% of RMSF patients. The histopathology of RMSF illustrates a constellation of mechanisms, namely vascular injury, that involves predominantly lymphocytes and macrophages rather than the neutrophil-rich vasculitis seen in immune complex diseases.

Rickettsiae infect and damage blood vessels throughout the body (Figure 3). The general pathophysiologic effects of the vascular infection are the consequences of increased vascular permeability and include edema in the tissues surrounding the leaky blood vessels. The specific distribution and extent of the vasculitis largely determine the manifestations of illness in an individual. It culminates with shock due to peripheral vascular collapse.

Enzyme activities, particularly those of phospholipase A and protease, have been proposed as rickettsial pathogenic mechanisms. *R. rickettsii*-mediated cell injury is reduced by compounds which have been reported to inhibit phospholipase activity (Silverman, 1992) or to block attachment of *R. prowazekii* to erythrocytes (Austin and Winkler, 1988).

Members of the spotted fever group spread rapidly from cell to cell by an unknown mechanism. Actin filaments (Heinzen et al., 1993) associated with one pole of intracellular rickettsiae with tails greater than 70 μm in length were seen extending from rickettsiae, implicating these structures in the movement of *R. rickettsii* from cell to cell. A survey of virulent and avirulent species within the SFG rickettsiae demonstrated that all formed actin tails. Typhus-group rickettsiae, which do not spread directly from cell to cell, lacked F-actin tails entirely or exhibited only very short tails.

Glucose-6-phosphate dehydrogenase deficiency, a sex-linked genetic condition found in 12% of American black males, predisposes individuals to RMSF of enhanced severity, and, in some patients, a fulminant course leading to death within 5 days or less after disease onset.

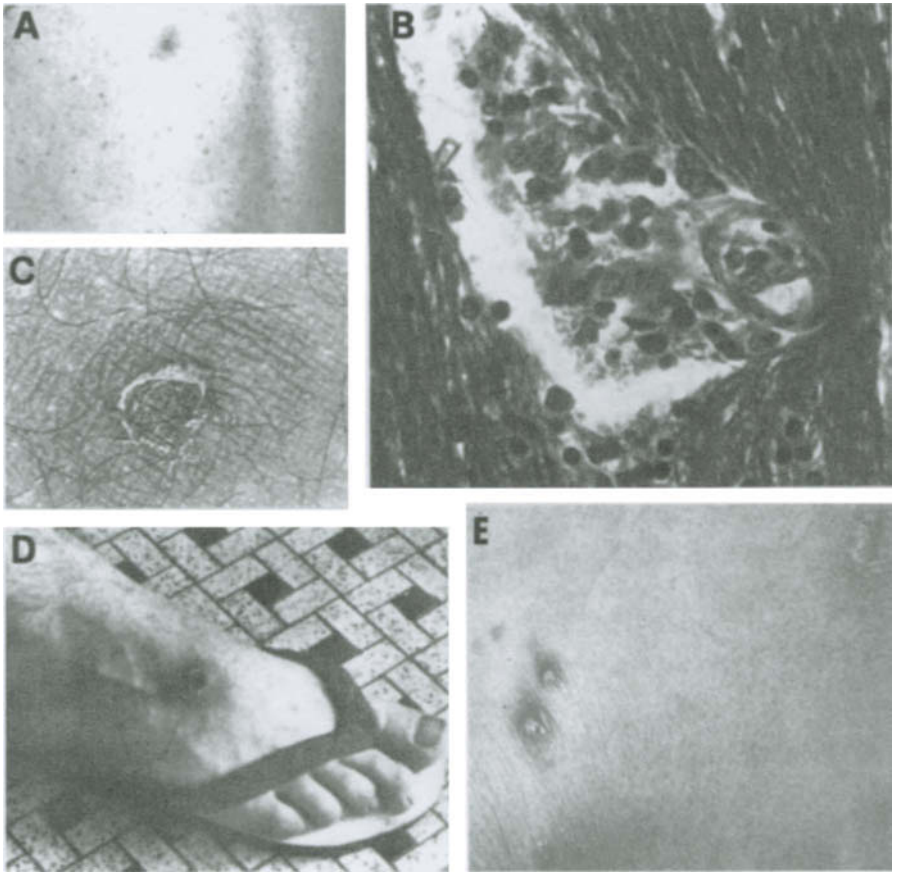

Figure 3. A. RMSF seventh day of illness. Eruption with macules and petechial elements. B. RMSF vasculitis in a small blood vessel showing prominent perivascular clusters of mononuclear inflammatory cells. C. Mediterranean spotted fever showing a healing stage of the initial infection, with a central scab and surrounding erythema. D. The eschar of scrub typhus. (Reprinted by permission, Brown, G.W. (1988), Biology of Rickettsial Diseases, Walker, D.H., Ed., CRC Press, Inc., Boca Raton, FL.) E. Cat scratch disease showing papular lesion at scratches. (a, b, c, e, reprinted by permission, Farrar, W. Edmund et al. (1982), Infectious Diseases, Gower Medical Publishing, London.)

Clinical Diagnosis

The incubation period from the inoculation to onset of the symptoms ranges from 2 to 14 days and averages 7 days. Onset of the disease can be either abrupt or gradual in nature (Walker, 1989). During the first 3 days, the most prominent clinical manifestations are fever, rash, malaise, and severe headaches often accompanied by muscle aches, anorexia, nausea, vomiting, abdominal pain, and photophobia.

The rash usually appears on day 3 to day 6 of illness (Figure 3). It begins on the wrists and ankles and extends throughout the body including the palms and soles, with relative sparing of the face. However, the rash is not present in every individual and sometimes the rash is evanescent. Initially, the rash only consists of lesions 1 to 5 mm in diameter where dilation of the small blood vessels imparts a pink color to the skin in and surrounding the foci of rickettsial vascular infection. Later in the course, particularly in severely ill patients, a pinpoint hemorrhage occurs in the center of the pink spot, where the damage caused by the intense rickettsial infection is most pronounced. In Mediterranean spotted fever and Rickettsial pox (Figure 3), a characteristic black eschar is found at the site of the insect bite. Usually a firm red papule appears that turns vesicular and eventually develops into an eschar.

Encephalitis occurs in 26 to 28% of patients with RMSF and the symptoms include confusion, stupor or delirium, ataxia, coma, and seizures. Coma occurs much more frequently in fatal cases than in nonfatal cases. Patients with severe RMSF are likely to develop acute renal failure. Metabolic wastes such as urea and creatinine accumulate in the blood. Renal blood flow is curtailed and acute renal failure indicates a poor prognosis. The initial clinical picture of RMSF may suggest a diagnosis of viral encephalitis, meningitis, or meningococcemia with early meningitis.

Immune Response

Immunity against rickettsiae is complex and involves several effector mechanisms that are interconnected by regulatory feedback mechanisms.

Protection in animals has been seen only when immune serum was administered before infection or when rickettsiae were treated with antibodies before inoculation. The immune serum does confer partial immunity for a short time period.

T cell-mediated immunity is of primary importance for the successful clearance of rickettsiae from infected hosts. Passive transfer of T cells from an immune host will confer protection to a native animal. The mechanism of cellular immune response is supported by the delayed-type hypersensitivity or proliferation of immune T-cells in the presence of a specific antigen. However, athymic mice die of fulminating rickettsial disease although the mice produce significant quantities of rickettsia-specific T cell independent antibody after infection (Gage and Jerrells, 1992).

Laboratory Diagnosis

Isolation of rickettsiae, if performed, should be done in a facility having a containment class 3 facility. Isolation is performed using cell culture, and it can be achieved in 4 to 7 days. Cultured rickettsiae are identified provisionally by staining with immunofluorescence and the Gimenez method. Rickettsial DNA can be detected in acute-phase blood specimens by PCR technology (Tzianabos et al., 1989).

Skin biopsy (Walker, 1989) to identify *R. rickettsii* in petechial lesions is done by selecting a classic petechial lesion centered in an erythematous maculopapule and removal of this sample of skin with a 3-mm punch under local anesthesia. RMSF is diagnosed when three or more fluorescent structures compatible with rickettsiae are identified in the blood-vessel wall in the dermis. A positive result is diagnostic. A negative result does not preclude infection. The sensitivity of detection decreases if the biopsy is obtained after the initiation of the antibiotic treatment.

Serologic testing remains the most frequently used approach to diagnosis, although it usually fails to identify rickettsioses early enough to affect the management of individual patients. It must be regarded as a retrospective confirmation of the clinical diagnosis. One should note that antibodies from a rickettsial group, produced as a result of infection with one rickettsial antigen, will cross-react with antigens of the rickettsiae from the same group. It is so extensive that diagnostic tests usually cannot speciate the causative agents, but are an indicator of infection with a given antigen group. On the other hand, cross-reactivity between groups, e.g., is not extensive and, even when cross-reaction occurs, the differences in titer are large enough to differentiate between infection with the two rickettsial groups.

