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THE SECRET LIVES OF THE PATHOGENIC MYCOBACTERIA

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■ **Abstract** Pathogenic mycobacteria, including the causative agents of tuberculosis and leprosy, are responsible for considerable morbidity and mortality worldwide. A hallmark of these pathogens is their tendency to establish chronic infections that produce similar pathologies in a variety of hosts. During infection, mycobacteria reside in macrophages and induce the formation of granulomas, organized immune complexes of differentiated macrophages, lymphocytes, and other cells. This review summarizes our understanding of *Mycobacterium*–host cell interactions, the bacterial-granuloma interface, and mechanisms of bacterial virulence and persistence. In addition, we highlight current controversies and unanswered questions in these areas.

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INTRODUCTION

We dance around in a ring and suppose
The Secret sits in the middle and knows

Robert Frost

The majority of the >50 species that comprise the genus *Mycobacterium* are non-pathogenic environmental bacteria related closely to the soil bacteria *Streptomyces* and *Actinomyces*. However, a few species are highly successful pathogens, including *Mycobacterium tuberculosis, M. leprae*, and *M. ulcerans*, the causative agents of tuberculosis, leprosy, and Buruli ulcers, respectively. Their key to success lies at least in part with their ability to establish residence and proliferate inside host macrophages despite the antimicrobial properties of these cells (41, 52). The host mounts a complex immune response involving both innate and adaptive components that often sequesters the pathogen in organized structures called granulomas. However, the pathogenic mycobacteria are extraordinarily adept at establishing long-term infections that can manifest as acute or chronic disease or be clinically asymptomatic with the potential to resurface later. Understanding the factors that contribute to this long and complex relationship between pathogen and host is essential to our ability to modulate its clinical outcomes.

At least eight *Mycobacterium* genome-sequencing projects are at or near completion (http://www.tigr.org/; http://www.sanger.ac.uk/). Comparison of these genomes will likely reveal important clues regarding the evolution and pathogenic mechanisms of mycobacteria. This review highlights some of the interesting features and prevalent beliefs about tuberculosis and other mycobacterioses. Our comparative approach focuses on the importance of in vivo studies, and we discuss

mycobacterial diseases in the context of their host range, pathology, and pathogenesis. We highlight commonalities and differences that potentiate understanding of their pathogenic mechanisms, describe recent studies of mycobacterial virulence factors that illuminate mechanisms of pathogenesis, and concentrate on recent advances and current outstanding issues.

THE CAST

M. tuberculosis Complex

The *M. tuberculosis* complex consists of *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. bovis*, and *M. microti*, which are closely related organisms sharing >99% identity at the nucleotide level for some loci. However, they differ significantly in morphology, biochemistry, host range, and disease patterns in experimental animals (21). *M. tuberculosis* kills nearly three million persons annually (50); although in parts of Africa, *M. africanum* causes more cases of tuberculosis than *M. tuberculosis* (117). In contrast, infection by *M. canettii* appears to be rare. Although humans are the only natural hosts for *M. tuberculosis*, experimental animal models exist. *M. bovis* has a broad host range, producing tuberculosis in several mammals including humans and cattle, and was a major cause of human tuberculosis prior to pasteurization of milk. The pathology and course of *M. tuberculosis*, *M. bovis*, and *M. africanum* disease in humans are similar. *M. microti* is a pathogen of voles but is avirulent in humans and mice. The attenuated *M. bovis* derivative BCG and *M. microti* are equally efficacious as live tuberculosis vaccines (79).

ANIMAL MODELS OF TUBERCULOSIS *M. tuberculosis* infection of humans results in a variety of outcomes, in terms of both disease and pathology. Organized immune structures called granulomas are produced that evolve morphologically during the course of infection with the formation of areas of necrosis called caseum and the deposition of fibrin and calcium. Various animal models are used to study at least some of these aspects of *M. tuberculosis* pathogenesis in humans. Mice are most commonly used for reasons of cost, convenience, their amenability to genetic manipulations, and the availability of inbred strains and immunological reagents (121). However, tuberculosis in the mouse model differs from human disease in several important respects. In susceptible strains, bacterial burdens can be very high, reaching 10^6 per lung, and are never cleared. Moreover, mouse granulomas do not caseate or calcify. Guinea pigs exhibit many pathological features similar to those seen in humans, but unlike humans they are exquisitely sensitive to a progressive pulmonary infection (107). Rabbits display pathology more characteristic of human disease, ranging from spontaneous healing to caseous and cavitary pulmonary lesions. However, most rabbits are resistant to *M. tuberculosis* infection and were used to distinguish between *M. tuberculosis* (mild illness, eventually cleared) and *M. bovis* (severe, lethal disease) (42). Lurie bred rabbits that were

highly susceptible to *M. tuberculosis* (42), but unfortunately this strain was lost. The rabbit model is being revived and susceptible strains may be redeveloped (Y.C. Manabe, A.M. Dannenberg Jr. & W.R. Bishai, personal communication). Nonhuman primates are also used to model *M. tuberculosis* infection (108, 172), and intratracheal inoculation of cynomolgus macaques leads to a spectrum of outcomes similar to that seen in humans (J. Flynn, personal communication).

EVOLUTIONARY RELATIONSHIPS *M. tuberculosis* was believed to have evolved from the broad-range *M. bovis*, an unintended consequence of the domestication of cattle (87, 157). However, recent genomic analyses have shed new light on the evolutionary lineage within the *M. tuberculosis* complex. Two studies mapped the distribution of a series of DNA deletions (regions of difference, RD) among closely related mycobacteria (21, 110). Both argued that *M. tuberculosis*, with the fewest regions deleted, is the most ancestral of all *M. tuberculosis*-complex organisms, and that *M. bovis*, with the most regions deleted, evolved most recently. *M. africanum* and *M. microti* represent intermediates in this new evolutionary scenario. These analyses are compelling; however, they beg the question of how *M. bovis* evolved its broader host range during a process dominated by the loss of DNA. Interestingly, analysis of one of the RDs, RD1, has revealed a role in virulence (see Bacterial Factors Involved in Pathogenesis, below).

M. tuberculosis appears much more polymorphic than was previously appreciated. Because single nucleotide polymorphisms in coding regions are extremely rare in *M. tuberculosis* (112, 156), its genome was thought to be remarkably stable, and researchers have generally discounted the idea that differences in tuberculosis disease presentation, progression, or pathology might stem from differences among *M. tuberculosis* isolates. However, recent high-density oligonucleotide array analysis detected an average of 2.9 deletions per strain in 15 of 16 clinical *M. tuberculosis* isolates (88). In total, 25 unique deletions representing >76 kb, or 1.7%, of the H37Rv genome were reported. Also, a comprehensive comparison of the genomes of H37Rv (virulent laboratory strain) and CDC1551 (recent clinical isolate) reported 74 large sequence polymorphisms (LSP; insertions or deletions unique to one strain relative to the other) and over 1000 single nucleotide polymorphisms (62). Comparison of 169 clinical isolates for 17 of the LSPs revealed significant strain-to-strain variation. Thus, despite the scarcity of single nucleotide polymorphisms, there appears to be substantial heterogeneity among clinical *M. tuberculosis* isolates. However, the total heterogeneity observed is less than that observed for some other bacterial pathogens (27, 141).

M. marinum and *M. ulcerans*

It is common in bacterial pathogenesis to study a close relative of a human-restricted pathogen in its natural host. With this approach, it is possible to study a pathogen "at the top of it's game" and many points relevant to human disease can be explored. For example, infection of susceptible mice with the mouse pathogen

Salmonella typhimurium is widely used to model typhoid fever in humans, which is caused by *Salmonella typhi* (61). Similarly, *M. bovis* and *M. marinum* in their natural hosts are used as surrogates of *M. tuberculosis*. *M. bovis* cattle disease is of considerable economic and public health importance in its own right, and the study of *M. bovis* infection in cattle provides a natural host model of one of the closest relatives of *M. tuberculosis* (113). However, experimental infection of cattle is expensive and cumbersome and the broad host range of *M. bovis* can be exploited to study disease in smaller animals such as rabbits (42).

