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Microbial Pathogenesis of *Mycobacterium tuberculosis*: Dawn of a Discipline

Review

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"If we ask further what significance belongs to the results gained in this study of tuberculosis it must be considered a gain for science that it has been possible for the first time to establish the complete proof of the parasitic nature of a human infectious disease, and this of the most important one. So far such proof was established only for anthrax, while in a number of other infectious diseases in human beings, for example relapsing fever, wound infections, leprosy, gonorrhoea, it was only known that parasites occur simultaneously with the pathogenic process, but the causal connection between the two has not been established. It may be expected that the elucidation of the aetiology of tuberculosis will provide new viewpoints for the study of other infectious diseases."

—Robert Koch, 1882

Tuberculosis (TB) is a bacterial infectious disease caused by the obligate human pathogen, *Mycobacterium tuberculosis*. Mycobacteria are a distinctive rod-shaped bacteria that share a common property of a lipid-rich cell wall that avidly retains Carbol fuchsin dye even in the presence of acidic alcohol (acid fast staining). Despite longstanding intense efforts to control this disease, Tuberculosis remains an expanding global health crisis that mandates new therapeutic and preventative strategies. Although a wealth of information has been gathered about *M. tuberculosis* over many decades of research into the bacteriology, cell biology, biochemistry, and immunology of this infection (Barksdale and Kim, 1977; Ratledge and Stanford, 1982; Kubica and Wayne, 1984; Bloom, 1994; McKinney et al., 1998; Hatfull and Jacobs, 2000), investigation of this bacterial pathogen has lacked the multidisciplinary approach that has evolved from the investigation of genetically tractable and fast growing bacterial pathogens such as *Salmonella*, *Yersinia*, *Shigella*, and *Listeria*. These organisms, among others, have given rise to the integrated discipline of microbial pathogenesis (Finlay and Falkow, 1997), also known as cellular microbiology (Cossart et al., 1996). This discipline probes the molecular interactions between specific microbial products and the host cell, predominantly through the examination of defined bacterial mutants. Until recently, this type of analysis has not been possible with *M. tuberculosis*, mostly because of our inability to genetically manipulate this organism and the consequent lack of defined mutants with specific phenotypes. These barriers have been surmounted such that multidisciplinary investigation into *M. tuberculosis* pathogenesis is now possible, an enterprise that has accelerated dramatically due to the completion of the genome sequence of *M. tuberculosis* (Cole et al., 1998). This review will outline present efforts to understand the pathogenesis of this unique bacterial infection, focusing primarily on insights gained from recently isolated *M. tuberculosis* mutants with altered growth kinetics in experimental animals. This review is not intended to comprehensively detail the incredible breadth of the mycobacterial literature, but rather to highlight and interpret recent advances and to point to future directions.

The Ongoing *M. tuberculosis* Epidemic

The global burden of *M. tuberculosis* infection is overwhelming. In addition, Bovine Tuberculosis (caused by *Mycobacterium bovis*) and Leprosy (caused by *Mycobacterium leprae*) remain significant global health problems. Recent estimates of Tuberculosis disease burden documented approximately 1.86 billion people infected with *M. tuberculosis* with 16.2 million cases of active disease. These active cases resulted in approximately 2 million deaths in 1997, an average global case fatality rate of 23% (Dye et al., 1999). These impressive statistics are even more daunting when compared to the relative rarity of lethal TB in the industrialized world due to effective antibiotic therapy. Fully drug-sensitive *M. tuberculosis* infection in an immunocompetent patient must be treated for at least 6 months with multiple antibiotics to achieve reliable clinical cure rates (Council, 1974; Cohn et al., 1990). The financial and logistical barriers to administering these drug regimens in the areas where TB is most prevalent are enormous. Furthermore, microbial resistance to commonly used antituberculous drugs is increasingly common, both in primary isolates and as a result of failed treatment (Surveillance, 1997). Therefore, development of new drugs active against *M. tuberculosis*, particularly drugs that would allow shorter courses of therapy, is a major priority of *M. tuberculosis* investigation. In addition, BCG, the widely administered live attenuated vaccine against TB, is inconsistently effective in adults (reviewed in McKinney et al., 1998). Therefore, understanding the pathogenic strategies of *M. tuberculosis* on a molecular level will allow both the development of new drugs and the design of either live attenuated or subunit vaccine candidates to combat this global health emergency.