In recent years, the principal tests that use antigens of *R. rickettsii* for confirmation of the diagnosis of RMSF are the microimmunofluorescence (Micro-IF) (Philip et al., 1976) and latex agglutination (Hechemy et al., 1980). Antibodies to *R. rickettsii* are detected 7 to 9 days after onset of illness.

The micro-IF test is performed on whole rickettsiae and is generally accepted as the best serologic test presently available for diagnostic and seroprevalence purposes.

The latex agglutination test relies on a solubilized protein-carbohydrate complex rickettsial antigen, coated on latex beads. The latex test is more discriminatory than the micro-IF for establishing the diagnosis of a recent infection and titers fall to nondiagnostic levels within 2 months. The latex test therefore should not be used to study the prevalence of antibodies.

Therapy

The greatest factor in reducing the morbidity and mortality of RMSF and other rickettsial diseases is treatment with appropriate antimicrobial agents early in the course of illness. In the years which preceded the availability of antirickettsial

drugs, the mortality rate was 23%. In recent years the mortality rate has been as low as 3%. The treatment of RMSF, typhus, scrub typhus, and ehrlichiosis has not changed appreciably since the introduction of tetracycline and chloramphenicol. For adults, appropriate therapy includes doxycycline 100 mg orally twice a day, tetracycline 500 mg four times a day, or chloramphenicol 50 to 75 mg/kg intravenously in four divided doses. Antimicrobial therapy should be continued for approximately 5 to 7 days and for at least 2 to 3 days after resolution of the fever.

TYPHUS GROUP

Few diseases in history may lay claim to the degree of global impact produced by epidemic typhus.

The typhus fever comprise a group of closely related infectious diseases that occur in different parts of the world. Two species of the genus Rickettsia belonging to the typhus group are pathogenic for humans. They include *Rickettsia prowazekii*, transmitted by the human body louse and causing epidemic typhus, Brill Zinsser (BZD) or recrudescent typhus and sylvatic typhus, and *Rickettsia typhi* (formerly known as *R. mooseri*) transmitted by the flea and causing murine or flea-borne typhus. These diseases are very similar clinically and pathologically, although epidemic typhus can be fatal.

Epidemiology

Epidemic Typhus

R. prowazekii, the etiologic agent of epidemic typhus, is transmitted to man by the body louse *Pediculus humanus* (Boyd and Neldner, 1992). Epidemic typhus is a disease of the cold months, when heavy clothing and poor sanitary conditions are conducive to lice activities. As the body louse bites a human it defecates. The rickettsiae are present in these feces and may be rubbed into the wound. Lice do not propagate the infection by the bite or mouth parts per se. Human infections may also be acquired by inhalation of dust containing infected louse feces or by crushing a louse on the skin. The microorganisms are not transmitted transovarially. Humans are the reservoir for *R. prowazekii*. The louse acquires *R. prowazekii* from a rickettsemic patient during the feeding process and becomes infective with rickettsiae 5 to 7 days later; this infection leads to death of the louse after 1 to 3 weeks.

At present, the known distribution of louse-borne epidemic typhus includes scattered foci in mountainous regions of South America, the Himalayan regions, and mountainous or highland Africa. BZD is believed to represent the interepidemic reservoir of epidemic typhus.

Sylvatic Typhus

In 1975, it was discovered that eastern flying squirrels (*Glaucomys volans*) carry *R. prowazekii* causing sylvatic typhus. This indigenous typhus corresponds to the territory of the flying squirrel. This illness tends to be less virulent than epidemic typhus and is not associated with a seasonal preference. These findings establish that humans may not be the only reservoir of *R. prowazekii* as originally thought (McDade et al., 1980).

Endemic Typhus

R. typhi, the etiologic agent of endemic typhus, is principally a disease of urban areas (Azad, 1990). Areas of high disease prevalence are usually associated with grain storage and large rat populations. Unlike most other major arthropod-borne infections, murine typhus can be essentially a household infection because of its intimate association with commensal rats *Rattus rattus* and *Rattus norvegicus* and their fleas *Xenopsylla cheopis*. The classical transmission cycle for murine typhus is rat-flea-rat and accidentally rat-flea-man. The rats display no evidence of infection and have a normal life span. Fleas do not appear to be harmed by their infection. Once infected, fleas remain infected for life.

The disease can be year round if climatic conditions in these foci are favorable for survival of rats and their ectoparasites. The majority of cases occur during the late spring and early autumn or whenever warm and humid climates prevail.

R. typhi is spread to man by contamination of the wound with infected feces, which are deposited on the skin of the host at the time of feeding, by contamination of the respiratory tract, or conjunctivae of the host with infected flea feces. Rubbing the rickettsia into the skin promotes entry into the host. *R. typhi* are hardy organisms and may remain viable in fecal matter for years provided that the humidity and temperature are appropriate. Beside the classical rat-flea cycle, recent studies suggest the involvement of an opossum-flea cycle. Endemic areas include South West United States, Mexico and Central America, the Balkans, West Africa, and southeast Asia.

Bacterium

The typhus fever bacterium resembles the spotted fever bacterium in morphology. The G + C content of DNA is 28.5 – 29.7 mol.% and the genome size is 1.1×10^9 daltons.

Typhus group rickettsiae grow in the cytoplasm of eukaryotic cells (Figure 2). During the exponential growth very few bacteria exit from the infected cell to set up secondary infections in neighboring cells. Instead they grow and fill the cytoplasm until, presumably, the host can no longer support the growth of the parasite. The host cell then bursts and hundreds of bacteria are released to initiate infections in many new host cells. It appears that phospholipase A stimulates the

host cell to internalize the rickettsiae and to provide a means for those rickettsiae to escape the phagosome and be released into the cytoplasm (Austin and Winkler, 1988).

Immunity to endemic typhus also confers protection from epidemic typhus. Heat-sensitive epitopes of protein estimated having 120 kD (SPA) were reported to be the most important antigenic stimulus for protective immunity (Dasch and Bourgeois, 1981).

Pathology

Lesions can be present in the heart, brain, testis, and muscles. Cerebrovascular events may be precipitated by blood-vessel thrombosis. Perivascular infiltration with lymphocytes, plasma cells, histiocytes, and polymorphonuclear leukocytes occurs with possible necrosis of the vessel. The vasculitic process typified by the petechial lesions in the skin takes place in the systemic vasculature as well. Consequently, blood vessels may present with skin necrosis or gangrene of an extremity. Early leukopenia is present with leukocytosis occurring later. Disseminated intravascular coagulation with platelet consumption and prolonged bleeding times have been described. Neuropsychiatric findings of paranoid behavior and terrifying hallucinations may be present, and the mental state may fluctuate. Most of these symptoms resolve following defervescence. If typhus is untreated, defervescence occurs over a 4-day span after 2 to 3 week of illness. The fatality rate has varied widely from 10 to 60% for epidemic typhus. Mortality is enhanced by poor nutrition and initially poor general health. Endemic typhus rarely is fatal. Children usually experience a less virulent course than adults. Owing to the degree of similarity between *R. prowazekii* and *R. typhi*, there exists cross-immunity for either disease among survivors.

Infection with *R. prowazekii* results in deposition of these organisms in lymphoid tissue, where they remain indefinitely in a "latent state" where the patient appears to have recovered. At some point later in the life of the recovered patient, an unknown factor triggers a recurrence of typhus symptoms known as BZD.

Clinical Diagnosis

The incubation period of endemic and epidemic typhus is about 1 to 2 weeks with an average of 10 days. Onset of symptoms is acute with high fever usually 104° to 107° F and unremitting headache. The fever rises rapidly and may be unresponsive to antipyretics. Rash, myalgias, chills, weakness, and constipation are common. Eye findings include conjunctivitis and photophobia. Bradycardia is seen in early typhus followed by tachycardia later on in the disease course. Hypotension is also frequently present (Boyd and Neldner, 1992). The rash usually presents between the 4th and 7th day of symptoms and may be preceded by a faint nondescript erythema. The eruption initially consists of nonconfluent erythematous macules beginning on the trunk or axilla. Lesions may become palpably purpuric,

or frankly hemorrhagic. Typhus rash rarely involves the palms or soles. It is usually centrally distributed on the trunk.

The course of BZD and of sylvatic typhus is usually milder than that of epidemic typhus, and its duration is shorter. Symptoms begin abruptly with a headache and fever, although the temperature is usually not as high. Defervescence occurs at or before 2 weeks with a rapid recovery shortly thereafter. The immunologic anamnestic response in BZD occurs rapidly resulting in early IgG production.

Laboratory Diagnosis

The laboratory diagnosis of the typhus fever group antigen is based on the detection of antibodies to the group antigen. The micro-IF and latex tests are the test of choice. They use typhus antigen as described under the spotted fever section. Neither test can differentiate between epidemic and endemic typhus. All the conditions that apply to both tests for the spotted fever group antigen also apply to the typhus fever group antigen.

