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Themes in Bacterial Pathogenesis

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Introduction

The speed of progress in understanding how bacteria cause disease is providing novel insights and perspectives on pathogens and the pathogenesis of bacterial infections at an almost overwhelming rate. As the tsunami of antimicrobial resistance threatens our long-standing expectation that we can successfully treat bacterial infections with existing antimicrobial drugs, understanding how bacterial pathogens of animals cause disease is of fundamental value in designing new and better ways to counter infections. Combined with rapid diagnosis of specific infections, novel antimicrobial treatments based on understanding the unique weaknesses of pathogens, such as those discussed in Chapter 6, can be targeted in ways that could overcome the inherently highly untargeted and resistance-enhancing nature of most current antimicrobial therapies.

Although an overview of the basic themes in bacterial pathogenesis provides a conceptual skeleton for the extensive details of individual pathogens and their interaction with the host given in later chapters, understanding of virulence and pathogenicity is changing rapidly. The fundamental concepts have withstood the test of time, but new knowledge has brought the complexities of host–pathogen interactions into sharper focus and has identified both important broad new topics as well as nuances not recognized previously. Although more is understood about bacteria, especially through the application of genome sequencing and related technologies such as RNAseq (Chapters 3, 4), bacterial infections seem to be increasing and changing, especially those associated with increased antibiotic resistance, driven both by exposure to increasingly powerful antibiotics and by changes in affected patient populations. Numerous anthropogenic activities, including antibiotic use at both therapeutic and subtherapeutic concentrations, may be driving bacterial evolution and the selection of pathogens adapted to changed circumstances (Chapter 2). Against the background of stunning advances in technologies, there is increasing recognition of the poor general application of well-established simple infection control techniques, such as hand washing to reduce the transmission of infection in people and in animals in clinical settings. The fight against bacterial infections requires the disciplined use of hard-earned knowledge, not simply the development and application of new technology.

The Basic Elements of Bacterial Pathogenesis

The basic elements in the establishment of infection by a bacterial pathogen (Figure 1.1) are well established. These are:

1. *Association* (colonization, invasion, or other ways of entry into the body).

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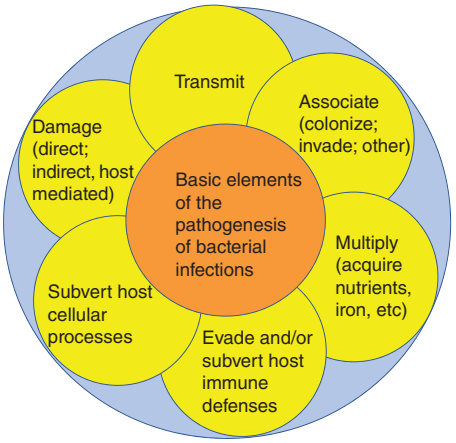

Figure 1.1 Basic processes in the pathogenesis of bacterial infections. The processes are more stages than steps, occurring often simultaneously, but also progressively and dynamically, integrated by regulatory processes responsive to signals provided by the host and environment. "Regulation" is shown in the sea of blue in which the processes are embedded. For highly virulent, destructive pathogens, "subversion of host cellular processes" does not occur, whereas it is critical for intracellular pathogens.

2. *Multiplication* (after nutrient, iron, etc. acquisition) to significant numbers at the site of infection and/or spread to other sites.
3. *Evasion* of host innate, sometimes acquired, immune defenses.
4. *Damage* to the host, either directly through subversion of cellular processes, or indirectly through host responses to the pathogen or its products.
5. *Transmission* from the infected animal to other susceptible animals, so that the infection cycle can continue.

As would be expected for carefully regulated systems, the infection process is a dynamic continuum rather than a clear series of steps, but breaking it down into progressive steps allows ease of understanding.

Pathogen Association with the Host

Successful colonization of the skin or a mucosal surface of the host is usually the first prerequisite of the infectious process. Some organisms need to employ motility and chemotaxis as well as resistance to acid and bile to reach their target host cells. Initial contact between bacterial pathogen and host cell is usually mediated by fimbrial or non-fimbrial adhesins on the bacterial surface. Binding may result either in extracellular colonization or in internalization of the pathogen. The adhesins bind to specific host cell surface receptors, and both host and organ specificity of infection are determined by differences among animals in cellular receptors for the bacterial adhesins. For example, the *Listeria monocytogenes* adhesion molecule internalin A (InlA) promotes uptake of the bacterium into intestinal epithelial cells by binding to E-cadherin. InlA binds to human and rabbit E-cadherin and causes disease in these species; however, it fails to bind to mouse E-cadherin and so does not readily cause disease in mice. Interestingly, Wollert et al. (2007) showed that by making two substitutions in InlA, they could increase the binding affinity to mouse E-cadherin by 10 000-fold and thereby establish experimental infection in mice. New host adaptations of different infections arise by similar naturally occurring mutations.