M. marinum, one of the closest relatives of the *M. tuberculosis*-complex organisms (165), is emerging as a model for *M. tuberculosis* pathogenesis (7, 45, 47, 67, 134, 154, 163). *M. marinum* grows optimally at 33°C and causes superficial lesions in humans called fish tank, or swimmer's granulomas, which are pathologically indistinguishable from dermal *M. tuberculosis* lesions (166). Consistent with its lower optimal growth temperature, *M. marinum* causes a tuberculosis-like granulomatous infection and disease in fish and frogs, natural hosts that are readily studied in the laboratory. Zebrafish (*Danio rerio*) are a particularly attractive model system because they are genetically tractable and have both innate and adaptive immunity, and because the embryos are transparent, which allows for real-time imaging of infection not afforded by other systems (45). Other genetically tractable model hosts that are susceptible to *M. marinum* include *Drosophila* spp. and the slime mold *Dictyostelium discoideum* (47, 154). The *Drosophila* model offers the potential to employ genetics to study the contribution of innate immunity, and the *Dictyostelium* model allows for the genetic manipulation of the host to identify host determinants of *Mycobacterium*-macrophage interactions.

M. ulcerans is the causative agent of Buruli ulcers, which are on the rise in certain areas of the tropics such as West Africa. The bacteria secrete a cytotoxic polyketide toxin that produces extensive, painless, necrotic, and noninflammatory ulcers where the bacteria grow extracellularly. *M. ulcerans* is studied using a guinea pig dermal model (71).

The relationship between *M. marinum* and *M. ulcerans* illustrates the complexity of the genomic and pathogenic relationships of this genus. These two species are closely related to each other, as judged by 16S rRNA analysis, lipid profiles (165), and sequence comparisons of housekeeping and structural genes (159). *M. ulcerans* strains are less diverse than *M. marinum* strains, which can be typed into subgroups, suggesting that *M. ulcerans* is an evolutionary derivative of *M. marinum* (159). Moreover, a new isolate has been reported recently (31) that appears to be a missing link between *M. ulcerans* and *M. marinum*, lending support to this idea. Despite their similarities, these two species have important phenotypic differences. First, *M. marinum* grows relatively rapidly with a generation time of ∼4 h, whereas the generation time of *M. ulcerans* and the *M. tuberculosis*-complex organisms is >20 h. Second, similar to other pathogenic mycobacteria, *M. marinum* can live within host macrophages (20, 134), whereas *M. ulcerans*, which produces large ulcers, is possibly the only pathogenic *Mycobacterium* species that does not have a significant intracellular existence (71). With respect to modes of transmission, it

is interesting that *M. marinum* likely has an environmental niche, being repeatedly isolated from temperate waters in swimming pools where it has caused outbreaks (96). Although *M. ulcerans* has never been cultured directly from the environmental waters that are associated with human infections, a recent study found that aquatic insects of the family *Naucoridae* can transmit *M. ulcerans* to mice via biting and that insects isolated from the wild in areas where Buruli ulcer is endemic harbor the bacteria in their salivary glands (102). Furthermore, feeding these aquatic insects with grubs infected with *M. ulcerans* but not *M. marinum* results in sustained colonization of the salivary glands. Thus, these two genetically related pathogens have evolved distinct pathogenic traits and occupy distinct environmental niches.

M. leprae

This species is the causative agent of leprosy, a highly variable disease with a spectrum of clinical manifestations that range between two polar forms (149). At one end is a paucibacillary condition known as tuberculoid type, which is associated with active cell-mediated immunity and granuloma formation. Nerve damage found in tuberculoid type leprosy is thought to be a consequence of immune-mediated killing of Schwann cells and subsequent loss of the myelin sheath responsible for protecting nerve axons. At the opposite end of the spectrum is a multibacillary condition known as lepromatous type leprosy in which a weak cell-mediated immunity response does little to abate bacterial proliferation, resulting in heavily infected and inflamed perineurium. *M. leprae* has resisted all attempts at in vitro culture and must be grown in either the nine-banded armadillo (*Dasypus novemcinctus*), a natural host, or in the mouse footpad (149). Completion of the *M. leprae* genome sequence was especially welcome for this elusive pathogen (35). Several startling features were immediately apparent. The 3.27 Mb *M. leprae* genome is severely contracted from the 4.4 Mb *M. tuberculosis* genome, having only ~1600 open reading frames, as compared to 4000 in *M. tuberculosis*. Half the genome is composed of pseudogenes, intact orthologs that are present in *M. tuberculosis* and other mycobacteria. Whole metabolic pathways have been purged. This massive gene decay is unprecedented in sequenced genomes and likely explains why the leprosy bacillus is resistant to axenic culture. Recent advances in culture techniques for Schwann cells and Schwann cell/axon cocultures have given rise to detailed investigations of *M. leprae*/Schwann cell interactions in vitro (77, 136) (see Bacterial Factors Involved in Pathogenesis, below).

M. avium Complex

The *M. avium* complex (MAC) is a group of related environmental mycobacteria, including *M. avium* subspecies *avium*, *paratuberculosis*, and *silvaticum*, and *M. intracellulare* (26, 85). MAC bacilli are extremely widespread in nature. They are common in surface waters and soils and are frequently isolated from water taps (54). Transmission is not known to occur between hosts but exposure to MAC via tap water is common, and those with MAC disease are often

immunocompromised. MAC organisms are intrinsically drug resistant, making infections difficult to eradicate. MAC bacilli show remarkable genetic plasticity. In addition to numerous insertion sequence elements scattered throughout the genomes, at least three independent morphotypic switching systems have been described (27, 86). There is recent evidence that strains infecting humans differ from those infecting chickens (26). The strains isolated most frequently from humans are *M. avium* subsp. *avium* and *M. intracellulare*. In addition, *M. avium* subsp. *paratuberculosis* is a significant pathogen of livestock (Johne's Disease) and has been implicated in Crohn's disease, a debilitating inflammation of the intestines in humans (81).

FIRST ENCOUNTERS—THE MACROPHAGE

Intracellular pathogens likely evolved from environmental bacteria that initially invaded and survived in protozoa (such as amoebae), thus using them as the training ground for their battles with host phagocytes in later evolutionary time (133). Although both pathogenic and nonpathogenic mycobacteria can enter cultured eukaryotic cells with similar facility, only the pathogenic species can survive and replicate therein (151). This requirement of replication in cultured cells for pathogenicity in vivo has been corroborated by the analysis of isogenic attenuated mutants, where mutants deficient in growth in tissue culture macrophages are attenuated in vivo (14, 23, 134). Furthermore, growth in environmental amoebae also distinguishes pathogenic from nonpathogenic species (33).

Mycobacteria and Macrophages

A central role for the macrophage in tuberculosis has been long established on the basis of serial histological studies of infections in experimental animals (41). Curiously, throughout the infection the macrophage plays contradictory roles as both the primary unit of cellular defense and the primary site of bacterial replication. They are likely the first host cells to respond to invading mycobacteria and yet aid in their subsequent dissemination. In the rabbit and mouse models of pulmonary tuberculosis, alveolar macrophages have been implicated in the transport of aerosolized bacteria from the lung to deeper tissues (41, 93, 164). Recent real-time imaging studies of live *M. marinum*-infected zebrafish embryos observed infected macrophages migrating from the bloodstream or hindbrain ventricle to deeper tissues (45). Also observed was the transfer of bacteria from one macrophage to another via membrane tethers, and uninfected macrophages migrating from afar to engulf a dead infected macrophage, thus creating new infected macrophages.

Mycobacteria and Other Host Cells

Other host cells, both immune and nonimmune, contribute to the establishment of systemic tuberculosis infection (127). *M. leprae* strains have been found in both

macrophages and Schwann cells (149) (see Bacterial Factors Involved in Pathogenesis, below). Both dendritic cells (17, 70, 76, 162) and M cells (164) have also been found to phagocytose mycobacteria, and the former likely play a significant role in the transport of mycobacteria. Similarly, neutrophils are present early at sites of tuberculous infections as well as in mature granulomas (63, 127). Experiments in mice to determine their role in curtailing *Mycobacterium* infection have yielded disparate results (125, 150). Even the study showing a significant role for neutrophils in controlling infection found no organisms within these cells, which suggests that their role may be indirect (125).