GENUS *ORIENTIA*: SCRUB TYPHUS GROUP

The genus *Orientia* encompassed one species with many serovars. Scrub typhus, also called tsutsugamushi disease, or "mite disease," is caused by *Rickettsia tsutsugamushi*. The rickettsiae are transmitted by the bite of the larval stage of certain trombiculid mites or 'chiggers.' The disease has been known for ages to physicians in China and Japan as an illness associated with the bite of a red mite and contracted in flood plains of rivers.

Epidemiology

Scrub typhus is known to be endemic over a wide area of Asia, but does not occur in Africa, the Middle East, or the Americas. The distribution of this rickettsia coincides with the distribution of trombiculid mites of the *Leptotrombidium deliense* group and wild rats of the genus *Rattus*.

All trombiculid mites are parasitic only in the larval or chigger stage. The chigger attaches to a host and feeds on tissue juices and serum exudates for 2 to 12 days but not on blood. Upon repletion, the chiggers drop to the forest floor, where after a few days they metamorphose to a pupa-like stage—to an eight-legged nymph—then to adult stage. Neither nymphs nor adults are parasitic.

The larva feeds only once on a host. It is postulated that infection in chiggers is by transovarian transmission, rather than infections acquired from a rodent host. The trombiculids serve as reservoirs as well as vectors. Chiggers are not host-specific and any species of mammals or birds may become infested and may acquire *O. tsutsugamushi*.

Bacterium

O. tsutsugamushi (Figure 2) seems to be a little larger than the other rickettsiae, and the size is in the range of 0.5 to 0.8 μm in width and 1.2 to 3.0 μm in length. *O. tsutsugamushi* seems to have a fragile outer envelope (Tamura et al., 1991). This property is clearly distinctive from the other species of rickettsiae. Very little is known about the actual chemical composition or function of scrub typhus rickettsial components. The complement fixation test revealed antigenic heterogeneity. Proteinase-K digested samples did not form any LPS-like bands in PAGE analysis. Also, it appears that *O. tsutsugamushi* lacks peptidoglycan and LPS, components which usually impart rigidity to the cell wall. Surrounding the cytosol of the bacterium is the cytoplasmic membrane, a typical lipid bilayer, and around this is the outer membrane, which is characteristic of all Gram-negative bacteria. Between the inner, cytoplasmic membrane, and the outer membrane, a clear space can be discerned by electron microscopy. *O. tsutsugamushi* usually multiplies in the host cell cytoplasm. The G + C content of DNA from *O. tsutsugamushi* falls within the 28.5 to 30.0 mol.% range.

Pathology

Variation in virulence in a number of prototype strains in mice appears to correlate with severity of human disease. It ranges from inapparent or mild to severe or fatal infections.

The presence of an eschar at the tick bite site is characteristic of scrub typhus. Rickettsiae have been demonstrated in the endothelium of an eschar. The rash contains edema in the papillary dermis, hyperemia, perivasculitis, and platelet thrombi and is rarely hemorrhagic. Patients without an eschar often have generalized lymphadenopathy. Widespread vascular injury occurs in all organs, particularly the skin, brain, kidneys, and myocardium. Vascular damage is not usually as severe as is seen in louse-borne typhus or RMSF. Renal failure may be more common in patients who are deficient in glucose-6-phosphate. The cause of the death can be due to encephalitis, respiratory and circulatory failure.

Clinical Diagnosis

The classical features of the disease when present are: eschar (Figure 3), rash, and adenopathy. Scrub typhus often presents without the characteristic features of the eschar and rash. The incubation period is 6 to 21 days. Onset of disease is marked by fever and headache, and patients frequently complain of generalized body and backache, cough, and gastrointestinal disorders. Usually, this nonspecific syndrome is all that develops, and many patients recover spontaneously after a few days. Some patients experience more prolonged illness, and a small number <1% die if untreated. Certain clinical features are characteristic if present. The eschar may develop at the site of the infecting bite. It progresses from a small red papule to a

punched-out ulcer, usually covered with a hard black scab. The eschar is often present in areas that are easily overlooked during a cursory examination, e.g., the groin, between the buttocks, in the belt area, and in the axilla. Over 50% of patients do not have a recognizable eschar.

Immune Response

Isolates exhibit considerable antigenic diversity and are classified into three main serotypes, namely, Karp, Gilliam, and Kato. Several serotypes have been recovered from a single specimen. In humans, immunity after infection lasts for 1 to 3 years against the homologous strain but is short-lived (less than 1 year) against heterologous strains (Hanson, 1988).

Studies on the host immune response to *O. tsutsugamushi* have indicated that clearing of intracellular rickettsiae requires cellular immune response. Structurally intact and degenerating rickettsiae were found in phagosomes of polymorphonuclear leukocytes (PMN), but only intact rickettsiae escaped phagosomes and entered the glycogen-rich cytoplasm.

Laboratory Diagnosis

An indirect peroxidase (IPA) assay and an indirect IFA for serodiagnosis of scrub typhus are used to measure the antibodies to rickettsial antigen (Kelly et al., 1988). For diagnostic purposes a pooled antigen is as effective as using the separate strains to detect specific *O. tsutsugamushi* antibody. PCR was developed to detect scrub typhus DNA in clinical specimens. The primer pair used for PCR was designed from the nucleotide sequence of the gene encoding the 56 kD antigen of the Gilliam strain.

GENUS COXIELLA

In 1935, in Brisbane, Queensland, Australia, some employees of a meat-packing plant developed an acute febrile illness. The pattern of illness suggested a single entity about these employees, which was named "Q" fever for query fever. The agent was transmissible from human blood and urine samples to guinea pigs but could not be cultivated on the usual bacteriological media. Subsequently, this organism was shown to be the same organism as the Nine Mile Creek, Montana organism described by Cox. A new genus *Coxiella* was established; the bacterium was named *Coxiella burnetii*. Only one species is in the genus *Coxiella*.

Epidemiology

The reservoir for *C. burnetii* is ticks and the domestic ungulates, e.g., cattle, sheep, and goats (Babudieri, 1959). *C. burnetii* seems to thrive within these ecologic niches apparently with minimal or mild overt injury to the host. Infected animals

shed the desiccation-resistant material in urine, feces, milk, and in birth products. Viable organisms are present in the soil for periods of up to 150 days. Man appears to be a dead end for *C. burnetii*. Man is infected by inhalation of infected aerosols, which can travel a long distance. Cases of Q fever in humans can be linked with exposure to infected domestic animals and its products by milk.

Bacterium

C. burnetii (Figure 4) is a small, rod-shaped bacterium approximately 0.25 μm in width by 0.5 to 1.25 μm in length. Organisms grown in chicken embryo yolk sacs or in tissue cultures are readily stained by the Gimenez method or by modification of the Giemsa stain (Thompson, 1988). The ultrastructure of the envelope resembles that of Gram-negative bacteria. The organism possesses a LPS complex and a muramic acid-containing peptidoglycan structure. In the cytoplasm, a central nucleoid mass with fine radiating fibrils often confers a rather distinctive appearance to the cytoplasmic ultrastructure. The genome size is 1.1×10^9 daltons and has a guanine + cytosine ratio of 42 mol %.

Phase variation of the surface LPS of *C. burnetii* depends on its source. Phase I, the virulent phase, is that seen in animal and human hosts with established infection. Phase II occurs after serial passage in the laboratory. Both LPS phases differ in

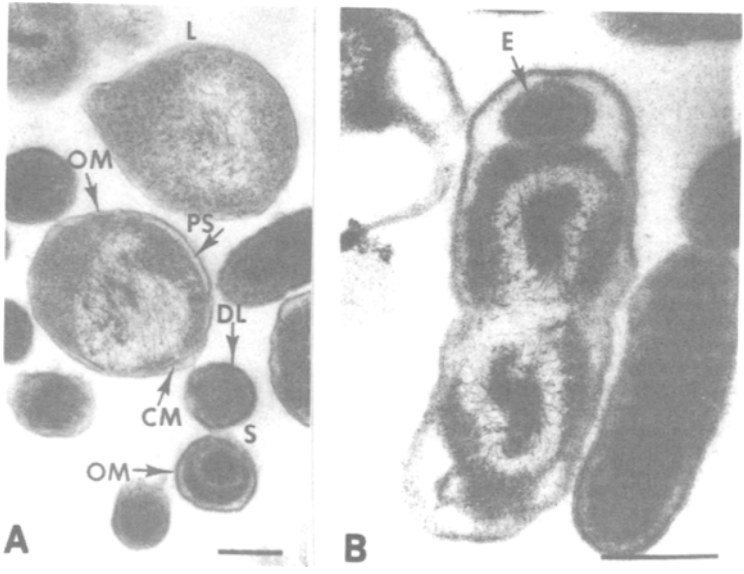

Figure 4. A. Electron micrographs of thin sections of *C. burnetii* cells. The cytoplasmic membrane (CM) and outer membrane (OM) are separated by a periplasmic space (PS). Dl, Dense layer. B. Formation of the endospore (E). (Reprinted by permission, McCaul & Williams, J. Bacteriol., 147, 1063, 1981).

amino acids and neutral sugar content, immunogenic surface proteins, surface charge, cell density, and resistance to phagocytosis by macrophages and lymphocytes (Baca and Paretsky, 1983). Only phase I elicits the localized Schwartzmann phenomenon, the classic marker of endotoxin activity.