As many receptors are developmentally regulated, age specificity may also be determined by the receptor that a pathogen binds to. Well-established examples are known in K99 (F5) pili of porcine and bovine enterotoxigenic *Escherichia coli* (ETEC), which bind to the intestinal epithelium of neonatal animals, and in F18 pili of porcine ETEC, which bind to the intestinal epithelium of recently weaned pigs.

Bacterial pathogens, including those associated with wound infections, may bind to extracellular matrix molecules such as fibronectin, collagen, laminin, or other proteins possessing RGD (Arg-Gly-Asp, arginine-glycine-aspartic acid) sequences for binding of eukaryotic cell membrane integrins. Bacteria may use "invasins" to mediate their uptake into non-professional phagocytic host cells

after attaching to molecules on the cell surface and activating host cell signaling to facilitate their entry, often through host cell cytoskeletal rearrangement. An excellent example of this is found in the adherence to and invasion of M cells by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. The outer membrane protein invasin produced by these bacteria binds to β1 integrin on the surface of M cells and triggers uptake of the bacteria in a zipper-like internalization process (Hauck 2002). This entry provides the bacteria with access to the lymphoid tissue, and to draining lymph nodes, in which the bacteria are well equipped to multiply.

Facultative intracellular pathogens may deliberately target macrophages, for example by entering through complement or other lectin-binding receptors and thus avoiding the oxidative burst that might otherwise kill them. Remarkably, the safest place in the body for these organisms is a macrophage, since these pathogens subsequently interfere with phagosome maturation.

After initial association with the host, bacterial pathogens need to evade host defenses and to multiply to numbers sufficient for the infection to be self-sustaining rather than to be aborted by the host response. The "defensins" or "protectins" involved in the evasion–multiplication process can be divided into those involved in defense against innate immune mechanisms and those involved in defense against specific immune mechanisms.

Innate immunity can be overcome in a wide variety of ways (discussed throughout the book, in particular Chapter 5). The lack of available iron that restricts the growth of many bacteria within the body is an important defense mechanism because iron is critical for iron-containing cofactors for enzymes required for primary and secondary bacterial metabolism. This limitation is often overcome by the iron-acquisition systems of pathogens.

Many organisms, particularly those that cause septicemia and pneumonia, have prominent, usually carbohydrate, capsules that help the organism resist phagocytosis in the absence of antibodies. Some capsules mimic host matrices so that the organisms are unrecognized by phagocytes. The lipopolysaccharide (LPS) molecules of some Gram-negative bacteria can protect them from the membrane attack complex of complement or from the insertion of antimicrobial peptides. Some bacteria, such as streptococci, can break down complement components through C5a peptidase or other proteases. Other bacteria may destroy or impair phagocytic cells through their leukocidins such as the RTX (repeats in the structural toxin) toxins or enable bacteria to survive inside phagocytes through enzymes such as superoxide dismutases or catalases.

Acquired immunity can be overcome in numerous ways (Chapter 5). These include the ability to degrade immunoglobulins with enzymes such as the immunoglobulin A (IgA) proteases, or the ability to alter the antigenicity of cell surface components such as fimbriae or outer membrane proteins. Bacterial superantigens can dramatically upregulate certain T cell subsets with specific Vβ regions, which may result not only in a cytokine storm, which confuses the immune system, but also in the deletion of these cells from the immune repertoire.

Pathogen Damage to the Host

Bacterial damage to the host is usually essential for immediate or long-term acquisition of the nutrients that the bacterium needs to thrive and to continue its pathogenic lifestyle. Infection does not always lead to disease, which is only one of the possible outcomes of bacteria–host interaction. Other outcomes include commensalism, latency, or quiet parasitism.

Among the wide variety of "offensins" produced by bacteria are many different types of toxins. Toxins can be classified in different

though not fully satisfactory ways, with that based on activity the most logical. Type I toxins, the membrane-acting toxins, bind to cell surface receptors to transduce a signal that results in the activation of host cell pathways, leading to aberrant cell metabolism. Examples in *E. coli* include the heat-stable enterotoxin, STa, which binds to the receptor for guanylyl cyclase, resulting in hypersecretion due to excessive levels of cyclic guanosine monophosphate (cGMP), and the cytotoxic necrotizing factor toxins, which activate Rho guanosine triphosphatases (GTPases), resulting in cytoskeletal rearrangements. The superantigens fall into this class. Type II toxins, the membrane-damaging toxins, include the membrane channel-forming toxins using the β-barrel structure (e.g. *Staphylococcus aureus* α-toxin), channel-forming toxins involving α-helix formation, the large range of thiol-activated cholesterol-binding cytolysins, and the RTX toxins. Type II toxins that damage membranes enzymatically also include the phospholipases of many bacteria. Type III toxins, the intracellular toxins, are toxins that enter and are active within the cell. These are often active-binding two-component toxin molecules. Examples include the adenosine diphosphate (ADP)-ribosyl transferases (e.g. the *E. coli* heat-labile enterotoxin, LT), the N-glycosidases (e.g. the Shiga toxins), the adenylate cyclases (e.g. the *Bordetella bronchiseptica* adenylate cyclase toxin), and the metalloendoproteases of the clostridial neurotoxins.