Natural History of *M. tuberculosis* Infection in Humans

In order to understand the pathogenesis of *M. tuberculosis* infection on a molecular level, it is essential to understand the clinical behavior of this pathogen in its only

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natural host. Because *M. tuberculosis* has no known reservoir outside of man, the environments to which the organism must respond are defined solely by its natural history within its host. Tuberculosis is a disease that is almost exclusively transmitted by aerosolized droplets containing infectious *M. tuberculosis.* These droplets are generated by the cough of a person with *M. tuberculosis* lung infection and are inhaled by an uninfected person. The inhaled bacilli lodge in the terminal air spaces of the lung where they enter and replicate within alveolar macrophages. The initial, or primary, infection with *M. tuberculosis* involves replication of the organism at the initial pulmonary site of infection, spread to local lymph nodes within the lung, and eventual dissemination of infection to remote sites in the body. Despite this successful initial parasitization of the human host, the primary infection is almost invariably asymptomatic in adults. With the onset of the host immune response, active bacterial replication and dissemination are controlled. Although the human immune response against *M. tuberculosis* is highly effective in controlling the primary infection, the organism is almost never eradicated. Thus, *M. tuberculosis* is foremost among bacterial pathogens in its ability to establish and maintain latency, a period during which the infected person does not have clinically apparent Tuberculosis, but harbors *M. tuberculosis* organisms able to reactivate at a later date. The only clinical evidence of *M. tuberculosis* infection during latency is delayed type hypersensitivity against mycobacterial antigens, demonstrated by a tuberculin skin test. Reactivation of the latent *M. tuberculosis* infection often occurs in apparently healthy people, and very frequently in people who are immunosuppressed as a result of diseases such as AIDS.

Reactivation of Tuberculosis most commonly occurs in the lung, but can involve any organ. The disease is a chronic wasting illness characterized by fever, weight loss, and in the case of pulmonary reactivation, cough. The cough of Tuberculosis is both a symptom of chronic pulmonary inflammation and the mechanism by which the organism disseminates to new hosts. Many of the symptoms of Tuberculosis, including the tissue destruction that eventually liquefies infected portions of the lung, is mediated by the host immune response against *Mycobacterium tuberculosis* rather than a direct toxicity of the bacterium itself. Understanding the interplay between the host immune response and bacterial pathogenic strategies remains a major goal of investigation into *M. tuberculosis* pathogenesis.

Based on this understanding of the clinical behavior of *M. tuberculosis*, the organism must have evolved to successfully execute several distinct but sequential pathogenic strategies. First, the organism must successfully replicate within host macrophages, a property shared with other intracellular bacterial pathogens but apparently achieved through unique mechanisms that are discussed below. Second, the organism must either resist the host immune response or modify this immune response to allow the host to control bacterial replication without sterilization. Third, *M. tuberculosis* must be able to persist within its host in a relatively inactive state, retaining the potential for reactivation. All of these stages of infection represent distinct environments to which the organism must respond through the coordinate regulation of multiple genes. Although we know relatively little about the molecular events that mediate these three pathogenic stages, the recent successful isolation of defined mutants, and their careful characterizations, suggests that *M. tuberculosis* employs novel strategies to achieve both replication and persistence.

M. tuberculosis and the Macrophage

M. tuberculosis primarily infects macrophages. In contrast to other bacterial pathogens that avoid phagocytosis as a specific pathogenic strategy, *M. tuberculosis* is promiscuous in its use of multiple cell surface receptors to gain entry into macrophages (Ernst, 1998; reviewed in Aderem and Underhill, 1999). These receptors include the mannose receptor, complement receptors, and Fc receptors. Once inside the host macrophage, *M. tuberculosis* resides within a membrane-bound vacuole. It is clear that *M. tuberculosis* modifies the maturation of this phagosomal compartment in order to enhance its own intracellular survival (Armstrong and Hart, 1971; Clemens and Horwitz, 1995). This altered phagosomal maturation is associated with alterations in the protein content of the vacuole including altered Rab GTPase composition (Via et al., 1997; Clemens et al., 2000), exclusion of the vacuolar proton ATPase with consequent lack of acidification (Sturgill-Koszycki et al., 1994), and retention of a protein designated TACO (Ferrari et al., 1999). Uptake of mycobacteria by macrophages and subsequent retention of TACO on the phagosome appears to be dependent upon the accumulation of host cell–derived cholesterol in the plasma membrane at the point of bacterial entry (Gatfield and Pieters, 2000). These experiments and others yield a relatively detailed view of the biochemical events that accompany mycobacterial vacuolar trafficking within the macrophage. However, the specific bacterial proteins or cell envelope components that interact with the host cell to perturb these processes are completely unknown. In addition, we do not understand the relationship between altered intracellular trafficking of *M. tuberculosis* in macrophages and development of the granulomatous pathology characteristic of *M. tuberculosis* infection. Therefore, a major goal of future studies will be to understand the relationship between specific *M. tuberculosis* products, intracellular survival within macrophages, and the interrelationships between these altered macrophage responses and the complex multicellular pathology of the infection that mediates tissue destruction and clinical symptoms.