Although glucose, pyruvate, and some tricarboxylic acid cycle intermediates can be metabolized by *C. burnetii*, glutamate is a preferred substrate (Thompson, 1988). *C. burnetii* is a phagolysosome pathogen. It does not escape from the phagosome upon internalization. The stages of intracellular growth and development occur within phagolysosomes. Endospore-like structures have been identified on the basis of morphology and ultrastructure. Nothing is known regarding the resistance or refractivity of these dense bodies. They do not contain dipicolinic acid, and their capability for transmission of genetic inheritance remains unknown (McCaul and Williams, 1981). High-voltage electron microscopy and three dimensional reconstruction revealed that, in heavily infected cells in time culture, *C. burnetii* appears to reside in one giant, one-lobed, or multilobed vacuole. The infected cells have no significant difference in cell cycle or population doubling time from uninfected cells.

A plasmid termed QpH1 exists in both antigenic phases of the Nine Mile strain. Another plasmid QpRS is larger and may be found in strains associated with chronic disease such as endocarditis (Minnick et al., 1991).

Pathology

It is presumed that the bacterium spreads via the bloodstream to infect mononuclear phagocytes in organs where lesions have been observed, including liver, bone marrow, and spleen. *C. burnetii* enters a cell passively, multiplies within the cytoplasmic vacuole, and ultimately destroys the cell. Some of the necrotic changes associated with infection by this organism may be caused directly by the organism or may result from release of lysosomal enzyme (Reimer, 1993).

Fifty percent of the individuals do not develop overt clinical disease. Illness that does occur can be divided into acute and chronic infection. The gross findings resemble those of other bacterial pneumonias except that the alveolar cells are mostly histiocytes rather than polymorphonuclear leukocytes. Severe *Coxiella* pneumonia is characterized by gross consolidation and microscopic interstitial pneumonia and alveolar exudates. Alveolar sputa are thickened by infiltrates of prominent macrophages accompanied by lymphocytes, plasma cells, and PMNs. Hemorrhage and extensive areas of necrosis suggesting vascular injury may be present. Biopsies show primarily granuloma formation. The granuloma may be nonspecific or may have a more distinctive doughnut appearance, with a central clear space surrounded by inflammatory cells and fibrin.

The most prominent disturbance in chronic Q fever is Q fever endocarditis. Most cases involve the aortic or mitral valve in patients with preexisting valvular disease or prosthetic valves. Infection remains probably inactive since they have occurred

many years after apparent exposure to *C. burnetii*. Lesions on native valves have been described as including small perforations of the valve, multiple small, pale yellow to brown vegetations, small calcific nodular sclerosis.

Clinical Diagnosis

Acute Infection

After an incubation of up to 6 weeks, the clinical symptoms include high fever ≥ 38.5° C, chills with rigors, severe headache and/or retroorbital pain, general malaise, and myalgia. Chest pain, nausea, and vomiting may also be present. Physical signs of infection may include hepatomegaly and splenomegaly. No rash is present.

Q fever pneumonia may occur as an atypical pneumonia or a rapid progressive pneumonia or just a systemic febrile illness (Reimer, 1993). The radiographic features of Q fever are variable and similar to those of atypical pneumonia. Atypical pneumonia can be of non-pneumococcal origin or a pneumonia with a dry cough or a cough productive of mucoid sputum. Such patients have negative blood cultures. Most cases are self-limited with symptoms resolving in 1 to 2 weeks without further complications. A small number of patients, probably fewer than 1% of those infected with *C. burnetii*, do not clear the organism and develop chronic disease long after the initial illness or exposure.

Chronic Infection

The major feature in chronic Q fever is endocarditis. Symptoms begin gradually and may take a long time to develop. The longest proven interval between the original attack of Q fever and the onset of endocarditis is 7 years. Endocarditis tends to occur in older patients with the majority being female. Because symptoms initially might be mild, medical care is not sought. Q fever endocarditis may present itself as fever of unknown origin, or as culture-negative subacute endocarditis.

Manifestations of Q fever endocarditis in descending order are: fever, splenomegaly and hepatomegaly, purpuric rash, arterial emboli, and anemia. Hepatic transaminases and alkaline phosphatases are usually elevated. There are no pathognomonic symptoms or signs. In contrast to other causes of endocarditis, blood cultures are negative. Q fever should be part of the differential diagnosis of atypical pneumonia, culture-negative endocarditis, and granulomatous hepatitis.

Immune Response

After initial infection, antibodies are usually detected, and cell-mediated immunoresponse as measured by skin and lymphoproliferative response also occurs. Cell-mediated immunity plays an essential role in the final elimination of the organism and prevention of the chronic manifestations. Failures of specific cell-

mediated responses to *C. burnetii* have been associated with the development of chronic infection (Reimer, 1993). The antibody response appears to be closely associated with the acute phase of the disease. Antibody of the IgM class is first formed to phase II antigens soon after infection, and IgA and IgG are formed soon after. IgM response to phase 1 antigen begins during convalescence and may persist at low levels for up to 2 years from onset. IgG and IgA responses to phase 1 antigen are produced during chronic manifestations of active chronic Q fever.

Antibodies to *C. burnetii* appear to promote the uptake of the organism by macrophages and polymorphonuclear leukocytes. Phase I organisms appear to be less effectively phagocytized and killed after ingestion than phase II organisms. Since both killing and proliferation of the organism occur intracellularly, phagocytosis can have either a positive or deleterious effect to the host.

Laboratory Diagnosis

The diagnosis of *C. burnetii* is based mainly on the detection of antibodies to phase I and phase II antigens. Routine cultivation by clinical laboratories is not recommended because of the high transmissibility of the organism. The serologic assays that are performed are the complement fixation test, the IFA, and the ELISA. The overall sensitivities are 94, 91, and 78% for ELISA, IFA, and CF respectively (Peter et al., 1987). The specificity was similar for all three tests. The stage of illness can generally be determined by the ratio of phase II to phase I antibodies. In acute disease, the ratio is >1, in subacute disease >1, and in chronic disease <1. Individuals with previous infection may remain seropositive for a long time. Except for the high titers to phase I antigen seen in endocarditis, a single positive titer cannot be used to establish a diagnosis. PCR was developed to detect *C. burnetii* DNA in clinical samples using primer from the DNA sequence of the gene of the superoxide dismutase enzymes of *C. burnetii*.

Therapy

A number of antimicrobial agents have been used to treat infection caused by *C. burnetii* (Raoult, 1993). The most active antimicrobials and combination of antimicrobials based on *in vitro* studies using L-929 mouse fibroblast cell lines are: rifampin, sulfamethoxazole-trimethoprim, tetracyclines and their analogs, and quinolones. In acute infection, tetracyclines appear to shorten the duration of the fever by 50%. Cases of endocarditis have been treated with a combination of sulfamethoxazole-trimethoprim, rifampin, and quinolone/doxycycline. It is recommended that patients with endocarditis be treated with the antibiotics for at least 3 years or until antibody titers fall below an arbitrary value. Valve replacement has frequently been required.

GENUS EHRLICHIA

Ehrlichiosis is a disease caused by members of the genus *Ehrlichia*. They parasitize circulating mononuclear phagocytes or granulocyte of humans and a variety of domestic and wild animals. *Ehrlichia canis*, the type species of the genus *Ehrlichia*, has been known since 1935. *E. canis*, the prototype organism, has a close relationship with *Ehrlichia sennetsu*, the causative agent of human sennetsu fever in Japan, first identified in the 1950s. In the United States, the first human case of ehrlichiosis caused by an *E. canis*-like bacterium was reported in 1987. It was identified as *Ehrlichia chaffeensis* (Anderson et al., 1991). All the above species parasitize the mononuclear phogocytes. More recently (reviewed by Dumler and Bakken, 1995) a phylogentically distinct organism that infect human granulocyte was discovered. The disease was named human granulocytic ehrlichiosis (HGE) to distinguish it from the ehrlichia that invade human mononuclear phagocytes (HME). Genomic analysis has shown that the HGE organism is more closely related to the ehrlichias causing disease in equines, e.g., *Ehrlichia equi.*

Epidemiology

Both human ehrlichiosis apparently are transmitted by ticks. However, a definitive vector and reservoir have not yet been identified. In the United States, laboratory-based surveillance has identified 300 cases of human ehrlichiosis from 20 states during the period of 1986 to 1991. Older persons appear to be predisposed to more severe illness.

Most patients are from rural areas. Most illnesses are reported between May and July, and few patients report a history of tick bites or exposures to tick within 3 weeks of onset of illness. However, the strong seasonality of disease and the residence of a large proportion of the patients in rural areas support the premise that human ehrlichiosis is primarily a tick-borne infection.