Mycobacteria and Epithelial Cells

In vitro studies using alveolar epithelial cell monolayers have implicated these cells in the translocation of bacteria across the lung epithelium both directly and by inducing macrophage migration and transcytosis (13). A recently described *M. tuberculosis* mutant also implicates epithelial cell interactions in pathogenesis (128). Bacilli disrupted in the *hbhA* gene encoding the heparin-binding haemagglutinin showed diminished adhesion to a pneumocyte (lung) cell line while preserving adherence to cultured macrophages. Mutant bacteria grew well in the lungs of BALB/c mice infected by the intranasal route but were 100-fold reduced in spleen colonization. Intravenous infection demonstrated no inherent inability of the bacteria to colonize the spleen, which suggests that *hbhA* contributes to dissemination. However, a conflicting study (111) finds that *hbhA* mutants have a stronger growth defect in lungs of C57BL/6 mice than previously reported (128). Second, initial colonization of the spleen occurs at a frequency comparable to wild-type, but the *hbhA* mutant was subsequently eliminated (111). Thus, both groups find a requirement for *hbhA* in mouse infection, and elucidation of its exact role awaits further study. These two studies highlight the fact that different results can be obtained using different pathogenesis models (in this case mouse strains), a theme that recurs in this review. Furthermore, even if HbhA mediates dissemination, it may still be due to indirect effects of HbhA-pneumocyte binding such as the production of signals that mediate macrophage recruitment, activation, or trafficking similar to those seen in the transcytosis studies described above (13).

Entry into Macrophages

In vitro assays with cultured macrophages have revealed that pathogenic mycobacteria can use a variety of receptors to gain entry (52, 131), which suggests that the bacteria have evolved a fail-safe system to reach their preferred destination within the host. However, it is also possible that different receptors are expressed at various phases of infection, so that the ability to use multiple host receptors represents a series of adaptations to complete a successful infection cycle (52). An inevitable question is whether the different routes of entry produce different consequences for the bacteria-host interaction. This is the case for the intracellular pathogens *Leishmania* and *Toxoplasma*, where entry via one receptor leads to the establishment of a replicative phagosome, whereas entry via another leads to death

of the organism (52, 133). Experiments using cultured cells have failed to reveal such differences in intracellular survival of mycobacteria entering via different receptors (52). Of course, in vitro experiments may fail to discern differences that impact the infection in vivo such as differences in cytokine production by other cells. In addition to cellular receptors, plasma membrane cholesterol appears to be essential for entry into both macrophages and neutrophils (68, 129). Although the requirement for cholesterol appears specific to *Mycobacterium* entry (68), there is controversy as to whether its role is in direct binding to the mycobacteria or allowing for appropriate receptor interactions (68, 129).

The *Mycobacterium* Phagosome

Studies of infected cultured macrophages have furnished a detailed understanding of the biology and biogenesis of the *Mycobacterium* phagosome. Experiments using *M. tuberculosis*, *M. avium*, and *M. marinum* have yielded similar results (7, 140). In brief, the phagosome communicates with early endosomes of the host endocytic machinery and acquires specific components from both cell surface plasma membrane and early endosomes, but fails to fuse to lysosomes and become acidified (140). Biochemical studies have revealed that this limited acidification is due to reduced incorporation of the vacuolar proton ATPases into the phagosome (140). In addition, there is recent evidence that the endoplasmic reticulum is recruited to nascent phagosomes containing inert particles or *Leishmania* and makes a major contribution to the phagosomal membrane (66). This surprising result explains how antigens of vacuolar pathogens might be presented to T lymphocytes via the MHC Class I processing machinery that is located in the endoplasmic reticulum membrane. It is not known if the endoplasmic reticulum participates in the formation of *Mycobacterium* phagosomes, but *Mycobacterium* antigens are processed by MHC Class I (63). Host-pathogen interactions may also be mediated by *Mycobacterium* proteins and lipids that are released from the phagosome into the cytoplasm or the endocytic network of the infected cell (9–11, 14). Bacterial lipids have been observed in neighboring uninfected cells, presumably transferred via exocytosed vesicles (9, 11).

Host Proteins Associated with the *Mycobacterium* Phagosome

Current research is focused on the generation and maintenance of the *Mycobacterium* phagosome and the molecules that regulate its trafficking (140). The quest for host proteins that specifically associate with *Mycobacterium* phagosomes has yielded two candidates: coronin 1/TACO, an actin-binding protein whose *Dictylostelium* homolog is required for phagocytosis (60, 148), and Gal3/Mac2, a protein expressed on the macrophage cell surface and associated with the Golgi and nucleus (8). However, cells from a Gal3/Mac2 mutant mouse supported *Mycobacterium* infection to the same extent as wild-type mice, and infection of Gal3/Mac2 knockout mice actually resulted in higher bacterial loads than in wild-type mice (8). Similarly, a recent study using the *M. marinum-Dictyostelium* model questions the requirement for coronin in *Mycobacterium* phagosome maintenance (154).

Similarly, although coronin was found to associate with *M. marinum*-infected phagosomes in wild-type *Dictyostelium*, bacterial replication was higher in the coronin mutant. The caveat of this study is that there are several mammalian coronin isoforms (with potentially different functions), and it is not clear if the *Dictyostelium* coronin studied corresponds to coronin 1/TACO. In any case, the coronin and Gal3/Mac2 studies highlight the importance of combining genetic and biochemical approaches to determine the role of various host components in the context of in vivo infection. Genetically tractable models, such as *Dictyostelium*, *Drosophila*, and zebrafish, should facilitate the identification of candidate host determinants that can be tested in more complex mammalian systems (45, 47, 154).

Mycobacterium Phagolysosome Fusion: In Vitro and In Vivo Studies

The demonstration of the avoidance of phagolysosome fusion by *Mycobacterium* as a bacterial survival mechanism is also based on in vitro data (140). This phenomenon is observed in all pathogenic species studied (*M. tuberculosis*, *M. avium*, and *M. marinum*) except the mouse pathogen *M. lepraemurium*, which resides predominantly in phagolysosomes during replicative infection (78). Moreover, in macrophages activated by gamma interferon, mycobacteria were found predominantly in phagolysosomes and were killed (147, 170). However, other experiments suggest that *Mycobacterium* spp. can survive in acidified compartments. When mycobacteria and *Coxiella burnetii* were coinfected into cultured macrophages, the majority of the mycobacteria colocalized with the *Coxiella* in acidified compartments, without significant loss of viability (75). Also *M. tuberculosis* infection of freshly isolated human alveolar macrophages revealed that the majority of phagosomes containing intact bacteria were in intimate contact with lysosomes (19). Furthermore, when opsonized, *M. tuberculosis* enter into macrophages via Fc-receptors, most bacteria survive and even replicate in phagolysosomes (4). It is conceivable that Fc-receptor-mediated entry operates in later phases of infection after specific antibodies have been generated (63).

There is also evidence to suggest that *Mycobacterium* spp. survive in phagolysosomes within macrophages in granulomas. Recently, transmission electron microscopy was performed over an extended time course to analyze phagosomes in granulomas of *M. marinum*–infected frogs (20). Approximately 60% of the intact bacteria resided in phagolysosomes, and the level of phagolysosomal fusion correlated with the level of macrophage activation. It is possible that the bacteria within phagolysosomes are on an inexorable pathway to death, but this seems unlikely, as the overwhelming majority of bacteria appear intact. It seems more likely that the bacteria have adapted to be able to live within a phagolysosome. Consistent with this hypothesis, several *M. marinum* genes are specifically activated, not by residence in single macrophages, but only upon aggregation of the infected macrophages (30, 45, 134). In any case these data suggesting that survival in a phagolysosome is possible in granulomas provide an in vivo correlation for the studies of Fc-mediated entry (4). Therefore it is possible that mycobacteria have at least two sets of adaptive mechanisms: restriction of phagolysosomal fusion

early in infection and adaptation to phagolysosomal fusion within the activated macrophages of granulomas later in infection. In this context it is important to note that the intracellular pathogens *Leishmania*, *Cryptococcus*, and *Listeria* can occupy distinct compartments in infected cells under different situations in vivo and in vitro (16, 57, 160).