Tissue damage and impairment of host function is often due to the inflammatory response mounted by the host in response to infection with a bacterial pathogen, recognition of different pathogen-associated molecular patterns (PAMPs) and activation of the host's pattern-recognition receptors (PRRs). Sepsis represents an extreme case in which hyperresponsiveness to LPS and/or other host signaling molecules unleashes an excessive inflammatory response, resulting in vascular damage, hypotension, and multiple organ damage. The inflammatory response mounted by the host may also provide a point of entry for certain invasive enteric pathogens.

Pathogen Transmission from the Host

Although not often considered in a discussion of bacterial pathogenesis, a crucial feature of bacterial pathogens is their ability to use their pathogenic nature to ensure transmission from the host, either back into their environmental reservoir or directly to other susceptible hosts. Depending on the infection, further transmission to animals may be immediate or involve many years.

An important aspect of transmission involves bacterial infections of animals, which are important primarily because of the transmission of organisms from animals to humans. In some cases, as with Shiga toxin-producing *E. coli* O157:H7, the bacteria are normal flora in the intestine of ruminants, in which they do not cause disease; however, they do induce severe disease following transmission to humans. A similar situation exists for *Campylobacter jejuni* and most serotypes of *Salmonella* in poultry. Efficient transfer from their reservoir hosts to accidental host occurs directly through contamination of foods of animal origin and indirectly through fecal contamination of water and the environment.

Regulation is Critical in Orchestrating Pathogenesis

The outcome of infection is dependent on complex multistep processes involving the host, pathogen, environment, and their interactions. Although much is known about virulence and virulence-associated genes in bacterial pathogens, and about the diseases they cause, how bacteria orchestrate the process at a regulatory level in terms of timing, response to different host signals, environments, and structures, is an area for considerable further investigation.

Bacteria have an astounding ability to sense and to respond rapidly to their environment.

Bacteria–host–environment communication systems important in pathogenesis may involve combinations of bacterial type III secretion systems (T3SS), type IV secretion systems, host cell cytoskeletal rearrangement, quorum sensing, two-component regulatory systems, stress responses and regulatory RNAs. Studies of T3SS have identified a conservation of the secretion apparatus and a remarkable diversity in the effector functions mediated by the systems in extensively investigated bacterial pathogens such as *Salmonella*, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Yersinia*, among others. The effectors of the T3SS are virulence factors that interact with specific host cell structures and factors that set off complex host cell pathways.

Pathogenic bacteria need to be aware of their environment to know when to deploy their virulence genes. Cues to bacterial location are as diverse as temperature, pH, growth phase, nutrient availability, oxygen levels, ion concentrations, and quorum sensing molecules, or often combinations of these cues. Depending on the environment, some virulence genes may be upregulated while others are downregulated, only to reverse as the environment changes. The regulation of virulence genes is highly complex, with several regulators controlling the expression of a particular virulence gene and with coordinated and dynamic regulation of genes whose products are required under the same conditions (Bervoets and Charlier 2019; Barrientos et al. 2021; Felden and Augagneur 2021; Ishii and Eguchi 2021) although obtaining an integrated view of these interactions is in its infancy (Huang et al. 2019).

Concepts of Bacterial Virulence are Being Refined

How bacteria have evolved to cause disease in animals is truly remarkable in variety and complexity. Given the wide range of bacterial pathogens in different animal species, veterinary microbiologists have insights into this variety, dating back to the start of the microbiology revolution. The astounding ways in which such tiny packages cause so many different diseases is the subject of this book.

Understanding Virulence and Pathogenicity

Understanding infection and the definitions of virulence and pathogenicity has a long and fascinating history. Earlier definitions of virulence and pathogenicity derived from historic studies of classic bacterial pathogens ("Koch's postulates"), many of which have been successfully controlled by immunization, hygiene, or antimicrobial drugs. Koch's postulates, developed to define causation in infectious disease, ruled for 100 years (Wassenaar and Gaastra 2001). Early understanding of virulence and pathogenicity was primarily pathogen-centered and often does not work as well for opportunist pathogens, where host factors are more important than pathogen factors, as it does for primary pathogens. A pathogen is a microbe capable of causing host damage, pathogenicity is the capacity of a microbe to cause damage in a host, and virulence is the relative capacity of a microbe to cause such damage (Casadevall and Pirofski 1999). A virulence factor (virulence determinant) was defined by Casadevall and Pirofski (1999) as a component of a pathogen that damages the host. Pathogenesis is the process through which bacteria cause disease.