Animal Models of *M. tuberculosis* Replication and Persistence

As detailed above, the natural history of *M. tuberculosis* infection in humans has distinct phases of replication, dissemination, establishment and maintenance of latency, and reactivation. There is no adequate animal model for this complex natural history. The mouse is frequently used for experimental *M. tuberculosis* infection due to the ease of housing in biosafety level 3 facilities, well characterized inbred strains including knockout mice, and the abundance of commercially available reagents. Other animal models, including guinea pigs and rabbits, have contributed greatly to our under-

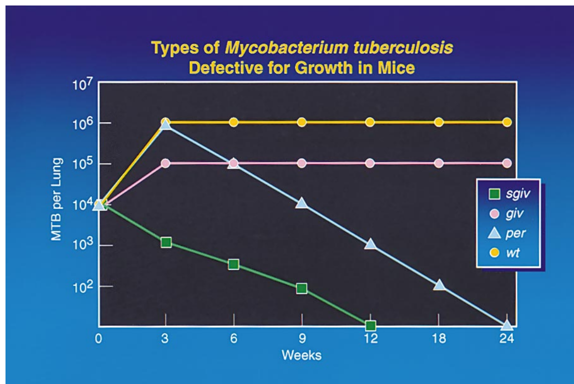

Figure 1. Mouse Model of *M. tuberculosis* Growth and Persistence Illustrating Wild-type and Mutant Phenotypes

Wild-type *M. tuberculosis* replicates for 2–3 weeks in the lungs of mice until the onset of specific immunity. After this point, the organisms persist at a constant titer. Three types of theoretical mutant phenotypes could be anticipated. Growth in vivo (*giv*) mutants are defective for replication in the initial stage of infection. Severe giv (*sgiv*) mutants do not replicate at all in vivo. Finally, persistence mutants (*per*) replicate normally but decline in titer after the onset of host immunity. Specific examples of each mutant class have recently been isolated and are discussed in the text.

standing of *M. tuberculosis* infection in animals, including vaccine efficacy (Dannenberg, 1994; McMurray, 1994). Unfortunately, the immune systems of these animals, while capable of controlling *M. tuberculosis* replication, cannot reduce the viability of *M. tuberculosis* such that a latent state ensues. The time course of the murine *M. tuberculosis* lung infection is shown in Figure 1. After replicating logarithmically for two or three weeks, the organisms are controlled by the onset of specific immunity. A prolonged stage of persistence then ensues during which a constant titer is maintained and the animal is relatively free of symptomatic disease. During this persistence phase, there is minimal bacterial replication (Rees and Hart, 1961). Although the large numbers of bacilli in this phase of the murine infection clearly do not mimic the latent state in the human host, the constant titer does represent an equilibrium between the persisting pathogen and the host immune response. It is likely that a distinct set of *M. tuberculosis* gene products mediates this persistence in mice; gene products that may be important for paucibacillary latency of the human host. Consequently, when evaluating mutants of *M. tuberculosis* that may be defective for in vivo growth, several theoretical phenotypes are possible. Mutants could fail to grow at all in vivo (*sgiv* mutants), or replicate substantially less than wild type (*giv* mutants). Other mutants might replicate normally in vivo, but then fail to persist (*per* mutants) (Figure 1). As will be detailed below, both of these types of mutants have now been identified, and thus far these *giv* and *per* phenotypes are genetically mutually exclusive.