GRANULOMA FORMATION: HOST VERSUS PATHOGEN

Features of *Mycobacterium* Granulomas

Once the bacteria are transported into the deeper tissues by macrophages and perhaps other phagocytic cells, additional macrophages gather at individual infected foci to form granulomas. The pathological definition of a granuloma is an organized collection of differentiated macrophages with a characteristic morphology (1). Granulomas are formed in response to persistent intracellular pathogens, such as *Mycobacterium*, *Schistosoma*, and *Brucella*, or to foreign bodies. Although only the presence of macrophages is required for the pathologists' definition of granulomas, tuberculous granulomas in humans and mice have a large complement of T lymphocytes, some B lymphocytes, dendritic cells, neutrophils, fibroblasts, and extracellular matrix components (63, 127). Although the role of all the accessory cells in the granuloma has not yet been clarified, certain T lymphocyte subsets play an unequivocal role in the maintenance of the granuloma and restriction of the bacteria both in human infection as well as in mouse models of *M. tuberculosis* (63). Virtually all pathogenic mycobacteria (with the exception of *M. ulcerans*) produce granulomas in their vertebrate hosts, and here we discuss similarities and differences in their structure and composition in selected host-pathogen combinations and their impact on infection outcome.

Granulomas likely begin as aggregates of mononuclear phagocytes that surround individual infected macrophages (1, 45). These macrophages become activated, a transformation reflected by an increase in their size and subcellular organelles, ruffled cell membranes, and enhanced phagocytic and microbicidal capabilities (1, 20, 41). A common feature of all *Mycobacterium* granulomas is the further differentiation of the macrophages into epithelioid cells that have tightly interdigitated cell membranes in zipper-like arrays linking adjacent cells (1, 20). In many cases, several macrophages fuse to form giant cells, which are also seen with other persistent infections, particularly with viruses. In a mouse model of *M. tuberculosis* infection, T lymphocytes and other immune cells are recruited early during the process of granuloma formation (139). However, the use of a zebrafish embryo model of *M. marinum* infection has revealed that lymphocytes are not required for the initial aggregation of macrophages and their transformation into epithelioid cells (45).

Caseous Necrosis

Another striking feature of certain tuberculous granulomas is the presence of areas of caseous necrosis, regions of acellular debris that have a distinct pathological

appearance (1, 41). The traditional view of a human granuloma is that of a central core of necrosis surrounded by epithelioid cells with a peripheral cuff of lymphocytes (41, 127). Mycobacteria are located within macrophages of granulomas and in larger numbers within the central caseous region, when present (20, 41, 163). Human *M. tuberculosis* granulomas can also be fibrotic and calcified, and such lesions only occasionally contain live bacteria (see Bacterial Persistence, below), which suggests that the presence of these components may be related to healing or healed lesions.

In its natural host (humans), *M. tuberculosis* produces highly complex granulomas that can have all the features described above at various points in their evolution. Interestingly, even within a single infected host, granulomas (presumably arising from the same initial infection event) can be at different stages of evolution (43). Whereas epithelioid cells are a feature of all tuberculous granulomas, the presence of caseous necrosis, presumably formed by the lysis of infected cells, is highly host species–specific (20, 41, 63). Human granulomas can caseate and indeed this is necessary for transmission, as it is the rupture of the liquefied caseum loaded with bacteria that allows for person-to-person transmission of pulmonary tuberculosis (43). The actual mechanism of caseation remains unclear. Mice experimentally infected with *M. tuberculosis* form granulomas that do not caseate, whereas rabbits and guinea pigs do (42, 107, 121). Similarly, *M. marinum* produces noncaseating granulomas in the leopard frog (*Rana pipiens*) (20), but it produces both caseating and noncaseating granulomas in certain toads, goldfish, zebrafish, and humans [(20, 163, 166); D. Beery & L. Ramakrishnan, unpublished results)]. In some instances, both caseating and noncaseating granulomas can be found within the same fish (D. Beery & L. Ramakrishnan, unpublished results). In the case of *M. marinum*, where it has been possible to follow infection long term in natural host species, there appears to be no appreciable difference in infection outcomes in frogs and fish, which do and do not caseate, respectively (135, 163). Bacteria in the caseum can be visualized by the standard modified Ziehl-Neelsen staining techniques; however it is not clear how many are alive. In the context of human pulmonary cavitary tuberculosis, at least some of the bacteria in the caseum must be viable, as they are transmissible. This issue of viability has been addressed recently with molecular methods. Granulomas from resected lung of humans with caseous tuberculosis have been subjected to in situ hybridization for various mycobacterial targets (58, 59). *M. tuberculosis* DNA was found throughout the lesions, and RNA, a much better indicator of live bacteria, was only found associated with macrophages and giant cells in non-necrotic zones.

Lymphocytes in *Mycobacterium* Granulomas

Another host-specific difference in granuloma composition is the relative number of lymphocytes present. All mammalian species studied recruit large numbers of lymphocytes to *M. tuberculosis* granulomas, which are critical for the maintenance of the architecture and for the restriction of the infection (42, 43, 63, 107, 121).

M. marinum human granulomas also contain a high density of lymphocytes [(166); L. Ramakrishnan, unpublished results], whereas frog and fish granulomas do not despite being largely similar otherwise [(20, 163); D. Beery & L. Ramakrishnan, unpublished result]. Only occasional lymphocytes are found in the majority of frog and fish lesions, with few lesions having the cuff of lymphocytes common to mammalian tuberculous granulomas [(20); D. Beery & L. Ramakrishnan, unpublished results]. Yet lymphocytes also play an important role in amphibian tuberculosis, because frogs treated with hydrocortisone resulting in depletion of blood lymphocyte counts are highly susceptible to *M. marinum* infection. These frogs exhibit poorly formed multibacillary macrophage aggregates and die (135). Because frogs and fish appear to be able to contain mycobacterioses as well as mammals do, with the recruitment of fewer lymphocytes, it is possible that the greater number recruited into mammalian lesions represents an excessive and unnecessary immune response. Alternatively, it is possible that the immune systems of ectotherms have evolved to control infection with fewer lymphocytes called into action.

Influence of Specific *Mycobacterium* Virulence Determinants on Granuloma Composition

Finally, it is important to ask whether the granuloma benefits the host, the bacterium, or both. The traditional view is that the host uses the granuloma to wall off and prevent spread of the bacteria via the interdigitation of cells, formation of an extracellular matrix, fibrosis, and calcification (1, 41, 63). Individuals unable to form granulomas succumb to disseminated tuberculosis. However, it has also long been believed that the host granulomatous response may actually mediate the pathology and consequent morbidity associated with tuberculosis (41, 63). There is new evidence that specific *Mycobacterium* determinants influence the type of host response. Some attenuated mutants influence the cellular composition of lesions. For instance, an *M. tuberculosis* mutant with altered mycolic acid composition was found to result in lymphocytic rather than macrophage aggregates in a mouse model (73). Similarly, infection with an attenuated PE-PGRS *M. marinum* mutant resulted in many lesions consisting predominantly of loose aggregates of immature macrophages rather than mature interdigitated macrophages (20). Perhaps the most compelling evidence for the impact of bacterial determinants on the pathology of the granuloma comes from the analyses of the *M. tuberculosis* WhiB3 and SigH mutants (see Bacterial Factors Involved in Pathogenesis, below).

BACTERIAL PERSISTENCE

As typified by *M. tuberculosis*, *M. leprae*, *M. marinum*, and *M. avium*, the pathogenic mycobacteria are all capable of establishing long-term infections. Although *M. tuberculosis* has received the most attention, it is anticipated that all these organisms employ similar strategies to persist. Epidemiological evidence suggests that

exposure of humans to *M. tuberculosis* via the aerosol route can lead to several potential outcomes. These include clearing the infection early (presumably by mechanisms of innate immunity without leaving an immunological or radiological fingerprint), developing disease soon after infection (referred to as primary or postprimary tuberculosis), or developing a subclinical or asymptomatic infection (referred to as latent tuberculosis) with the potential to transition to active disease subsequently (69, 124). The diagnosis of latent tuberculosis is based on the presence of a T lymphocyte–mediated immune reaction to a dermal injection of processed proteins from *M. tuberculosis* culture supernatants called purified protein derivative (PPD) (69). A long-term study of rates of PPD conversion and subsequent development of progressive pulmonary tuberculosis was conducted in Norway from 1937 to 1944 (69). Although over 50% of PPD-positive individuals had radiological evidence of tuberculosis lung lesions, usually seen soon after PPD conversion, only 11% developed progressive pulmonary tuberculosis in the seven-year observation period. Importantly, the risk of developing progressive disease was overwhelmingly greater in the first few months after PPD conversion, with a cumulative rate of ∼10% for the first year diminishing to ∼1% in years three to seven. The World Health Organization estimates that 1.86 billion people (32% of the world's population) are infected with *M. tuberculosis*, with 16.2 million cases of active disease while the rest are asymptomatic but PPD positive (50). Therefore, the question of how *M. tuberculosis* persists, and for how long, is central not only for fundamental biological interest but also to shape policies and programs for tuberculosis treatment and control.