Bacterium

The genus *Ehrlichia* contains five species plus a new, yet unnamed species, that causes HGE. They are small pleomorphic, obligately intracellular bacteria that parasitize the cytoplasmic phagosomes of monocytes or granulocytes.

Individual ehrlichiae are called elementary bodies (Figure 5) (Ristic et al., 1991; Rikihisa, 1991). They are small Gram-negative bacteria known as elementary bodies, about 0.5 μm in diameter. They are usually coccoid or ellipsoid, but pleomorphism is frequently observed. After phagocytosis, the elementary bodies begin to grow and divide by binary fission within the confine of the phagosome. Ehrlichiae survive intracellularly by inhibiting phagosome-lysosome fusion. After 3 to 5 days after infection (Figure 5), small number of tightly packed elementary bodies 1.0 to 2.5 μm in diameter are observable as pleomorphic inclusions called initial bodies. After 7 to 12 days, additional replication occurs and the initial bodies

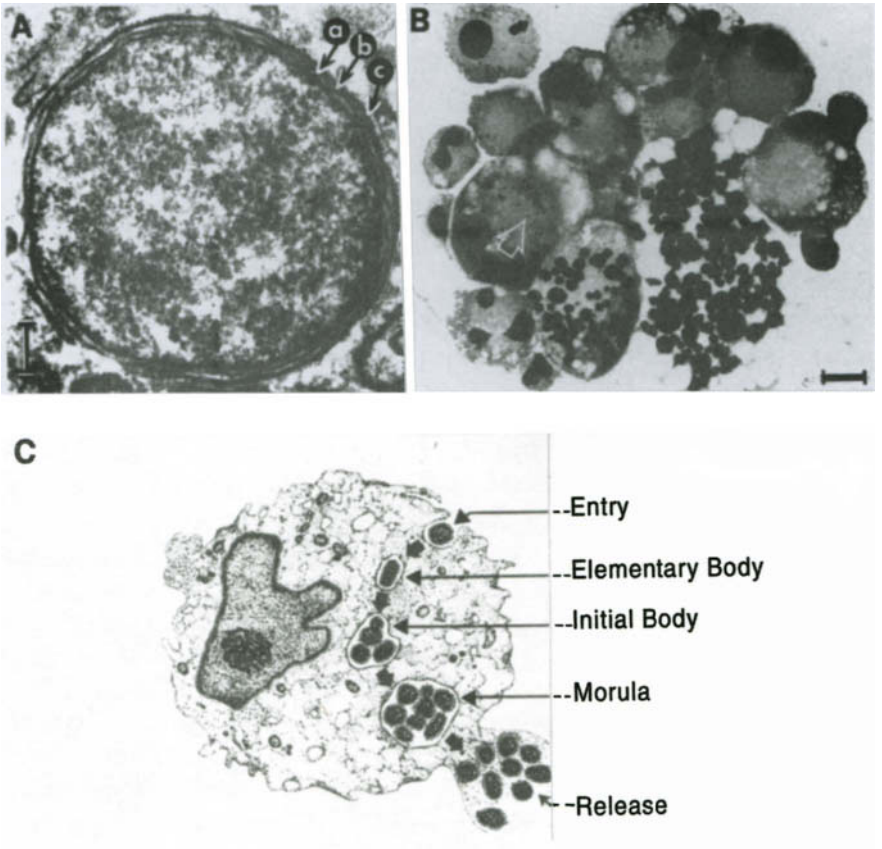

Figure 5. A. *E. risticii* in the cytoplasm of a macrophage in the large colon of a horse. *E. risticii* is tightly enveloped by the host membrane (A) and has its own outer (B) and inner (C) membranes. ×87,600. Bar = 0.1 μm. (Reprinted by permission, Rikihisa et al., Clin. Microbiol. Rev. 4, 286, 1991). B. Canine monocytes cultivated *in vitro* and heavily infected with *Ehrlichia canis*, as viewed by light microscopy. One of many large morulae (arrow); smaller inclusions (initial bodies), open arrow (bar = 10 μm). C. Schematic representation of the growth cycle of ehrlichiae in an infected cells. (b and c Reprinted by permission, McDade, J. Infect. Dis. 161, 609, 1989).

mature into an inclusion that has a morula configuration. When the infected cells rupture, the morulae break up into elementary bodies. Ehrlichiae derive some ATP from their catabolic activities and utilize glutamate.

The 16S rRNA sequence comparisons indicate that the human ehrlichiosis agent *E. chaffeensis* is closely related to *E. canis* (98.2%) and more distantly related to other *Ehrlichia* species. In contrast, the HGE's is more closely related to *E. equi* (98.3%).

Pathology

Ehrlichiae are leukotropic rickettsia that have a predilection for blood monocyte (Figure 5), thus mimicking infectious mononucleosis (Ristic et al., 1991) or granulocyte.

In fatal canine *E. canis* infection, the extensive invasion of parenchymal organs, and perivascular cuffing, by plasma cells, particularly of the lungs, meninges, and kidney cells are among the most prominent pathological manifestations. Lymphocytes of infected dogs exert a cytotoxic effect upon autologous monocytes.

Clinical Diagnosis

Human ehrlichiosis ranges from a mild infection to a severe life-threatening fatal disease (Ristic, et al., 1991). Both the monocytic and granulocytic ehrlichosis produce similar clinical symptoms.

Ehrlichosis is usually a mild debilitating disease. Affected individuals usually recover spontaneously after 1 or 2 weeks of illness. The symptoms and signs of this disease are somewhat similar to those of infectious mononucleosis. A recent study in the United States on sera from 98 patients suspected to have infectious mononucleosis but were negative by the monospot test revealed that 30% had anti-ehrlichial antibodies.

Patients often present with an acute febrile illness often exceeding 102°F. Other common symptoms of ehrlichiosis include myalgia, headache, and nausea. The rash in ehrlichiosis is fleeting and is seen in only about 30% of the patients.

Laboratory Diagnosis

Microscopic examination of peripheral blood smears may reveal occasional inclusion bodies in leukocytes. The inclusions are round or ovoid and 2–5 μm in diameter.

The IFA and ELISA are the tests of choice for the detection of antibodies to the ehrlichial organisms. The kinetics of IgG and IgM responses showed that specific IgM is short lived, falling to undetectable levels by 60 days after infection (Dawson et al., 1991). In contrast, specific IgG persists for more than one year. Because of the apparent lack of crossreactivity in serologic testing with patient sera from cases with monocytic and granulocytic ehrlichiosis, two antigens are used to detect the respective antibodies in patient sera. *E. chaffeensis* and HGE organisms are used to detect antibodies for cases of monocytic and granulocytic ehrlichioses in the United States. Other laboratory findings include leukopenia, thrombocytopenia, and abnormal liver function tests.

PCR for *E. chaffeensis* and HGE organism detection in specimens was developed to amplify DNA segments specific to *E. chaffeensis* and the HGE organism respectively.

GENUS BARTONELLA (ROCHALIMAEA)

Until recently all isolates of human pathogens identified as belonging to the genus *Bartonella (Rochalimaea)*, first described by Rocha-Lima in 1916, were limited to the etiologic agent associated with trench fever. During World War I, trench fever morbidity was considered to be the most prevalent, with 1,000,000 military personnel affected by this incapacitating febrile syndrome. After World War II, it largely disappeared although there have been sporadic reports of it in the literature.

At present the genus *Bartonella (Rochalimaea)* contains three species of importance to humans: *B. quintana, B. henselae*, and *B. elizabethae*. *Bartonella* species are being associated with four clinical syndromes: bacillary angiomatosis (BA), bacillary peliosis hepatitis (BPH), relapsing fever with bacteremia, and cat scratch fever (CSD). Cognizance of the populations at risk for *Bartonella (Rochalimaea)* infections has expanded from adults infected with HIV to transplant patients to immunocompetent adults and children (Schwartzman, 1992).

Epidemiology

The trench fever agent *B. quintana* is transmitted by a louse vector from the human reservoir. Another possible arthropod vector of *Bartonella (Rochalimaea)* is the tick. It was shown that bites from ticks preceded diagnosis of *B. henselae* bacteremia in two patients. There appears to be a significant epidemiologic association between traumatic exposure to a cat (bite or scratch) and development of BA and associated diseases. Currently no evidence of an insect vector has been shown to induce BA. There is also an epidemiologic association between owning a cat with fleas and developing CSD in immunocompetent patients. This finding may indicate that the cat flea should be added to the list of arthropod vectors that could transmit *Bartonella (Rochalimaea)* species to humans. The frequency of CSD increases in the fall and winter months in temperate climate and appears as epidemics and intrafamilial microepidemics, particularly in families with cats or kittens.