Systems for Generating Mutants and Transferring Genotypes

Mycobacteria began to enter the genetic world with the successful cloning and expression of genes of *M. tuberculosis* (Young et al., 1985a) and *M. leprae* (Young et al., 1985b; Jacobs et al., 1986). Genetic tools for the mycobacteria have been difficult to develop because of the inefficiency of transformation and homologous recombination. Thankfully, many of these obstacles have been surmounted such that transformation with episomal and integrating plasmids (Jacobs et al., 1987; Snapper et al., 1988) and efficient transposon mutagenesis are now possible (McAdam et al., 1995; Bardarov et al., 1997; Pelicic et al., 1997). The use of conditionally replicating plasmids and counterselectable markers have greatly facilitated gene replacements through allelic exchange (Pelicic et al., 1996; Pavelka and Jacobs, 1999). In addition, the recent use of conditionally replicating mycobacteriophages (Bardarov et al., 1997) to mediate allelic exchange (Glickman et al., 2000, 2001) shows tremendous promise. The combination of these efficient mutagenesis strategies of the *M. tuberculosis* genomic sequence coupled with rigorous complementation of mutant phenotypes provides an unparalleled opportunity to define the molecular bases for unique properties of the mycobacteria.

Distinctive Features of the *M. tuberculosis* Genome Sequence

The complete nucleotide sequence of the circular chromosome *M. tuberculosis* strain H37Rv was completed in 1998 (Cole et al., 1998) and has dramatically accelerated investigation into *M. tuberculosis* pathogenesis. Several novel features of the *M. tuberculosis* genome are evident and deserve mention due to their possible relationship to pathogenesis. This topic has been reviewed recently (Brosch et al., 2000).

Approximately 9% of the *M. tuberculosis* genome consists of two related families of genes that have been named the PE and PPE families (Cole et al., 1998). These names derive from the presence of conserved proline-glutamate (PE) or proline-proline-glutamate (PPE) residues near the N terminus of the predicted proteins of the family. These predicted proteins share a conserved N-terminal domain of ~110 amino acids (PE) or 180 amino acids (PPE) with divergent C-terminal sequences (Brosch et al., 2000). A subfamily of the PE family has been designated the PGRS proteins based on C-terminal extensions rich in a repetitive glycine/alanine (GA) motif. A recent search for *Mycobacterium marinum* promoters that were active within granulomas of host frogs identified two members of the PGRS family (Ramakrishnan et al., 2000). Deletion mutants of these genes in *M. marinum* were impaired for replication within macrophages and reached lower titers in frog tissues at 8 weeks compared to wild-type organisms. These results confirm the suspected importance of this gene family based on genomics. In addition, one member of the PPE family of proteins is necessary for in vivo growth of *M. tuberculosis* (Camacho et al., 1999). It is presently unclear whether the common sequence motifs of these gene families reflect related functions of the proteins or represent a mechanism of antigenic variation, as has been suggested (Cole et al., 1998).

A large portion of the coding capacity of the *M. tuberculosis* genome is putatively involved with lipid biosynthesis or lipid degradation. One of the most prominent classes of genes represented in the genome are homo-

logs of the fatty acid β oxidation system. *E. coli* is able to metabolize fatty acids as a sole carbon source through the action of the Fad system, comprised of FadA, FadB, FadD, and FadE (Clark and Cronan, 1996). This system catalyzes the β oxidation of fatty acids to produce acetyl-CoA, which then enters the Krebs cycle in the presence of an active glyoxylate bypass. The first step in the β oxidation cycle is coupling of the fatty acid to coenzyme A, catalyzed by FadD (acyl-CoA synthetase). This protein is encoded by a single copy gene in the *E. coli* chromosome and is able to process fatty acids of heterogeneous carbon lengths (Kameda and Nunn, 1981). The *M. tuberculosis* genome contains 36 FadD and 36 FadE homologs (Cole et al., 1998). Many of these FadD genes appear to be transcribed in operons with polyketide synthases, suggesting that they may be involved in lipid synthesis, rather than degradation. Indeed, FadD28 is necessary for synthesis of phthiocerol dimycocerosate both in *M. tuberculosis* (Cox et al., 1999) and BCG (Fitzmaurice and Kolattukudy, 1998). Nevertheless, the presence of multiple homologs of the canonical β oxidation machinery suggests that *M. tuberculosis* assimilates lipids as important carbon sources. Supporting this notion are studies demonstrating that *M. tuberculosis* harvested from mouse tissues preferentially metabolizes fatty acids as carbon sources (Segal and Bloch, 1956; Wheeler and Ratledge, 1988). Thus, the abundance of β oxidation machinery may indicate that *M. tuberculosis* is equipped to utilize a diverse array of lipids as carbon sources during infection and that the structural diversity of the lipids available in vivo necessitates a diversity of FadD enzymes. The sources of these lipids are not completely clear, but could originate in host tissues (Barclay and Wheeler, 1989) or from the abundant lipids in the cell envelope that could be catabolized under conditions of nutrient limitation.