Detailed understanding of virulence and specifically its genetic basis was importantly updated by Falkow (1988) based on identification of its molecular basis ("Falkow's molecular Koch's postulates"). In these "molecular Koch's postulates," the property under investigation should be associated with pathogenic strains of a species, inactivation of the virulence trait gene(s) leads to measurable loss of pathogenicity, and reversion or allelic replacement of the gene restores pathogenicity. This important advance was

still largely pathogen-centered and focused on a narrow range of virulence determinants such as the exotoxins of *Corynebacterium diphtheriae*, a pathogen of largely historic significance. It was dependent on measurable loss of pathogenicity. Although subtle changes might not be detected, this molecular approach has become a gold standard in virulence studies.

Ignoring for the moment host and environmental interactions with the pathogen as determinants of disease, developing understanding now clearly recognizes that bacterial virulence is multifactorial (Wassenaar and Gaastra 2001). This broader approach recognizes not only the "true" or "essential virulence genes" that are directly and demonstrably responsible for host damage, as described above, but also that pathogenicity involves "virulence-associated genes," which regulate essential virulence genes or are otherwise required for their expression, secretion, or processing, as well as "virulence lifestyle genes" that allow bacteria to colonize the host, evade host defenses, use host factors for survival, or survive intracellularly (Wassenaar and Gaastra 2001). An analogy to this multifactorial concept of virulence is to use of a gun in a robbery. The bullets can be considered the true virulence genes, the gun itself can be considered the virulence-associated genes, and the criminal using the gun can be considered the virulence lifestyle genes. Clearly, inactivation of any of these three elements will stop the bullets killing a victim, but ultimately it is the bullets (virulence factors) that kill. Recognition of these different elements will prevent some of the potential confusion that faulty interpretations of modern experimental methods can produce, such as identifying regulatory genes as virulence factors.

A more integrated understanding of virulence factors, which encapsulates some of the changing understanding of virulence, is that:

> Virulence factors refer to the properties (i.e. gene products) that enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease. Virulence factors include bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium, and hydrolytic enzymes that may contribute to the pathogenicity of the bacterium (VFDB, Virulence Factors in Pathogenic Bacteria database: www.mgc.ac.cn/VFs).

This definition of virulence factors captures important elements of virulence but fails to include critical aspects associated with virulence such as adaptation of metabolic pathways and cellular processes to the virulence lifestyle of bacterial pathogens, described by Letek et al. (2010) as "co-optive evolution." It does not recognize the often multifactorial but individually possibly redundant nature of different factors involved in host colonization or evasion of host defenses which, depending on the model used to investigate them, may not be identified.

How pathogens obtain nutrients in their host is a critical part of their ability and adaptation to cause disease and of their virulence lifestyle. The role of classical hemolysins in this regard has long been recognized, but the importance of obtaining nutrients is critical throughout the pathogenic process, even though this may not in the past have been viewed as vital to understanding pathogenesis and of how bacteria adapt their metabolism to their evolution as pathogens. One of Napoleon's aphorisms was that "an army marches on its stomach." For example, *Clostridium perfringens* is an intestinal inhabitant and often an enteric pathogen that produces a wide variety of glycoconjugate-and mucus-degrading exoenzymes as well as a vast array of other extracellular degradative enzymes. These include a glycopeptidase ZmpC found on a critical virulence plasmid that, together with a genome-encoded mucin-degrading glycopeptidase ZmpA, has been shown to be important in avian necrotic enteritis (Low et al. 2020;

Wade et al. 2020; Pluvinage et al. 2021). Thus, enzymes used specifically to degrade chicken intestinal mucus are not only a source of nutrients but also a way for the organism to expose the underlying intestinal mucosa for colonization by bacterial surface components. Intracellular bacteria can metabolically reprogram their host cell's metabolic pathways for their nutritional benefit (Escoli and Buchreiser 2019).

The application of powerful newer research techniques (Chapters 3, 4) continues to expand our understanding of virulence and of the host–pathogen relationship. For example, the diverse application of mass spectrophotometry-based proteomics are providing extraordinary insights into the host–pathogen relationship and its dynamics (Sukumaran et al. 2021). Its application, combined with other evidence, has identified the unexpected multiple functions ("moonlighting proteins") of enzymes that were thought to have classical housekeeping functions of little general interest (Jeffery 2019). For example, the glycolytic enzyme GADPH (glyceraldehyde-3-phosphate dehydrogenase), which can constitute 10–15% of total bacterial cell protein, has multiple functions including a role in host attachment and colonization by different bacterial pathogens (Jeffery 2019; Sirover 2021). The role of glycoconjugates on bacterial surfaces in bacteria–host interactions is being increasingly appreciated (Tytgat and de Vos 2016). The highly diverse nature of glycan-modified surface and secreted LPS, capsular polysaccharides, lipooligosaccharides, lipoglycans, peptidoglycan, teichoic acids, and glycoproteins give bacteria unique and specific ligands with which to interact with the host (Tytgat and de Vos 2016).