Bacterium

The organism (Figure 6) is a small, curved, Gram-negative rod. The cell size is estimated to be approximately 1 to 2 μm in length by 0.5 to 0.6 μm in width and to have a G + C content of 40.3 to 41.1 mol%. It is readily stained with Gimenez stain. Electron microscopy reveals that these bacilli possess a trilaminar cell wall and contain electron-dense granular material (Regnery et al., 1992). This bacterium does not utilize carbohydrates; both catalase and oxidase reactions are negative, and it does not hydrolyze urea and esculin, nor does it reduce nitrate. The bacterium has a relatively simple pattern of cellular fatty acid consisting of three major fatty acids, octodecanoic acid (C18:0, 54%), octodecenoic acid (C18:1, 18%), and hexadecanoic acid (C17:0, 17%). *Bartonella (Rochalimaea)* can be cultivated in

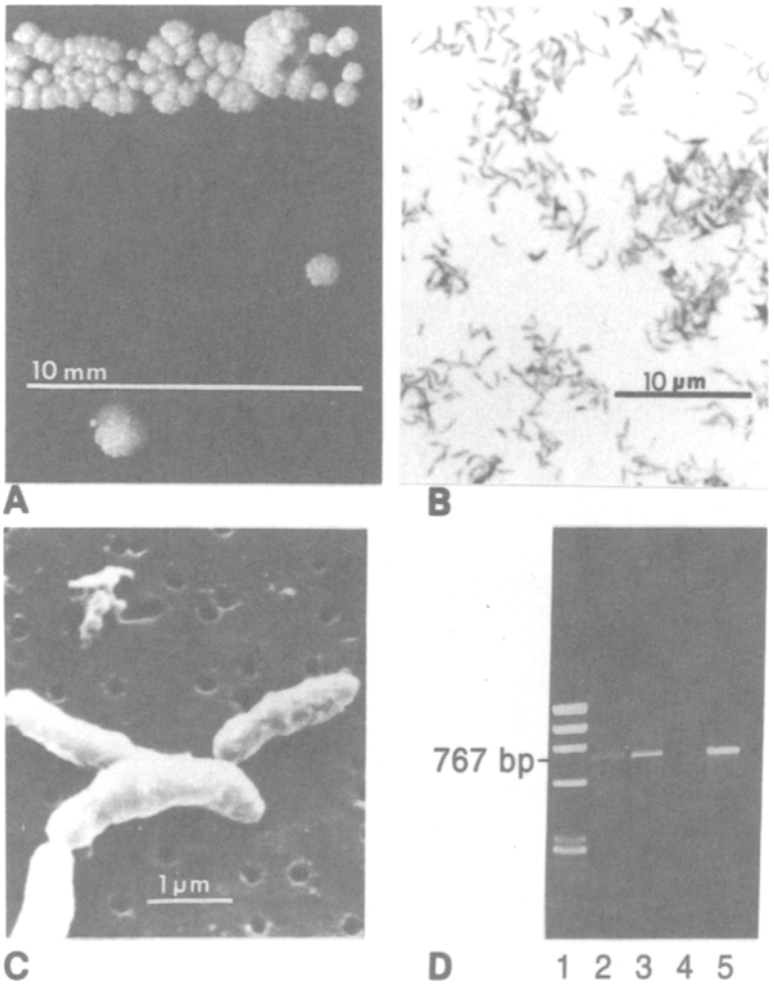

Figure 6. *B. henselae*: A. Growth on blood agar. Fourth-passage, 24-day-old Houston-1 isolate colonies were cultured on sheep blood-TSA bacteriological plates. B. Light microscopy of Gimenez-stained Houston-1 isolate. C. Scanning electron microscopy of glutaraldehyde-fixed *B. henselae* Houston-1 isolate. (Reprinted by permission, Regnery et al., 1992). D. Agarose gel electrophoresis of polymerase chain reaction (PCR) products amplified from DNA extracted from CSD skin test antigens. DNA used for PCR amplification was extracted from following sources: lane 2, CSD skin test antigen A; lane 3, CSD skin test antigen B; lane 4, DNA extraction blank (negative control); lane 5, 1 ng of purified DNA from *B. henselae*. Molecular size standards shown in lane 1 are (top to bottom) 1353, 1078, 872, 603, 310, 281/271, 234, 194, 118, and 72 hp. (Reprinted by permission, Anderson et al., J. Infect. Dis. 168, 1034, 1993).

cell-free culture. They are fastidious and slow-growing, when freshly grown from tissue specimens. Original colonies take 9 to 15 days to grow on BHIA and TSA supplemented with 5% rabbit blood in a 5% CO_2 humidified atmosphere at 35°C.

Pathology

The histopathology of lymph nodes in CSD is characterized by granuloma, nonspecific inflammatory infiltrate, and stellate abscesses. Organisms may be seen in sections that are stained with Warthin-Starry silver impregnation stain (Schwartzman, 1992).

HIV patients (Schwartzman, 1992; Koehler and Tappero, 1993) with BA develop multiple subcutaneous nodules characterized by angioproliferation, inflammatory infiltrate, and the presence of argyrophilic bacillary forms throughout the interstitium. HIV-infected patients may also have distinctive vascular lesions characterized by closely adherent cuboidal vascular endothelial cells, loose stroma, and epithelioid cells. BPH is characterized by a proliferation of cystic blood-filled spaces surrounded by fibromyxoid stroma in which one can see bacteria similar to those seen in BA.

Clinical Diagnosis

Cat scratch disease is generally characterized by localized lymphadenitis in an individual exposed to a cat scratch or an inoculation of the skin, eye, or mucous membrane (Schwartzman, 1992).

The typical CSD syndrome begins with the formation of a papule (Figure 3) at the site of inoculation. It appears from 4 to 6 days after animal contact. It progresses from a macule of 2 to 3 mm to a papule or pustule. Regional adenopathy develops within 7 to 50 days and in half of the cases this is the only symptom. The involved nodes are located in the head, neck, or upper extremities. The node is usually tender and may suppurate in a small number of patients. The episodes usually resolve spontaneously in 2 to 4 months without administration of specific therapy. Aspiration of suppurating nodes is recommended for relief of pain.

In HIV patients infected with *Bartonellas (Rochalimaeas),* BA has been reported to occur in every organ system (Schwartzman, 1992; Koehler and Tappero, 1993). BA occurs most frequently in the later stage of HIV. Dermal BA presents in several ways. It is the commonest form of BA. It has an enlarging red papule with some resemblance to a cranberry, often with a collarette of scale. Subcutaneous BA that appears as a flesh-colored subcutaneous nodule may be either fixed to subcutaneous tissues or freely mobile. These lesions have a vascular appearance and an erythematous base, and they bleed profusely when traumatized. The clinical diagnosis of these lesions is difficult; they have often been mistaken for Kaposi sarcoma. Diagnosis is only established after histopathologic evaluation of biopsied tissue.

BA of the gastrointestinal and respiratory tracts is characterized by lesions in the gastrointestinal tract and laryngeal involvement respectively. Osseous BA are

usually extremely painful and most frequently involve the long bones, especially the tibia, fibula, and radius. BPH is a disorder characterized by a unique vascular lesion associated with hepatomegaly.

Laboratory Diagnosis

At present IFA is the test of choice for the detection of antibodies to *Bartonella (Rochalimaea)* (Regnery et al., 1992). Examination of banked sera shows that *Bartonella (Rochalimaea)* antibodies are present up to 7 years before the development of BA. Sera from CSD patients showed high titers to *B. henselae.*

B. henselae can be isolated from the lymph nodes of CSD patients and from the blood and tissues of patients with BPH and BA using lysis-centrifugation technique. PCR assay for detection of *Bartonella (Rochalimaea)* nucleic acid from lymph node biopsies and aspirates has also been performed.

Therapy

Antibiotics in CSD are generally not indicated and particularly not useful. In contrast, for BA, and BPH, antibiotic therapy is effective and indicated.

HIV patients with *Bartonella (Rochalimaea)* infection should be treated with a prolonged course of antibiotic to avoid relapse. Treatment could be extended for the duration of the life of the patient. It is estimated that the minimum course should be at least 12 weeks. The antibiotic should be given intravenously for weeks, followed by prolonged oral therapy. The antibiotics of choice are: erythromycin, doxycycline, tetracycline, and minocycline (Koehler and Tappero, 1993).

CONCLUSION

The impact of rickettsial diseases on world health, although relatively low in terms of patient number in comparison with other infectious diseases, is nevertheless acute enough to warrant further investigations. This is further indicated by the discovery of the causative agents of CSD, BPH, BA, and the discovery of the causative agent of human monocytic ehrlichiosis and the discovery of granulocytic ehrlichiosis. These findings establish the rickettsiae as a part of the emerging infectious diseases.

Rickettsiae as obligate intracellular bacteria have DNA and RNA with a genome size half that of Escherichia and the same size as Neisseria, which are free-living bacteria. This makes them suited for molecular studies and for the study of host-parasite interaction. With the ability to manipulate DNA segments in these organisms, it will be possible to evaluate the effect of the loss or gain of specific genes. Virulence factors unknown in rickettsia could be identified, cloned, and evaluated.