M. tuberculosis Pathogenicity: Central Role of Lipid Effectors and Lipid Metabolism

The cell envelope of *M. tuberculosis* has been studied intensely for many decades due to its distinctive architecture and composition (Brennan and Nikaido, 1995; Daffe and Draper, 1998). The *M. tuberculosis* cell envelope differs substantially from the canonical cell wall structures of both gram-negative and gram-positive bacteria (Figure 2). In addition to the cell membrane and peptidoglycan layers found in other bacteria, the cell envelope contains a large hydrophobic layer of mycolic acids, long chain branched β hydroxyl fatty acids that exist either covalently attached to the cell wall or noncovalently attached in the form of Trehalose dimycolate (TDM). In addition to these mycolic acids, the cell envelope contains a wide array of distinctive lipids and glycolipids that associate noncovalently with the envelope. This vast array of lipids and glycolipids confers extreme hydrophobicity to the outer surface of the organism.

Many of these compounds have potent biologic activity when tested on eukaryotic cells in in vitro systems, raising the possibility that they may be important for pathogenesis. Lipoarabinomannan, a major cell wall associated glycolipid, has been extensively examined in vitro and can inhibit gamma interferon activation of macrophages (Sibley et al., 1988, 1990), induce TNF α release from macrophages (Chatterjee et al., 1992), and scavenge oxygen free radicals (Chan et al., 1991). TDM, a mycolic acid–containing glycolipid, can produce granulomatous inflammation and thymic atrophy when injected into mice (Ozeki et al., 1997) in addition to affecting membrane fusion in model systems (Spargo et al., 1991) and toxicity for mitochondria. The phthiocerol dimycocerosate family of complex lipids in the cell envelopes of pathogenic mycobacteria can suppress lymphocyte responses (Prasad et al., 1987; Vachula et al., 1989) and were recently shown to bind to peripheral nerve laminin and facilitate Schwann cell invasion by *Mycobacterium leprae* (Ng et al., 2000). Yet despite such an elaborate array of bioactive chemical entities in the cell envelope of pathogenic mycobacteria, detailed understanding of the role of each of these molecules in *M. tuberculosis* pathogenesis has been limited due to the lack of defined mutants defective in the synthesis of individual molecules. With the advent of efficient genetic systems for slow growing mycobacteria, rigorous genetic analysis of the role of the cell envelope in pathogenesis has recently begun.

Using signature-tagged mutagenesis to screen for mycobacterial genes important for in vivo growth, two groups identified a cluster of *M. tuberculosis* genes necessary for replication of *M. tuberculosis* in mice (Camacho et al., 1999; Cox et al., 1999). One of these groups also demonstrated that inactivation of two genes in this region abolished synthesis of phthiocerol dimycocerosate (PDIM), a complex lipid noncovalently attached to the mycobacterial cell envelope (Cox et al., 1999). In addition, a third mutant with the same in vivo phenotype contained a transposon insertion in a gene of unknown function with homology to membrane transporters (mmpL7). This mutant synthesized PDIM, but failed to localize the lipid within the cell wall such that, in contrast to wild type, the lipid was not present in the culture supernatant. In addition, all three mutants displayed replication defects only in the lungs, showing that synthesis and proper transport of PDIM is necessary for organ-specific replication of *M. tuberculosis*.

Further evidence for the role of an individual lipid molecule in *M. tuberculosis* replication in vivo came with the disruption of the *mmaA4* gene (Dubnau et al., 2000). MmaA4 is one member of large family of SAM-dependent methyl transferases that modify the mycolic acids of the *M. tuberculosis* cell wall by introducing cyclopropane rings and methyl branches (Barry et al., 1998). This mutant did not synthesize oxygenated mycolic acids, and therefore lacked two of the three major classes of mycolic acids present in the cell envelope. This mutant was impaired for initial replication in vivo, but then persisted normally at this lower titer (Dubnau et al., 2000), establishing a role for a specific subclass of mycolic acids in mycobacterial replication in vivo.