Another important theme in the emerging understanding of virulence revealed by recent metagenomic research is the role of the microbiota, particularly throughout the intestine, in chronic inflammatory diseases such as periodontal disease or inflammatory bowel diseases. These infections involve multiple host-adapted mucosal pathogenic commensals and complex relationships between these agents and the local innate immune system (Ellermann and Arthur 2017; Fabrice et al. 2021).

Bacterial virulence and the pathogenesis of disease is increasingly recognized as a truly complex, dynamic, multifactorial, changeable, and constantly surprising phenomenon. Such understanding is impossible to capture in a simple diagram or indeed in publicly accessible databases that list "virulence factors" (e.g. Sayers et al. 2019; Victors: http://www.phidias.us/victors; VFDB: www.mgc.ac.cn/VFs).

Conceptually however, as outlined in Figure 1.1, bacteria cause disease by a variety of means in an extraordinarily complex process that usually involves penetrating the host's protective barriers, evading deeper host defenses, multiplying to significant numbers, escaping innate and acquired immune defenses, and damaging the host, leading sooner or later to escape from the host and to continue the cycle (Figure 1.1). Although this overall concept of the pathogenic process is well established, the resurgence or emergence of infectious diseases in humans in recent years because of changes in host susceptibility (HIV infection, immunosuppressive drugs, implanted devices) emphasizes the importance of host factors in determining the outcome of encounters with microbes. Many people now die in hospitals from infectious agents that are not pathogens in healthy people. A parallel situation exists in many small-animal hospitals, especially in intensive care units. Similarly, the ability of some bacteria to develop rapidly or acquire antimicrobial resistance and then to emerge as significant problems in hospital or even community settings emphasizes the importance of environmental selection in determining the outcome of infection, as well as in shaping the adaptation of pathogens to different settings. Development or acquisition of resistance is one way in which clones of different pathogens can gain an edge in enhancing their fitness and success as pathogens. Virulence

does not occur in a vacuum but is often contextually dependent. In this case, antibiotic use in hospitals may remove the inhibitory effects of the normal microbial flora in reducing colonization by exogenous, resistant, bacteria. Furthermore, bacterial pathogens themselves may carry genes for bacteriocins that that assist host colonization and are an important part of their success as pathogens, even though these are not virulence factors. They are, however, important as part of the fitness of these pathogens for their niches. Selective pressures other than direct effects on the host may exert profound influences on the evolution and fitness of pathogens, discussed in Chapter 2 and other chapters of this book. Bacterial pathogens evolve in many ways to face many challenges to survival other than just successful encounter with a host (Chapter 2).

The aspects of bacterial virulence discussed above highlight the survival and successful further spread of pathogenic bacteria under potentially adverse conditions in the ecological niche(s) into which they have been introduced or to which they have adapted, and all the complexity that successful survival implies. From this perspective, antimicrobial resistance genes may contribute to virulence, since they are virulence lifestyle genes that allow survival in antibiotic-containing environments. It is thus not surprising to find that some resistance and virulence genes are linked on the same virulence plasmid.

For many years, animals have been used in experimental infections to better understand the virulence of bacteria. It is now unacceptable to use animals experimentally for this purpose in the ways that previous generations of researchers did. If deemed necessary, the use of animals to investigate bacterial pathogenesis must be under the highest standards of humane care. Increasingly, rather than using animals, the powerful techniques of pathogenomics (Chapter 3), such as whole genome sequencing for strain characterization, RNAseq for characterizing gene expression, and modern molecular epidemiology, and different ways of virulence assessment in vitro (Chapter 4) will be used to understand and characterize the basis of the pathogenicity of bacterial pathogens.

Virulence and the Host–Pathogen–Environment Paradigm

Recognition of the importance of the host–pathogen–environment is fundamental in understanding the emergence and spread of bacterial infections and the development of disease. Host factors predisposing to infection include genetic factors, age, sex, innate and acquired resistance, and nutritional status. Pathogen factors include species, strain or clone, challenge dose, and route of infection, whereas environmental influences include temperature, nutrition, humidity, and toxins (Langford et al. 2021). The interplay or "dance" of the interaction of these factors can be summarized dynamically in Figure 1.2 (and elaborated in Chapter 4, Figure 4.1). What the classic Figure 1.2 does not capture is the extraordinary complexity and dynamic nature of the interactions between host, pathogen, and environment, and the often-constant adaptation for optimal "fitness" of bacterial pathogens. As understanding of the pathogenesis of bacterial infections continues to develop, the complexity and the subtlety of its basis often reveals the continuing depth of our ignorance.