The extensive application of newer biomolecular techniques and tools may make possible the monitoring of a number of the clinical aspects of rickettsial diseases,

e.g., potential disease relapse and/or treatment failures. The application of these techniques in the clinical field will probably be used to more accurately determine the pathogenesis of rickettsial infections and determine the location of the organisms during persistent infection when there is no indication of disease.

The number of interesting rickettsial enigmas to solve is limitless from the most basic to the most practical research application.

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Chapter 19

Chlamydia

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MICROBIOLOGY

Chlamydia are Gram negative, nonmotile, obligate intracellular bacteria. They are classified under the order Chlamydiales, which contains only one family, the Chlamydiaceae, and one genus, *Chlamydia*. The genus *Chlamydia* contains three species pathogenic for humans; *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. *C. trachomatis* has been divided into 18 serovars based on immunologic characteristics. Different serovars of *C. psittaci* are likely to exist, but the number is not yet known. *C. pneumoniae* has one known serovar, strain TWAR.

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Chlamydia exhibit a unique, biphasic life-cycle with a smaller extracellular infectious form, the elementary body, and a larger replicating intracellular form, the reticulate body (Figure 1). The elementary bodies, which are metabolically inert and have a protective cell wall, attach to susceptible host cells and are phagocytized (Figure 2). Within the phagosome, they lose the cell wall, become metabolically active, and undergo reorganization into reticulate bodies. Chlamydia lack the ability to form energy stores and during growth and replication they obtain ATP from the host cell. They are therefore considered energy parasites. The reticulate bodies replicate by binary fission. They then revert to the elementary body form by a process of condensation, forming characteristic cytoplasmic inclusions. Release of the infectious elementary bodies by cell lysis or extrusion of intact inclusions allows infection of new cells and continuation of the life cycle.

Chlamydia are a distinct class of bacteria, but share some structural similarities with Gram-negative bacteria. Like Gram-negative bacteria, they have a trilaminar outer membrane composed of lipopolysaccharide and other membrane proteins similar to those found in *Escherichia coli*. Unlike Gram-negative bacteria, however, this cell wall does not contain peptidoglycan, which provides structural stability to the Gram-negative cell wall. In Chlamydia, stability of the cell wall in the extracellular form, the elementary body, is provided by disulfide cross-linking between cysteine residues both within and between outer membrane proteins.

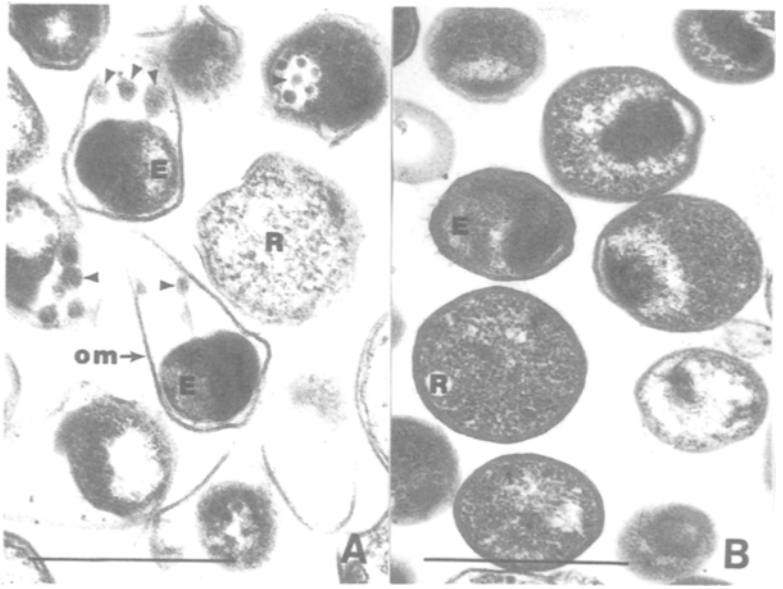

Figure 1. Electron micrograph of *Chlamydia pneumoniae* (A) and *Chlamydia trachomatis* (B). E = elementary body, R = reticulate body, om = outer membrane, arrowhead = small electron-dense bodies (mini bodies), bar = 0.5 μm.

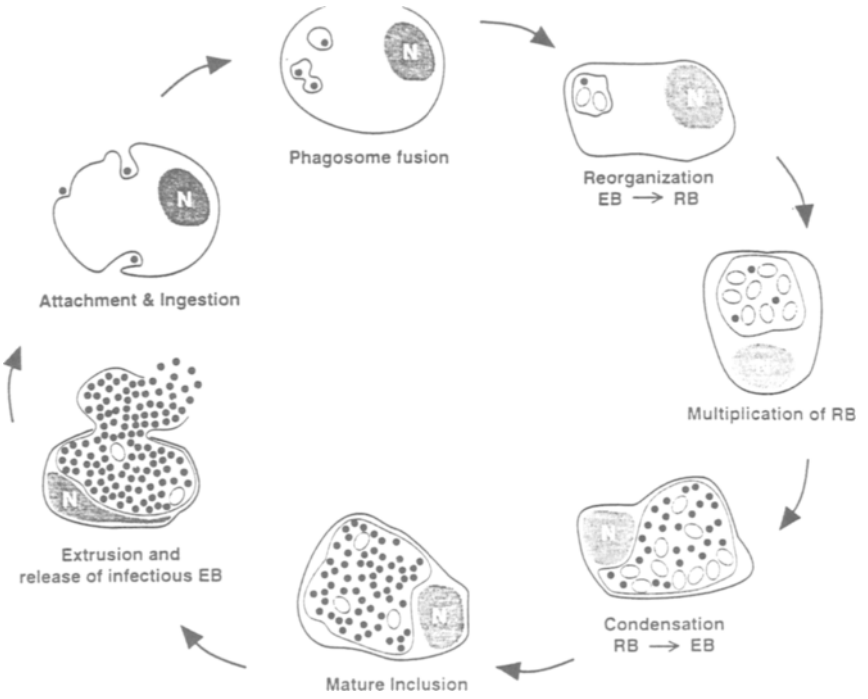

Figure 2. Life cycle of Chlamydia. From Jones, 1995.

The three chlamydial species can be differentiated based on DNA homology, appearance of the elementary body, staining characteristics of inclusions, antibiotic susceptibility, and the presence of extrachromasomal DNA (Table 1). The human strains of *C. trachomatis* are almost 100 percent homologous with each other and are between 5 and 20 percent homologous with *C. psittaci* strains. The *C. psittaci* strains exhibit between 10 and 60 percent homology with each other. *C. pneumoniae* shows less than 10 percent homology with either of the other two species. The reticulate bodies of all three species appear identical, and the elementary bodies of *C. trachomatis* and *C. psittaci* are round and similar in appearance, with little or no periplasmic space. The elementary body of *C. pneumoniae*, however, is pear-shaped and is surrounded by a relatively large periplasmic space. *C. trachomatis* produces a glycogen-like material within the inclusions which allows their staining with iodine. In contrast, *C. psittaci* and *C. pneumoniae* inclusions do not contain glycogen and do not stain with iodine. Of the three species, only *C. trachomatis* is sensitive to sulfonamides. *C. pneumoniae* does not appear to contain any extrachromosomal genetic material, while some strains of *C. psittaci* and most strains of *C. trachomatis* contain a plasmid.

Table 1. Characteristics of the Three Species of *Chlamydia*

	C. trachomatis	*C. psittaci*	*C. pneumoniae*
Elementary body	Round	Round	Pear-shaped
Iodine staining of inclusions	Yes	No	No
Sensitivity to sulfa	Yes	No	No
Number of serovars	18	Unknown	1 (TWAR)
Extrachromasomal DNA	Plasmid	Plasmid	None

The three species also cause distinct clinical syndromes, which are described briefly in the following section.

CLINICAL SYNDROMES AND PATHOGENESIS

Chlamydia trachomatis

C. trachomatis causes a variety of human infectious syndromes, including ocular, pulmonary, and genital tract infections. The organism is divided into 18 serovars based on antigenic variation in the major outer membrane protein. The L_1, L_2, and L_3 serovars are responsible for lymphogranuloma venereum, a sexually transmitted infection characterized by inguinal adenopathy and suppuration. The A, B, Ba, and C serovars are primarily associated with trachoma, an ocular infection that is the leading cause of preventable blindness in the world. Serovars D through K cause the majority of sexually transmitted infections, including urethritis, epididymitis, cervicitis and pelvic inflammatory disease.

Trachoma

Trachoma, one of the oldest recognized human infections, continues to be a major public health problem in many developing countries, particularly those in North Africa, sub-Saharan Africa, and Southeastern Asia. In those areas, infection is nearly ubiquitous and is spread among young children. In its initial stages, trachoma presents as a chronic follicular conjunctivitis. As the disease progresses, scarring of the conjunctiva occurs, and there is involvement of the cornea. In addition, as the inner surface of the lids become scarred the eyelashes turn in and abrade the cornea, resulting in ulceration, scarring, and visual loss. Many years are required for this process, and blindness therefore generally occurs more than 25 years after the peak of the active inflammatory process. Blindness occurs in 1 percent to 15 percent of trachoma patients.