Other classes of *M. tuberculosis* genes important for growth in vivo do not obviously relate to the cell envelope. Reasoning that secreted proteins would be important for *M. tuberculosis* virulence, Berthet and colleagues identified *erp*, a repetitive protein that is essential for normal replication of *M. tuberculosis* in macrophages and mice (Berthet et al., 1998). Mycobacteria encounter a chemically hostile environment inside the macrophage that includes both reactive oxygen and

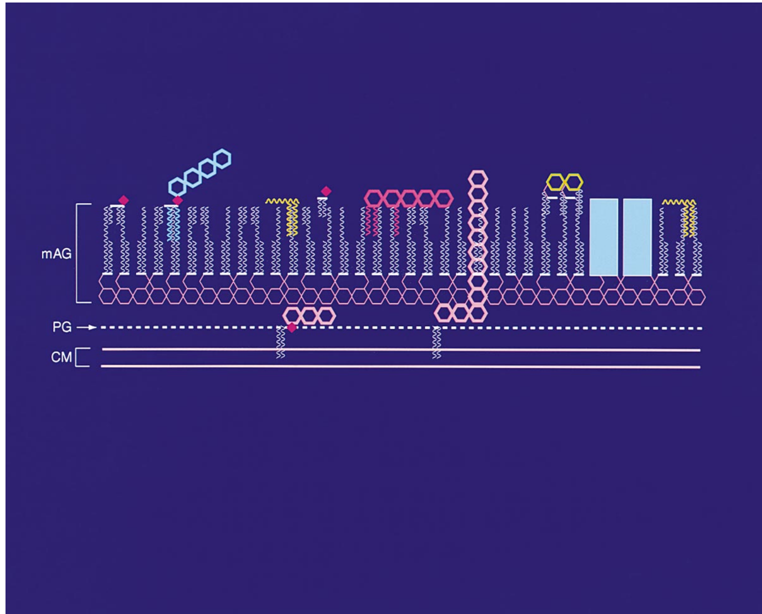

Figure 2. Schematic Diagram of the Cell Envelope of *M. tuberculosis*

Similar to classical bacterial cell walls, the cell envelope of *M. tuberculosis* contains a cell membrane (CM) and a peptidoglycan layer (PG). In contrast to other bacteria, *M. tuberculosis* has a thick hydrophobic layer of mycolic acids esterified to the cell wall, named the mycolyl arabinogalactan (MAG). A variety of unique lipids and glycolipids are noncovalently associated with the cell envelope and are depicted schematically, including Trehalose dimycolate, phthiocerol dimycocerate, and Lipoarabinomannan, among others. Many of these molecules are potent immunomodulators, suggesting a role in virulence that is discussed in the text. The depictions of these compounds is schematic and not intended to convey precise localization, which in many cases is not definitively known.

reactive nitrogen intermediates (Chan et al., 1992). *M. tuberculosis* produces a catalase-peroxidase (*katG*) that is often inactivated in isoniazid-resistant strains. These strains are less virulent for mice than wild-type strains, and this loss of virulence is corrected by a functional copy of *katG* (Li et al., 1998), establishing the importance of this gene for in vivo survival.

Persistence Genes of *M. tuberculosis*

Identification of the molecules that *M. tuberculosis* uses to persist within its host is of great interest. Understanding the mechanisms by which this organism can persist might allow the design of antibiotics that would specifically target persisting or latent bacilli, allowing shortening of chemotherapy. In addition, in the design of a rationally attenuated *M. tuberculosis* vaccine strain, mutations that affect persistence would prevent the vaccine strain from establishing latency and reactivating, an important safety consideration. A mutant that fails to grow or persist may also invoke a qualitatively different immune response and therefore be a more effective immunogen when used as a vaccine. Two classes of persistence genes have recently been identified.

The glyoxylate shunt allows bacteria and plants to grow on acetate or fatty acids as sole carbon sources. The glyoxylate shunt enzyme isocitrate lyase (Icl) is upregulated in *M. tuberculosis* exposed to anaerobic conditions (Wayne and Lin, 1982), stationary phase, and growth within macrophages (Graham and Clark-Curtiss, 1999; Honer Zu Bentrup et al., 1999). In addition, as mentioned above, previous work had shown that *M. tuberculosis* harvested from mouse tissues metabolizes fatty acids as a carbon source in preference to glucose, raising the possibility that *M. tuberculosis* is lipolytic in vivo and therefore would require the glyoxylate shunt for survival. Genetic proof that the glyoxylate shunt is required for *M. tuberculosis* survival in vivo was recently reported (McKinney et al., 2000). An isocitrate lyase mutant of *M. tuberculosis* replicated identically to wild type over the first 2 weeks of the infection, but then declined in titer after the onset of specific immunity. This study confirmed the importance of *icl* not only for in vivo survival of *M. tuberculosis*, but specifically for persistence. These results imply that *M. tuberculosis* must utilize acetyl-CoA as a carbon source for multibacillary persistence in mice, but not for active replication. This metabolic phenotype was dependent upon host gamma-interferon, suggesting a responsive relationship between bacterial in vivo metabolism and the host immune system (McKinney et al., 2000). As humans do not have a functional glyoxylate shunt, *icl* represents an attractive drug target.