Impact of Pathogens on Host Evolution

The impact of infection on the evolution of animal hosts can generally only be speculated upon but has likely been profound. The evolution of hosts and the individual pathogens that exploit them are inexorably linked (Brown et al. 2006). As just one of many examples, the target of the *Vibrio cholerae* toxin and *E. coli* heat-labile enterotoxin is the cystic fibrosis transmembrane conductance regulator (CFTR) protein, whose response to

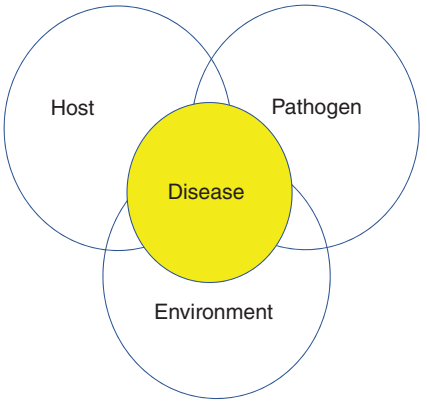

Figure 1.2 The classic host–pathogen paradigm of infectious disease. The outcome of an infection is always the result of the interaction of host, pathogen, and environmental factors. For "primary pathogens," pathogen factors are more important; for "opportunist pathogens," host factors are more important, and, for both, environmental factors may have a smaller or larger role. For example, many opportunist infections in animals involve mixed-agent infections because of the common host predisposition. Understanding the pathogenesis of a specific bacterial infection involves teasing out the numerous host–pathogen–environment factors involved and their relative importance in particular circumstances.

V. cholerae toxin leads to fluid outpouring in the intestine. The CFTR protein is necessary for fluid secretion in the intestine and in airways, and intestinal tissue from patients with cystic fibrosis fails to respond to the *V. cholerae* toxin. It has been suggested that the defects in the *CFTR* gene that provide resistance to cholera may have led to the maintenance of defective genes in the human population and the high frequency of the ΔF508 mutation (1 in 25); individuals who are homozygous for this mutation develop cystic fibrosis. The historical association of pathogens and their hosts, and the coevolutionary nature of this relationship, are also part of the host–pathogen–environment triad that determines the outcome of an infection.

At a far deeper level in evolutionary history, the continuous evolutionary struggle for survival between metazoal hosts and microbial pathogens since the dawn of time has driven the development of the different mechanisms of innate immunity including the responses of phagocytes to the multiple different signals of the presence of bacteria and other pathogens through detection of PAMPs by PRRs, the amoebocyte response of the horseshoe crab (*Limulus polyphemus*) to bacterial LPS, or of the signals of cell damage resulting from infection. The race has also driven the development of the acquired immune system.

Host–Pathogen Communications are Critical

The outcome of infection is dependent on complex multistep processes involving host, pathogen, environment, and their interactions. The tendency in the past has been for researchers to tackle problems of pathogenesis primarily by investigation of virulence attributes of the pathogen. One of the outcomes of this approach is that we now have an impressive catalog of virulence genes of bacterial pathogens, but we have a long way to go in understanding issues of regulation, timing, crosstalk, and interplay with host structures and physiology. In recent years, researchers have sought to redress this imbalance, and we have seen numerous investigations of pathogens in either their natural host environments or in settings in vitro that seek to simulate aspects of the in vivo environment. It is therefore not surprising that a major recent theme in pathogenesis research is that communication between bacteria, host, and environment is a critical aspect of pathogenesis. Studies in this field have led to a new branch of microbiology, namely cellular microbiology, which investigates bacterial signal transduction as a tool for characterizing host signaling pathways, based on discovery of the ways in which bacterial pathogens subvert these for their own benefit. Bacterial pathogens are expert cellular microbiologists.

An emerging theme in the 2010s has also been investigation and understanding of the

important role of microRNAs (miRNAs) and other non-coding RNAs in the host and bacterial pathogen relationship (Duval et al. 2017; Aguilar et al. 2019). Bacterial non-coding RNAs regulate host gene expression affecting multiple host functions and, in turn, eukaryotic miRNAs modulate bacterial gene expression. Identification of the targets of the bacterial and of the host miRNome, as well as of the regulons controlled by individual miRNAs, has identified important novel aspects of the host–pathogen relationship in many different types of pathogens, both in diverse host defenses against pathogens and in the subversion of different host defenses by pathogens. Detection of miRNAs in the circulation of infected animals is an accessible source in which to understand their potential host and pathogen role in infection. Understanding of the role of miRNAs in host defense and pathogen offense is a fertile field of current and future research.