It appears that recurrent infection is important in producing the inflammatory response that leads to scarring and tissue damage. In both human and animal models, initial infection in the eye resolves with little sequelae. However, recurrent

infection produces a much greater inflammatory response which causes scarring. It is therefore thought that multiple or persistent infection is required for progression of disease in trachoma.

Lymphogranuloma venereum (LGV)

LGV is a sexually transmitted disease caused by the LGV serovars of *C. trachomatis*. It is rare in the United States, but is endemic in areas of Africa, India, Southeastern Asia, South America, and the Caribbean. Initially, a primary lesion, a painless superficial ulcer or vesicle, forms on genital mucosa or adjacent skin. The secondary stage occurs days to weeks later and is characterized by lymphadenopathy and systemic symptoms. The lymph nodes involved are those that drain the primary lesion. In men with primary lesions on the penis or in the urethra, the inguinal nodes are usually involved and this is the most characteristic manifestation of the secondary stage. The inflammatory process then spreads from the lymph nodes into the surrounding tissue, forming an inflammatory mass. Rupture of the mass is associated with the development of loculated abscesses, fistulas, or sinus tracts. Without treatment, inguinal nodes eventually fibrose resulting in lymphatic obstruction and lymphedema of the external genitalia.

Urethritis, Epididymitis, Cervicitis and Pelvic Inflammatory Disease

C. trachomatis is the most common sexually transmitted bacterial pathogen in the United States. Sexual transmission results in the acute clinical syndromes of urethritis and epididymitis in men, and urethritis, cervicitis, and pelvic inflammatory disease in women. Although asymptomatic infections are common in men, *C. trachomatis* is also the cause of between 30 and 50 percent of cases of symptomatic nongonococcal urethritis. *C. trachomatis* is also a leading cause of epididymitis in men under age 35, and is usually associated with urethritis.

Most infected women are asymptomatic, but it is women who suffer the most serious consequences of genital chlamydial infections. Acutely, *C. trachomatis* can cause cervicitis, urethritis, and pelvic inflammatory disease. The majority of women with endocervical infection are asymptomatic or have only mild symptoms. Urethritis can occur in association with cervical infection or may occur as an isolated infection. Ascending infection of the endometrium or fallopian tubes (salpingitis) may occur after either symptomatic or asymptomatic cervical infection. It is estimated that approximately 8 percent of women with endocervical infection develop pelvic inflammatory disease, a term which refers to clinically suspected endometritis or salpingitis. Asymptomatic salpingitis is considered to be even more common than acute disease.

Both acute pelvic inflammatory disease and asymptomatic salpingitis can lead to the two major long-term complications of genital chlamydia infections, obstructive infertility, and ectopic pregnancy, which are consequences of inflammatory scarring and fibrosis of the fallopian tubes. As with trachoma, recurrent infection

appears to be important in the inflammatory response leading to these longer term complications. While acute chlamydial urethritis or cervicitis is characterized primarily by a polymorphonuclear cell response, reinfection or chronic infection is characterized primarily by a mononuclear cell response. The fibrosis which accompanies this chronic mononuclear inflammatory response is responsible for many of the long-term sequelae of chlamydial infection.

Perinatal Infections

Infant infection usually is acquired during passage through an infected birth canal. Approximately one third of infants born to infected women develop neonatal conjunctivitis, with an incubation period of approximately 5 to 21 days, and one-sixth develop late-onset pneumonia, with symptoms occurring between 3 and 19 weeks after birth. Unlike trachoma, inclusion conjunctivitis of the newborn is usually self-limiting and resolves without treatment. Inclusion conjunctivitis is less common in adults and is usually associated with genital infection and the presumed route of transmission is by autoinoculation via genital-hand-eye contact.

Chlamydia psittaci

Psittacosis is a relatively uncommon zoonosis which is usually acquired by exposure to infected birds. The organism is common in many types of birds, including parrots, finches, poultry, pigeons, pheasants, and seagulls, and is also found in mammals. Most patients with psittacosis have had some contact with birds, usually a pet. There are several strains of *C. psittaci*, and strains from turkey and psittacine birds are the most virulent for humans. This may account for the predominance of cases associated with poultry farming and contact with parakeets and parrots.

Infected birds may be either asymptomatic or obviously sick. The birds usually have gastrointestinal tract infection and it is likely that infection is spread by aerosol from infective droppings. Human to human transmission has been described but is uncommon. Clinical manifestations of infection range from a mild flu-like syndrome to a more severe illness with multiple organ involvement. Pneumonia is common and is the most characteristic manifestation of human psittacosis.

C. pneumoniae (TWAR)

Chlamydia pneumoniae (TWAR) is a recently recognized third species of *Chlamydia* that causes acute respiratory disease, including pneumonia, bronchitis, sinusitis, and pharyngitis. The organism was first isolated in 1965 from the conjunctiva of a Taiwanese child participating in a trachoma vaccine trial. The isolation was in the yolk sac of an embryonated chicken egg, the only method then available for growth of chlamydiae. In 1971, when cell culture methods became available, the organism (TW-183) was observed to form round, dense inclusions in

host cells in cell culture which were more similar in morphology to those of *C. psittaci* than *C. trachomatis.*

The organism's role as a human pathogen was not defined until 1983, when the first respiratory isolate (AR-39) was obtained from a university student in Seattle with pharyngitis. The strain name TWAR was derived from the laboratory designation of the first conjunctival and respiratory isolates (*TW*-183 and *AR*-39). In 1989, TWAR was established as a third species of *Chlamydia, C. pneumoniae.* Since only one strain or serovar of *C. pneumoniae* has been identified, at this time the strain name, TWAR, is synonymous with the designation *C. pneumoniae.*

C. pneumoniae infection is assumed to be transmitted from person to person via respiratory sections. Unlike *C. trachomatis*, sexually transmitted infection has not been described, and unlike *C. psittaci* no bird or animal reservoirs have been identified. Of the three *Chlamydial* species, *C. pneumoniae* is by far the most common cause of human infection. In the United States, it has been associated with approximately 10% of both outpatient and inpatient pneumonias, indicating that it is among the top five causes of community acquired pneumonia. Approximately 5% of bronchitis in adults is due to *C. pneumoniae.*

The organism has a worldwide distribution. In developed countries, infection appears to be uncommon before age 5 but is increasingly common in older children, with a peak incidence of acute infection as demonstrated by antibody conversion among children 5 through 14 years of age. By age 20 approximately 50 percent of persons have detectable levels of antibody to the organism and the seroprevalence increases to approximately 75 percent in the elderly. These prevalence rates exist despite the fact that infection induces only a transient antibody response (3–5 years after first infection), suggesting that most people are infected and reinfected throughout life. The majority of infections among children are either asymptomatic or mildly symptomatic while adults, especially the elderly, tend to have more severe disease. Pneumonia and bronchitis are the most commonly recognized clinical manifestations of *C. pneumoniae* infection. Other reported syndromes include sinusitis, pharyngitis, otitis media, endocarditis, myocarditis, erythema nodosum, and hepatitis.

An association between coronary artery disease and other atherosclerotic syndromes and *C. pneumoniae* infection has recently been suggested by both seroepidemiologic studies and by demonstration of the presence of the organism in atheromatous plaque. The initial study indicating a possible association between *C. pneumoniae* and coronary artery disease was performed in Finland, and showed that patients with coronary artery disease were significantly more likely to have serologic evidence of past infection with TWAR than were controls. Since that time, serologic studies from the United States and other countries have demonstrated similar findings among patients with coronary artery disease as well as patients with thickening of the carotid arteries. Morphologic and microbiologic evidence of the presence of *C. pneumoniae* in atheromatous plaques has been obtained by electron microscopic studies of coronary atheroma, and immunocytochemical staining and

polymerase chain reaction testing of coronary, carotid, and aortic atheroma. While these studies clearly associate TWAR organisms with atheromatous plaques, the role of TWAR infection in the pathogenesis of atherosclerosis is unknown.

SUMMARY

Chlamydia are among the most common bacteria to infect humans. The three species are genetically distinct and vary in the magnitude of their importance as human pathogens and in the clinical syndromes resulting from infection. *Chlamydia trachomatis* is of major worldwide importance, causing trachoma, the leading cause of preventable blindness in the world, as well as sexually transmitted genital infections which are important causes of infertility and ectopic pregnancy. *C. psittaci* is common in birds but is an uncommon cause of human infection. *C. pneumoniae* infects the majority of individuals by adulthood, is a leading cause of pneumonia, and has a worldwide distribution. Recent evidence suggests that *C. pneumoniae* may also be associated with atherosclerosis, an important cause of morbidity and mortality in adults.

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