Another class of persistence genes was recently discovered through the examination of mycobacterial colony morphology (Glickman et al., 2000). Screening for mutants of BCG that could not form serpentine cords, a colony morphology associated with virulence, the authors identified a novel methyl transferase, *pcaA*, putatively involved in the modification of mycolic acids in the cell envelope. Deletion of this gene in *M. tuberculosis* by specialized transduction revealed that *pcaA* synthesizes a single cyclopropane residue on the major mycolic acid of the cell envelope. These *pcaA* mutants also replicated normally, but were defective for persistence in mice such that bacterial titers dropped during chronic infection and the mice did not succumb. In addition, the inflammatory infiltrate in the lungs of mice chronically infected with the *pcaA* mutant was predominantly lymphocytic, in contrast to the granulomatous inflammation of wild-type infected mice. These results provided further evidence for the importance of specific molecules of the cell envelope in pathogenesis and implicated the fine structure of mycolic acid subclasses in the interaction of *M. tuberculosis* with the host immune system during long-term persistence in vivo. Both prior data (reviewed in Barry et al., 1998) and the *M. tuberculosis* genome sequence identified a large family of cyclopropane synthetases highly homologous to *pcaA*, each of which appears to synthesize a unique cyclopropane res-

idue in the cell wall (Glickman et al., 2001). The role of each member of this gene family in pathogenesis remains to be defined.

Despite the relative paucity of defined mutants of *M. tuberculosis* altered for in vivo growth, certain themes have already emerged. Lipid synthesis for the construction of the cell envelope, and lipid degradation by the bacterium for survival within the host, have emerged as centrally important. Before the isolation of specific mutants in cell envelope biosynthesis, the mycobacterial cell envelope appeared to be a redundant waxy coating that conferred extreme hydrophobicity to the organism through the combined presence of structurally diverse molecules. Now, one can view the mycobacterial cell envelope as an array of distinct lipid effector molecules, each with its own role in modulating the interaction of *M. tuberculosis* with its host. This novel pathogenic strategy is reflected by the distinct in vivo phenotypes of the mutants in genes required for PDIM synthesis, *mma4*, and *pcaA*. If these lipids were simply redundant members of an inert waxy coat, then the phenotypes of these mutants would be similar, yet they are distinct. In addition, the finding that PDIM is transported by mmpL7 suggests that the large family of mmpL proteins in the genome of *M. tuberculosis* may also be involved in lipid export. While further work is necessary to know whether all of the mmpL family members function to transport lipids to their correct locations within the cell envelope or to the extracellular environment, clearly mmpL7 is required for PDIM to appear in the media of in vitro grown organisms. Supporting this concept of exported lipid effectors, recent data demonstrates that mycobacterial lipids can be found within the cytoplasm of host macrophages, free of the intact bacterium (Beatty et al., 2000). It is tempting to speculate that the MmpL family of putative lipid transporters is involved in the export of lipid effector molecules into the host cell. This model is illustrated in Figure 3, where it is compared and contrasted to an analogous system from gram-negative pathogens. Gram-negative bacterial pathogens deliver protein effector molecules to the surface or the cytoplasm of the host cell through a protein export machinery known as type III protein secretion. Depending on the pathogen studied, these protein effectors have diverse effects on host cell function, including inhibition of phagocytosis, perturbation of signal transduction, cytoskeletal rearrangement (reviewed in Finlay and Cossart, 1997), and insertion of a bacterial receptor in the host cell membrane (Kenny et al., 1997). While there are no pathogenicity islands or type III secretion systems evident in the genome of *M. tuberculosis*, the mmpL system probably functions as an exporter of lipids into host cell membranes or to the host cell cytoplasm as pathogenesis effectors. Definition of the range of lipids exported, their subcellular localization within the host cell, the specific host cellular targets affected by exported lipids, and the consequences of this interaction all await further work. Nevertheless, the incredible chemical diversity of the lipids and glycolipids in the mycobacterial cell envelope is likely to mediate specific interactions with host ligands or membranes rather than existing solely to determine the hydrophobicity and rigidity of the bacterial cell wall.