Host cells also have elaborate mechanisms for identifying conserved bacterial structures and relaying this information to the pathways that respond to the presence of bacteria. PRRs on the surface of innate immune cells permit the recognition of infectious agents through their possession of PAMPs such as LPS, lipoproteins, peptidoglycans, and DNA with unmethylated CpG motifs. Included among the PRRs are the Toll-like receptors (TLRs), which are signal transduction proteins that, among other actions, trigger the secretion of cytokines through activation of the transcription factor NfκB. Signaling by TLRs occurs primarily through the adaptor molecule MyD88. Another adaptor molecule (Trif) is required for signals leading to the production of interferon-β following activation of TLR3 or TLR4. TLR3 detects double-stranded RNA; TLR4 recognizes LPS; and TLR2 recognizes lipoproteins, peptidoglycans, and lipoteichoic acid. Flagellin binds to TLR5 and causes the release of interleukin-8 (IL-8) from intestinal epithelial cells. Interestingly, TLR5 is expressed on the basolateral and not on the apical surface of intestinal epithelial cells, so that the alarm is sounded only when bacterial invasion has occurred, or bacterial products have reached this site. CpG-DNA interacts with TLR9, which is located intracellularly rather than at the cell surface.

The TLRs help to link the innate immune response with the acquired immune response, as macrophages and dendritic cells that contact pathogens become activated, causing the upregulation of costimulatory cell-surface molecules, as well as class I and II major histocompatibility complex molecules. Differential expression of TLRs on the various types of cells of the innate immune system and differences in the signals that are generated allow for a system in which the type of pathogen that is encountered is met with the appropriate type 1 or type 2 immune response. Innate immune responses that occur following binding of the pathogen to a TLR include killing of the pathogen through antimicrobial compounds, such as nitric oxide in macrophages and antimicrobial peptides at the surface of epithelial cells. Adaptive immune responses are influenced through the activation of B cell proliferation, release of chemokines, and adjuvant effects of the PAMPs. The numerous ways in which bacterial pathogens damp down proinflammatory responses induced by PAMPs (Garib et al. 2016; Brewer et al. 2019) or in numerous other ways interfere with and subvert the immune response is discussed in Chapter 5, and throughout this book.

Signaling that affects host programmed cell death pathways is a common aspect of pathogenesis of bacterial diseases, notably in protection against facultative and obligate intracellular bacteria (Imre 2020; Lacey and Miao 2020). Programmed cell death (apoptosis, necroptosis, or pyroptosis) can be triggered by interconnected pathways responding to signals including outer membrane proteins, LPS, lipomannans, lipoarabinomannans, lipoproteins, porins, and certain protein toxins. Apoptosis driven by caspases is usually anti-inflammatory since it does not trigger an

immune response whereas the two lytic forms of cell death release damage-associated molecular patterns (DAMPs) into the extracellular space and trigger an immune response (Imre 2020). Macrophages are highly vulnerable to apoptosis since it may be triggered by their possession of numerous TLRs for conserved bacterial surface components. Bacteria can induce apoptosis by stimulating proapoptotic molecules or inhibiting anti-apoptotic molecules (Demarco et al. 2020).

Apoptosis is a common feature of the pathogenesis of many intracellular pathogens described in this book. Apoptosis may provide benefits to the host by curtailing the innate immune response, thereby limiting damage due to excessive release of cytokines and destructive neutrophil enzymes. On the other hand, it may be of value to the pathogen by destroying host defense cells such as macrophages, thereby promoting invasion of tissues and prolonging infection. Efferocytosis, the engulfment of apoptotic infected cells by phagocytes, is a common means of destruction of many intracellular bacteria that is often inhibited by facultative intracellular bacteria (Behar and Briken 2019).

The role of inflammasomes as a host defense strategy and also as a target of subversion by intracellular pathogens has been the increasing subject of research in recent years (Brewer et al. 2019; Broz 2019). These intracellular pathogen PRRs are multiprotein complexes made of sensors, adaptors and effectors that act as host danger detection systems, targeting caspase-dependent cleavage of downstream proteins to activate innate immune defenses and initiating acquired immunity. Five different canonical sensor pathways have been described with a sixth noncanonical pathway activated by LPS in which caspases act as both sensor and effector (Brewer et al. 2019). Inflammasome activation of proinflammatory cytokine secretion leads to proinflammatory cell death (pyroptosis) and activation of immunity. Like their viral counterparts, intracellular bacterial pathogens have evolved different mechanisms to counteract inflammasome assembly and thus to preserve their replicative niche (Yu Garib et al. 2016).

The evolutionarily conserved eukaryotic signaling pathway Wnt has been studied for years as a regulator of metazoan embryogenesis, stem-cell maintenance and organogenesis, but it is only in the 2000s that it has been recognized as a mechanism of innate immune subversion by numerous intracellular and extracellular bacterial pathogens (Rogan et al. 2019). The manipulation of the Wnt pathway is discussed particularly in the context of intracellular pathogens (Chapters 20, 21) but is evident for numerous other pathogens (Rogan et al. 2019).