Latent Tuberculosis, Reactivation, and Reinfection

Latent tuberculosis has been the subject of several recent reviews (64, 100, 124), and we highlight new information and revisit certain old studies, the interpretations of which may merit reconsideration. As described above, some PPD-positive individuals can develop active disease after an asymptomatic interlude (69). In a study that combined epidemiological evidence with molecular fingerprinting data, reactivation of tuberculosis in an individual after 33 years of latent infection was convincingly demonstrated (95). Also, administration of isoniazid dramatically reduces the risk of active tuberculosis in PPD-positive individuals (37). However, studies suggest that latent tuberculosis is not necessarily a lifetime sentence, even in the absence of antibiotic therapy. In persons who died of causes other than tuberculosis, the majority of visible tuberculous lung lesions were sterile, as determined by an exquisitely sensitive assay (guinea pig inoculation) (55, 119). These findings suggest that at least some people can eliminate their infection and may account for the decreased risk of PPD-positive persons developing active disease over time (69).

Further complicating the issue is a new appreciation for the contribution of reinfection (24, 55, 155, 169) to the global burden of tuberculosis. Molecular fingerprinting techniques have documented reinfection with new strains, even in

immunocompetent individuals from both high and moderate incidence areas, which demonstrates that immunity to tuberculosis can be incomplete. These considerations argue that reactivation may not contribute to global disease burden as much as previously thought. Reactivation likely accounts for the great majority of active cases in areas of low tuberculosis transmission (91, 153). However, in areas where tuberculosis is prevalent, primary infection is a prominent factor and reinfection probably plays a greater role than was previously appreciated, especially among HIV-positive persons (24, 155, 169). Teasing out the relative contribution of reactivation and reinfection and the factors contributing to reactivation will provide a firmer scientific footing for efforts aimed at global tuberculosis control. For instance, it will determine whether drug treatment of PPD-positive individuals will play a role in diminishing transmission.

Location of Bacteria During Latency

Certain studies, both old and new, suggest that bacteria are present in apparently normal tissues outside of visible lesions. Two older studies used guinea pig inoculation to assess the presence of viable bacteria in apparently normal lung tissue samples from individuals who had tuberculous lesions but had died of other causes (56, 119). One study reported 45% of the normal tissues to yield viable bacteria (119), and the other found viable bacteria in less than 5% (56). The presence of bacteria in apparently normal lung has recently been re-addressed with molecular methods. An in situ PCR assay was used to detect *M. tuberculosis* DNA in apparently normal lung tissue from persons of unknown PPD status who died of causes other than tuberculosis (82). Tissues from 15 of 47 individuals from tuberculosis-endemic areas were positive for *M. tuberculosis* DNA, whereas all 6 samples from a low-prevalence area were negative. However, the presence of bacterial DNA does not speak to the presence of viable bacteria. Therefore, it is critical (albeit technically challenging) to extend these studies to estimate the viable bacterial burden in these superficially normal regions. Furthermore, in contrast to the two older studies (56, 119), the patients in this study were reported to have no evidence of tuberculous lesions anywhere in their lungs (82). The seminal Norway study on reactivation rates noted that progressive disease occurred only in those individuals who had first had a demonstrable primary lung lesion (69), calling the clinical relevance of the bacterial DNA detected in this recent study into question. In summary, while still leaving room for argument, these data together support a model in which most granulomas in the latently infected host become sterile. Viable bacteria may reside in apparently normal tissues but this also remains controversial.

State of the Bacteria During Latency

There is an ongoing debate about whether clinical latency results from bacteria achieving a metabolically dampened, nonreplicative state, or whether the low, constant bacterial numbers result from a dynamic equilibrium between bacterial replication and elimination by the host (124). The idea that latent tuberculosis

requires physiologically dormant bacilli is pervasive (74, 105, 173) and stems from the observations that clinical latency in humans involves few bacilli, apparently minimal pathology, and no disease symptoms. However, this proposal should be viewed cautiously given the lack of relevant data. The idea that the bacteria can enter a dormant state during human infection was strengthened by studies where diseased tissues staining positive for acid-fast bacteria did not yield bacteria on culture or the bacteria grew very slowly (100, 105, 124). However, it is important to reinterpret these studies in light of the fact that these tissues came from individuals with active tuberculosis, most of whom had received antituberculous antibiotic therapy immediately prior to lung resection, possibly rendering the bacteria more difficult to culture.

Another argument that has been used in support of a physiologically dormant bacterial state in vivo is the finding that *M. tuberculosis* is killed by antibiotics in vitro within a few days but it takes months to cure tuberculosis despite evidence of adequate antibiotic penetration to the infection site (105, 109). However, many infections take longer to cure than the time required to kill the causative agent in vitro, exemplified by the case of *E. coli* kidney infections. If one considers the relative in vitro generation times of *M. tuberculosis* (20 h) versus that for *E. coli* (20 min), it is hard to attribute the long time required to cure *M. tuberculosis* infection to a unique property of these bacteria in vivo. The very fact that isoniazid, an antibiotic that targets the *M. tuberculosis* cell wall, dramatically reduces the rate of reactivation tuberculosis in PPD-positive individuals (37) suggests that the bacteria must have some replicative activity during latency. Indeed, metabolically active but nondividing *E. coli* cells are resistant to penicillin, a cell wall antibiotic, whereas actively dividing cells are susceptible, providing the basis for penicillin selection used for a multitude of genetic screens.

Animal studies also provide results whose interpretations are complex. One experiment that is cited (74) to support the idea that the bacteria are not replicating during latency was performed in a mouse model by Rees & Hart (137). Viable bacterial counts and the numbers of acid-fast bacilli were compared during a several-month-long infection period. The number of bacteria enumerated by both techniques remained constant, and the authors argued that the bacilli must be in a nonreplicating state because the coexistence of bacterial death and replication would have resulted in an increasing number of microscopic bacterial counts over time owing to the accumulation of dead bacilli. Although these experiments were well executed, it is possible that the rate of bacterial death in the absence of antibiotic treatment is slow enough that the discrepancy between visualized and viable bacteria is not within the limits of detection. Indeed, several recent studies have argued for some level of bacterial metabolism during latency. *M. tuberculosis* mRNA in lungs of mice was detected in a drug-induced latency model (84, 122). Similarly, a large number of *M. marinum* genes involved in a variety of active cellular processes are highly expressed in seventeen-month-old granulomas of chronically infected frogs, even while the viable bacterial counts remain constant (30). Of course, bacterial gene expression is not necessarily proof of replication,

and global downregulation of bacterial gene expression within granulomas might have gone undetected because this study used the relatively stable reporter GFP. Furthermore, in a latent infection the bacteria need not all be in the same metabolic state, and the *M. marinum* frog study was done on bacterial populations from granulomas. Therefore it is possible that a subset of these bacteria were in an inactive state. In summary, the question of whether bacteria replicate during clinical latency remains unanswered.

Animal Models

In an effort to provide structure to this field, different animal models of tuberculosis latency (120, 137) were recently categorized as either "Cornell/drug-induced" or "low-dose/chronic" (145). In the Cornell model (104), mice are infected with *M. tuberculosis* and then drug treated (traditionally with isoniazid and pyrazinamide) for an extended period. Although no viable mycobacteria are detected immediately following treatment, disease reactivates in a fraction of the animals within several months (104, 145). This system models the state in humans in that the load of bacilli in infected organs is often undetectable. However, unlike in humans, drug intervention is needed to induce the latent state. Also, Cornell model experiments require several critical choices: route and dose of infection, type and timing of drug treatment, timing of recovery phase, and type of immunosuppressive drug used for recovery. Currently, no standard protocol exists and results depend materially on the parameters used to establish the latent infection (145). The low-dose (or chronic) model of latent tuberculosis grew from observations of mice inoculated with small numbers of *M. tuberculosis* (120). After an initial growth phase the bacillary burden in the lungs remains steady and the mice appear healthy until the disease reactivates as much as 18 months later. As with the Cornell model, immunosuppression (65) or innate immunodeficiency (144) leads to faster, more comprehensive reactivation. This chronic model resembles latency in humans in that it depends solely on the host immune response to contain the infection. However, unlike latent tuberculosis in humans this model results in a high bacillary burden. In addition, histological examination of chronically infected mice showed that lung damage accumulates steadily throughout the chronic period (138), and thus the disease is not really latent.