New host regulatory signals that are critical for virulence expression are being identified. For example, EHEC O157:H7 expresses the colonization genes encoded by the locus for enterocyte effacement (LEE) in response to a quorum-sensing regulatory molecule that was initially considered to be autoinducer 2 (AI-2) but was later shown to be a new autoinducer called AI-3 (Chapter 7). Both AI-2 and AI-3 require LuxS for their synthesis. Interestingly, the mammalian hormones epinephrine and norepinephrine had the same effect as AI-3 in activating the LEE-encoded genes. Furthermore, either exogenous AI-3 or epinephrine can activate the LEE genes in a *luxS* mutant, and epinephrine antagonists could block this activation. These data suggest that AI-3 and epinephrine may use the same bacterial signaling pathway in crosstalk between host and pathogen. Once the bacteria arrive at the host epithelium, epinephrine stimulates expression of the LEE genes, thereby allowing EHEC to attach to the intestine (Chapter 7).

Pathogenesis in the Post-Genomic Era

The vast influx of information from genome sequencing has revolutionized the science of pathogenesis, ranging from understanding

the most basic aspects of gene content to elucidating the regulatory networks of virulence gene expression, to investigating the global patterns of host response to infection (Chapter 4). Examining differences in specific genes between a pathogen and a closely related nonpathogen, or between the parent and its offspring with a specific null mutation, has been a valuable approach for identifying virulence genes. The rate of recognition of potential virulence genes is increasing dramatically as innumerable complete or almost complete genome sequences are available for thousands of bacteria, and genomic and RNAseq data are combined to identify hundreds of potential virulence factors simultaneously. However, these potential virulence factors will need to be tested individually to assess their roles in virulence.

The immensity and complexity of data that need to be analyzed are overwhelming. Initially, genomics brought us the concepts of the core genome (encoding the basic aspects of the bacterial biology) plus the dispensable and strain-specific genes for an isolate. As discussed in Chapter 3, we have moved beyond this and the pan-genome (the genetic information of a bacterial species) and the metagenome (the genetic information of a community of bacteria in a specific environmental niche) have moved from theoretical constructs to reality. Functional genomics can be used to investigate the transcriptome under specific conditions. Data from transcriptome studies are leading to a detailed understanding of memberships in virulence-associated operons and regulons and to the identification of the complex environmental cues that modulate virulence expression. Ideally, bacterial messenger RNA (mRNA) collected from infected tissues would be examined (Bourgeois and Smith 2020) but relatively low numbers of bacteria in most infected tissues, the small amounts of bacterial compared with host RNA, and the instability of bacterial mRNA still make this approach challenging for most infections (Chapter 4). To address this, it has been necessary to use in vitro conditions to simulate the in vivo setting. One of the challenges in these studies is to accurately simulate the host microenvironment but, currently, it is common for only one or two aspects of that environment to be examined in simulations.

As discussed in Chapter 4, comparative genomics involving comparison of open reading frames of genomes of pathogenic and closely related non-pathogenic species, or of highly pathogenic and less pathogenic strains, are a valuable starting point in the identification of virulence genes. A dramatic advance has been that genomic data can be used to probe not only bacterial metabolism in the host but also host changes in response to the presence of the bacteria. The enormous amount of data generated in these studies is a challenge for analysis and interpretation. The time at which a readout of mRNA is made is critical since too long a delay may reveal only the steady state after much of the series of responses by bacteria and host have been completed. These analyses identify genes expressed under certain conditions, and subsequent testing is needed to determine the subset of these genes that are essential for infection of the host and for disease. What may be apparently minor differences in experimental design, methodologies, and analysis will have a dramatic effect on the results, which can essentially be useless.

Data mining of complete and incomplete genome sequences has been used to generate valuable information on virulence-related genes in bacteria. There is, however, a large gap between genomic analyses and functional genomics. This is exemplified by the fact that only about 60% of the genes of *E. coli* and 56% of the genes of *Pseudomonas aeruginosa* have known functions. In addition, the presence of gene sequences does not necessarily mean that functional proteins are produced.

Newer rapid genome sequencing ability has permitted studies of metagenomics, thereby adding a new dimension to our capability to investigate pathogens in their natural

environments. This will be particularly valuable in host niches such as the intestine, which have rich microbial communities whose interactions with pathogens are critical to health and disease.

Gaps in Knowledge and Anticipated Directions

Understanding of bacterial pathogenesis and the host-bacterial interplay will continue to improve as research techniques become ever more powerful and encompass detailed understanding of the molecular basis of the host–pathogen relationship. Understanding will require collaboration of expertise across a wide variety of scientific disciplines. Application of this knowledge in terms of improved preventive and treatment strategies will continue to justify the need to understand the science behind this dynamic and evolving field.

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