Recent research offers hope that animal models can be improved. First, nonhuman primates currently being used to study other aspects of human tuberculosis [(39, 108, 172); J. Flynn, personal communication] may also provide a faithful model of latent tuberculosis in humans. Second, the C57BL/6 mouse that is commonly used in latency studies may be a poor choice, in part because the bacterial burden in the chronic state is too high. Recent studies with C57BL/6 × DBA/2 F1 hybrid (5, 130) suggest that low-dose inoculation of these mice results in a lower bacterial burden coincident with a subclinical infection that activates to fulminant disease upon immunosuppression. It will be important to determine the proportion of the mice that eventually succumb to infection and to document whether lung

pathology accumulates during the apparently latent phase, as was shown for the low-dose infected C57BL/6 mouse (138).

In Vitro Models

On the basis of the pervasive but unproven idea that bacteria during clinical latency are in a nonreplicating, metabolically inactive state, several researchers have attempted to produce growth-arrested bacilli using culture systems that manipulate temperature (46), pH (80), or nutrient starvation (15). Other factors potentially associated with the establishment and maintenance of latent tuberculosis include host-generated nitric oxide (NO) (115) and hypoxia (173). Of these, the role of reduced oxygen tension has received the most attention to date because tuberculosis infections are preferentially associated with the most-oxygen-rich sites within the body (2) and lesions in communication with open airways generally have a larger number of bacilli (25). Reactivation disease occurs most frequently in the upper lobes of the lung, thought to be the single most oxygenated regions of the body (2). Although replication of *M. tuberculosis* requires oxygen, the bacteria can survive for years without oxygen in vitro (25, 38). These findings have been interpreted to suggest that latent *M. tuberculosis* may exist in an oxygen-limited environment in vivo. However, it is important to note that direct measurements of granuloma oxygen tension have not been made.

On the basis of these observations, Wayne and others have explored the use of hypoxic culture conditions (referred to as the Wayne model) to generate nonreplicating persistent bacilli in vitro and to identify *M. tuberculosis* genes/proteins potentially important for development or maintenance of the latent state (173). As expected in the Wayne model, nonreplicating bacteria were found to downregulate many metabolic enzymes, but the activities of isocitrate lyase and glycine dehydrogenase were induced, which suggests an induction of the glyoxylate shunt pathway (173). Three subsequent studies have some bearing on the Wayne model. First, a mutation in the isocitrate lyase gene *icl* renders the bacteria attenuated in a mouse model but only at later (>2 weeks) times, which suggests that the glyoxylate shunt may indeed be activated at this stage (106). However, the mutant was not attenuated under hypoxic conditions in vitro. Another study showed that the *M. marinum* gene *ald*, which encodes glycine/alanine dehydrogenase, was induced in granulomas (30, 45) but was not induced by short-term hypoxia (30).

A recent approach combined hypoxic culture conditions with microarray readout to define the *M. tuberculosis* genes that respond rapidly to decreased oxygen tension (152). Expression of ~100 genes was altered significantly, although none of these corresponded to those identified in the Wayne model. Predicted functions for many of the 60 repressed genes indicate that low oxygen tension is associated with broad adaptation to reduced metabolic activity. In comparison, about two thirds of the 47 induced genes are of unknown function, which suggests that the adaptation to hypoxia is not yet well characterized. Several induced genes have postulated functions that could help promote survival in vivo. Among the hypoxia-induced loci is the predicted two-component response regulator Rv3133c, also called *devR*

and *dosR* (dormancy survival regulator) (18, 44, 152). Computer analysis identified a consensus DosR-binding motif, a variant of which is located upstream of nearly all *M. tuberculosis* genes rapidly induced by hypoxia, and DosR binding to the *acr* promoter has been demonstrated (123). Furthermore, *dosR* mutant bacteria fail to induce the hypoxic response genes (123, 152) and are significantly attenuated for survival in the Wayne model of hypoxic dormancy (18). Thus DosR appears to play a critical role in mediating expression of a series of hypoxia-induced genes, which have been termed the DosR regulon.

It is now clear that significant overlap exists between the *M. tuberculosis* responses to hypoxia and NO. NO is one of few host products known to be directly toxic to mycobacteria at clinically relevant concentrations (114), but at sublethal concentrations NO reversibly inhibits aerobic respiration and has a distinct role as a signaling molecule (103, 175). Recent work shows that, similar to hypoxia, low levels of NO reversibly inhibit growth of *M. tuberculosis* in vitro and that NO and hypoxia are additive in their inhibitory effects (M. Voskuil, D. Sherman & G. Schoolnik, unpublished data). Further, concentrations of NO that impede *M. tuberculosis* growth were shown to induce the DosR regulon in a DosR-dependent fashion. Similarly, a study of *S. typhi* genes induced by growth in cultured macrophages found that 70% were also induced by hypoxia, again suggesting a strong overlap between hypoxia- and NO-induced genes (40). In summary, although hypoxia has long been assumed to affect mycobacteria in vivo, it is entirely possible that NO, alone or in conjunction with hypoxia, is responsible for induction of the so-called hypoxia response genes. Indeed, whether bacterial respiration is inhibited by NO, by a lack of oxygen, or by a combination may be indistinguishable by the organism.

Several observations suggest that the DosR response has a role in vivo. The NOS2 locus encoding inducible NO synthase is expressed in *M. tuberculosis*–infected mouse (98, 146) and human lungs (32), and interfering with host NO production either by specific inhibitors (29, 51) or by gene disruption (98) greatly exacerbates tuberculosis infection. Genes of the DosR regulon are powerfully expressed when *M. tuberculosis* infects mice [(152a); M. Voskuil, D. Sherman & G. Schoolnik, unpublished data] or humans (58, 92). It will be interesting to test *dosR*-deleted *M. tuberculosis* strains in animal infection models. Because these instances of NO production and *M. tuberculosis* gene expression occurred in the context of active tuberculosis disease, the relevance of these data to latent tuberculosis is not clear. Like the other hypotheses based on in vitro models, the link between hypoxia, NO, DosR, and latency must be substantiated by an animal model that recapitulates human infection.

BACTERIAL FACTORS INVOLVED IN PATHOGENESIS

In recent years studies of mycobacterial pathogenesis have taken a tremendous step forward. A combination of new technologies, as well as old ones newly applied to mycobacteria, has made it possible to understand the biology of these enigmatic

organisms in greater detail. Although many genes required for growth in vitro and in vivo (e.g., genes involved in basic cellular processes) are potential antibiotic targets, they provide little information about the host-pathogen relationship. To find genes of selective importance in vivo, two kinds of genetic approaches have been used to identify genes important for virulence: expression screens and mutant screens.

Expression Screens

Genes expressed solely in the context of the host can be informative, as they provide clues concerning the conditions bacteria face in the presence of host immunity, and also the mechanisms utilized by the pathogen to circumvent host immune responses. Similarly, genes expressed in vitro that are repressed in vivo (for example, *Salmonella phoP*-repressed genes) highlight activities that, if expressed, subject the organism to eradication by the host (53).

Studies in cultured macrophages can approximate many of the host-pathogen interactions occurring in vivo, and genes expressed selectively therein have been reviewed (49, 168). However, granulomas contain a large number of other cell types whose influence on macrophage and granuloma physiology is complex. On the basis of the prediction that mycobacteria might express specific genes to aid their survival within granulomas, a GFP-reporter-based differential fluorescence induction screen was performed to identify *M. marinum* genes expressed in frog granulomas but not in bacteriological medium (30, 134). This screen identified two classes of genes: *mags* (macrophage activated genes), induced in granulomas and cultured macrophages, and *gags* (granuloma activated genes), induced solely in granulomas. Both classes encode proteins with a wide range of metabolic activities. The *gag*s fail to be activated in vitro under a variety of conditions predicted to mimic the granuloma (30); however, several are activated specifically upon aggregation of infected macrophages in the zebrafish embryo infection model. These findings validate the assumption that the complex physiological environment of the granuloma uniquely influences intracellular bacterial gene expression in ways that are not recapitulated in vitro.

Virulence Screens

In vivo expression screens yield important information about the environment, the genes isolated need not play a discernible role in virulence, particularly if the bacterium has evolved multiple strategies to aid its survival. Therefore, there has been a parallel effort to directly identify virulence genes by signature-tagged mutagenesis (3, 74, 168). More recently, Sassetti et al. (142) have combined a *mariner*-based transposon with microarray technology to determine the entire set of genes required for growth under a given set of conditions (TraSH, for transposon site hybridization). Preliminary results applying this method to a mouse infection model suggest that different genes are required at different times during infection, as the number of attenuating mutants increases with longer infection periods (143).

Virulence Determinants

The identification of genes involved in virulence is desirable for a better understanding of disease mechanisms and for the identification of potential targets for therapeutics. The very definition of a virulence factor can be controversial. In addition, the identification of a virulence defect depends on the specific assay used. From the pathogen's point of view, virulence may be measured by the number of organisms required to establish an infection, rate of bacterial growth, dissemination, steady-state bacterial load, long-term persistence, and even transmissibility. From the host's point of view, virulence may be measured by susceptibility to infection, tissue pathology, severity of disease symptoms, number of susceptible individuals in a population, rate of disease progression, and frequency of mortality. In reality, all these factors must be taken into account for a comprehensive assessment of the contribution of a particular virulence determinant. Thus far, *Mycobacterium* mutant analyses have yielded virulence determinants falling into at least four broad categories, and others will likely emerge. Some mutants are compromised in their ability to replicate from the onset of infection (e.g., *erp*, *phoP*, and PE/PGRS); others are compromised only later in infection (e.g., *pcaA* and *icl*). Recently, mutants defective for dissemination (e.g., *hbhA*; see First Encounters—The Macrophage, above) and mutants capable of normal replication that confer altered disease pathology have been identified (e.g., *whiB3*, *sigH*, and *sigC*). Other convenient ways to classify *Mycobacterium* mutants are based on their characteristics (proteins or lipids) and on their function if known (secreted protein, cell surface protein, enzyme or regulatory or transcription factors, to name only a few categories). A recent review has described many *Mycobacterium* mutants (74), and we focus on new mutants and describe new findings about previously described mutants.

Regulatory Mutants

Three newly described regulatory mutants alter disease pathology without affecting colony numbers.

WhiB3 Previously, Collins et al. (36) identified a missense mutation in the principal sigma factor, SigA (RpoV), of *M. bovis* that had no effect on growth in vitro but resulted in attenuation. These authors speculated that the attenuation was due to failure of the mutant SigA to promote virulence gene expression. Recently, Steyn et al. (158) used a yeast two-hybrid approach to identify WhiB3, a transcription factor that interacts with wild-type but not mutant SigA. *whiB3* mutations were generated in *M. tuberculosis* H37Rv and in a virulent *M. bovis* strain. Whereas both mutants grew well in vitro, they had notable defects in vivo. H37Rv *whiB3* mutants achieved normal bacterial loads in mice, but showed decreased lung pathology and corresponding slower time to death than wild-type strains. In a guinea pig model, H37Rv *whiB3* infection was comparable to that of the parent strain, whereas *M. bovis whiB3* was unable to grow, with a bacterial load 10^5 lower than the

wild-type. Interestingly, *M. marinum whiB3* is selectively expressed in vivo in frogs and in zebrafish embryos (30, 45).

SigH AND SigC *M. tuberculosis* SigH and SigC are related to the ECF (extracytoplasmic function) family of sigma factors. SigH mediates transcription of a variety of stress-response genes involved in heat shock, protein degradation, and redox cycling (89). Similar to the *whiB3* mutant, the *M. tuberculosis sigH* mutant grows to the same extent (in terms of colony-forming units) in lung and spleen as the parent strain but has greatly reduced lung pathology and 10-fold-reduced T-cell recruitment. Because C57BL/6 mice are more resistant than other mouse strains to *M. tuberculosis*, no difference in virulence of wild-type versus *sigH* mutant bacteria was observed during the 15-month assay period. However, genetically susceptible C3H mice died when they were infected with wild-type (median time to death of 52 days) but not *sigH* mutant bacteria (all mice still surviving at 171 days), indicating a clear difference in virulence. In most models of bacterial pathogenesis, heat-shock regulated factors such as chaperones, proteases, and redox enzymes are of fundamental importance for surviving host immunity. However, this study suggests that induction of these proteins is dispensable for mycobacterial survival in vivo and actually stimulates a more aggressive immune response. The authors reason that the modest cellular responses stimulated by the *sigH* mutant are sufficient to contain the infection and that the greater recruitment of lymphocytes in the wild-type infection may actually be detrimental, resulting in more significant pathology. More recently, a *M. tuberculosis sigC* mutant has been found to have a similar dissociation between in vivo growth and pathology (W.R. Bishai, personal communication).

The WhiB3, SigH, and SigC mutants raise several interesting points regarding the study of virulence in mycobacteria. First, they define a new class of virulence phenotype—reduced pathology/disease outcome with normal bacterial growth. The host immune system responds to more than the absolute number of organisms in the tissue, and likewise, a more robust T-cell response does not necessarily result in greater inhibition of bacterial growth. Whether the exaggerated pathology induced by wild-type bacteria is important for completion of the infection cycle (e.g., transmission) remains to be determined. Second, these approaches demonstrate the usefulness of multiple assays and animal models for virulence. By examining multiple host-pathogen pairs, phenotypes were found in one assay but not in another. These discrepancies underscore the importance of careful evaluation and provide the opportunity to learn from the differences. For example, *M. tuberculosis* H37Rv and *M. bovis* ATCC35723 have greatly varied needs for the *whiB3* gene. One possibility is genetic redundancy of *whiB3* function in *M. tuberculosis*. Similarly, C3H mice are known to be more susceptible to a variety of pathogens than C57BL/6 mice are, and the reasons for this difference are multifactorial (90). The failure of the *sigH* mutant to thrive in this background may provide insight into the mechanisms of C3H susceptibility.

Mycobacterial Cell Envelope and Virulence

Lipids play an important role in mycobacterial pathogenesis (74). Their role in growth and persistence is not surprising, as the importance of the lipid-rich cell envelope of mycobacteria has long been suspected and is supported by studies that show immunomodulatory activities of purified mycobacterial surface components on host cells and in vivo. There are both similarities and differences in the composition and arrangement of lipids in the various pathogenic mycobacteria. However, the role played by lipids in virulence has now been shown for *M. tuberculosis*, *M. ulcerans*, and *M. leprae*, which are all distinct from each other in pathogenesis and pathology. Several studies have shown that expression and regulation of *M. tuberculosis* lipids such as mycolic acids and phthiocerol dimycocerosate (PDIMs) are required for in vivo growth and persistence (74).

PhoP AS A *MYCOBACTERIUM* CELL WALL REGULATOR *M. tuberculosis* encodes nine two-component signaling systems, several of which have been postulated to play a role in virulence (126, 174). One in particular, the PhoP/Q system, has been shown to regulate virulence determinants in *Salmonella* spp. and other gram-negative organisms (53). As a transcriptional regulator, PhoP controls expression of genes involved in surface remodeling and adaptation to intracellular growth. Accordingly, both *phoP* null and constitutive mutants of *Salmonella* exhibit reduced viability in mice (53). On the basis of its central regulatory role in other systems, Perez et al. (126) generated an *M. tuberculosis phoP* mutant. This mutant was defective for growth in mouse macrophages and in mice. Recently, the *M. tuberculosis phoP* mutant was also shown to have a higher proportion of monoacylated mannose-capped lipoarabinomannan (ManLAM) and less multiacylated ManLAM than the wild-type parent strain. (97). This finding is interesting in light of reports that monoacylated ManLAM is less able to mediate *M. tuberculosis* immunosuppressive functions than the di- and triacylated forms (118). In gram-negative bacteria such as *Salmonella*, PhoP signaling has been shown to increase both the number and the length of lipid A fatty-acyl modifications, thus modifying the immunomodulatory activity of this molecule (53). Thus, it seems plausible that *M. tuberculosis* regulates the immunomodulatory activity of its ManLAM in an analogous manner through *phoP*-mediated acyl modifications.

PGL-1 A PDIM-derived phenolic glycolipid (PGL-1) produced by *M. leprae* has been credited with a number of immunomodulatory activities (116). Recently, PGL-1 was shown to mediate *M. leprae* binding to peripheral nerve laminin-2 of the Schwann cell basal lamina, and uptake by Schwann cells in a laminin-2-dependent manner, by a Schwann cell-axon coculture assay (116). These findings provide an explanation for *M. leprae* nerve predilection and further demonstrate that PGL-1 alone is sufficient for invasion of Schwann cells. More recently, PGL-1-mediated uptake of *M. leprae* was localized to nonmyelinated Schwann cells, whereas myelinated cells appeared to resist invasion (136). However PGL-1

itself could induce Schwann cell demyelination, thus promoting further *M. leprae* uptake and proliferation (136). However, another study (77) reported that *M. leprae* viability is enhanced at 33°C, thus allowing long-term (30 day) infection of Schwann cells and Schwann cell/axon cocultures. Under these conditions, which may better recapitulate conditions in vivo, *M. leprae* infected both myelinated and nonmyelinated Schwann cells, and no active demyelination was observed (77).

MYCOLACTONE Added to the list of lipid-derived molecules involved in mycobacterial virulence is the mycolactone of *M. ulcerans*, which has been characterized as a polyketide-derived macrolide synthesized by two separate polyketide synthases [(22, 71); P.L.C. Small, personal communication]. As seen for PGL-1 of *M. leprae*, the *M. ulcerans* mycolactone reproduces to a significant degree the pathology of the Buruli ulcer: noninflammatory, necrotic lesions that can spread throughout the dermis and into underlying adipose and muscle tissue. In a guinea pig model, mycolactone-induced cell death occurs by an apoptotic mechanism (72). This observation helps to explain the noninflammatory nature of the lesions, although polyketides also have both immunosuppressive and antibiotic properties. In contrast to *M. tuberculosis*, which is generally considered an intracellular pathogen, *M. ulcerans* grows extracellularly in the environment produced by its mycolactone. However, *M. tuberculosis* may also undergo a phase of extracellular growth in the liquefied caseum of granulomas (43), raising the possibility that *M. tuberculosis* employs a strategy similar to that of *M. ulcerans*, albeit at a different stage in the infection. The genome sequence of *M. tuberculosis* reveals a number of genes possibly involved in polyketide synthesis. Studies in liposarcoma cells suggest that although mycolactone is responsible for *M. ulcerans*–mediated cellular necrosis, additional secreted proteinaceous factors may contribute to host cell death via apoptosis (48). In summary, it seems likely that all mycobacteria use lipids, and lipid-linked macromolecules, as a barrier not only to host defense mechanisms but also to mediate their unique virulence traits.

Surface and Secreted Proteins

Several surface-localized and exported molecules play a role in virulence including Erp (14), HbhA (described above), and members of the PE-PGRS and PPE families.

PE/PE-PGRS AND PPE FAMILIES An intriguing feature of many *Mycobacterium* genomes is the presence of two highly unusual protein families, PE and PPE, which together constitute 10% of the *M. tuberculosis* H37Rv genome (34). The PE family is defined by the presence of a conserved 110-amino-acid N-terminal domain. Several members have an additional PGRS (polymorphic GC-rich repetitive sequence) domain of variable length, and others also have a unique C terminus (PE-PGRS-U). Finally, some members lack the PGRS and simply have a unique domain linked to the N-terminal PE. Less is known about the PPE family, which despite its similar name bears no homology to the PE family but has an analogous pattern of conserved and variable sequences. PE family members have been found

in all the sequenced *Mycobacterium* genomes, although their number is highly variable. *M. tuberculosis*, *M. bovis* BCG, and *M. marinum* have ~80–140 PE/PE-PGRS genes, whereas *M. avium*, *M. leprae*, and *M. smegmatis* have fewer than 10 genes (34, 35, 62) (http://www.tigr.org/; http://www.sanger.ac.uk/). In *M. leprae*, the few PE genes left appear to be pseudogenes, a trend shared by other *M. leprae* loci (35). At least some members of the PE/PE-PGRS family are surface localized (6), and the two families have been hypothesized to serve as reservoirs of antigenic diversity (34). At the very least they appear to be markers of genomic plasticity, as 50% of the sequence polymorphisms found between the two sequenced *M. tuberculosis* genomes involve PE or PPE genes (62).

Evidence is accumulating for the role of some PE/PE-PGRS and PPE proteins in virulence. *M. marinum* and *M. tuberculosis* PE and PE-PGRS genes are induced selectively in macrophages (30, 134, 167), and the *M. marinum* genes are confirmed to be expressed in granulomas (30, 45, 134). Mutants in the PE-PGRS genes identified in the *M. marinum* expression screen are attenuated in multiple infection models (45, 47, 134, 154) and can alter the composition of the infiltrates (20, 134). Recently, a TraSH screen for virulence mutants in a mouse infection model has identified two PE family members [(143); E. Rubin & C. Sassetti, personal communication]. Similarly, a mutant *M. tuberculosis* PPE family member with decreased growth in mouse lung has been identified (23). Also, several *M. avium* PPE family members are induced in macrophages (83). The role of PE/PE-PGRS and PPE proteins as virulence factors raises two issues. First, that attenuation results from deletion of individual members of these large families suggests a nonredundant function for at least some. Second, pathogens such as *M. leprae* and *M. avium* persist within granulomas despite having few or no functional copies of these genes, which suggests that these pathogens may have developed alternative strategies.

Use of Comparative Genomics in Virulence Determination

The mechanism of attenuation of *M. bovis* BCG, the world's most widely used vaccine strain, was a mystery until the completion of the *M. tuberculosis* genome sequence and the advent of whole genome microarray technology. Genomic comparisons of various BCG substrains identified a single ~9.5-Kb region from the *M. tuberculosis* genome, termed RD1, that was deleted in all the BCG strains but present in every *M. tuberculosis* strain examined (12, 99). Recently, two groups have used complementary approaches to establish that loss of RD1 was important in the attenuation of BCG (94, 132). Deletion of the RD1 region from virulent *M. tuberculosis* strain H37Rv resulted in reduced growth in cultured human macrophages comparable to that from the BCG strain (94). Also, the H37Rv::ΔRD1 and BCG bacilli grew slowly relative to wild-type *M. tuberculosis* in mice and produced little histopathology. Mice infected with virulent *M. tuberculosis* all succumbed to the disease by 40 weeks, whereas those infected with H37Rv:ΔRD1 were all alive and had gained weight. Thus, by all measures, results with H37Rv:ΔRD1 were indistinguishable from those with BCG.

In the other RD1 study, the *M. tuberculosis* RD1 region was introduced into a heterologous site on the chromosome of the BCG Pasteur strain, with a resultant increase in virulence in a mouse model of infection (132). The RD1-restored strain persisted longer in immunocompetent mice and grew to higher numbers in SCID mice than the BCG Pasteur parent did, although virulence was still reduced relative to *M. tuberculosis*. The failure of RD1 to restore full virulence may simply reflect the accumulation of additional mutations in the 80 years since attenuated BCG was first reported. The molecular mechanism(s) by which RD1 influences virulence is unclear, as none of the affected genes have known functions. Notably, they include the immunodominant *M. tuberculosis*–specific secreted antigens ESAT-6 and CFP-10, as well as PE and PPE genes, family members that have been implicated in virulence.

CONCLUDING REMARKS

Despite significant advances, critical gaps remain in our understanding of mycobacterioses. Important questions include how bacteria survive in host cells, particularly within a granuloma, how long and where do they persist during subclinical infection, and how do they exploit the host defenses for their survival. The molecular and genetic tools available for mycobacteria coupled with new *Mycobacterium* infection models ranging from *Dictyostelium* to primates make it possible to address many of these questions. The advent of comparative genomics will promote experiments to improve our understanding of these formidable microbial foes.

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