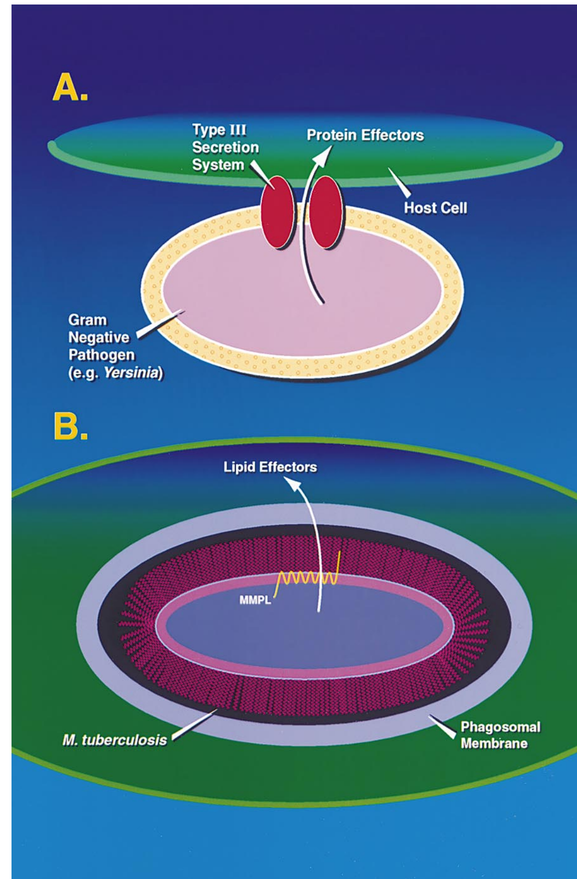

Figure 3. The Export of Lipid Effectors as a Novel Pathogenic Strategy of *M. tuberculosis*

(A) Schematic representation of a gram-negative pathogen exporting protein effectors into the host cell through a type III secretion system.

(B) *M. tuberculosis* within the phagocytic vacuole exports lipid effectors into the host cell membranes or cytoplasm. Lipids and glycolipids from the organism may modify the vacuolar membrane or interact with specfic ligands in the host cell cytoplasm. This lipid export is probably accomplished in part by the family of mmpL proteins, one of which transports PDIM, an *M. tuberculosis* lipid essential for virulence.

Host Factors in *M. tuberculosis* Infection

While this review has focused primarily on recently delineated microbial strategies of pathogenesis, many of the clinical symptoms and tissue destruction of *M. tuberculosis* infection are mediated by the host inflammatory response. The cornerstones of antimycobacterial immunity have been examined in experimental animals through use of knockout mice. Interferon Gamma is critical for the control of *M. tuberculosis* infection in mice (Cooper et al., 1993; Flynn et al., 1993), as are Interleukin 12 (Cooper et al., 1997), and CD4-positive T cells (Scanga et al., 2000). Beyond these pillars of antimicrobial immunity, defined polymorphic determinants of susceptibility to mycobacterial infection in mice are limited to *Nramp1* for BCG and the recently identified *sst*1 locus for *M. tuberculosis* (Kramnik et al., 2000). In humans, polymorphisms in the Interferon Gamma pathway clearly predispose to severe mycobacterial infections (Altare et al.,

1998; Jouanguy et al., 1999). However, the host genetic factors that affect more subtle aspects of *M. tuberculosis* infection in humans remain obscure. For example, why do the vast majority of people infected with *M. tuberculosis* contain the infection while a minority develop active disease? As the human genome sequence is applied to understand susceptibility to disease states with complex, nonmendelian inheritance, we may begin to understand the contribution of host genetic susceptibility to the varied manifestations of *M. tuberculosis* infection. Such knowledge might allow more accurate risk stratification for the development of active Tuberculosis among latently infected people and allow targeting of vaccination and chemotherapeutic strategies to these high risk groups.

Conclusion

In summary, *M. tuberculosis* pathogenesis investigation has recently entered a new and exciting phase. Through the efforts of a tremendous number of dedicated investigators, many of the formidable technical obstacles to *M. tuberculosis* investigation have been surmounted such that rigorous analysis of *M. tuberculosis* pathogenesis is now possible. Although our knowledge is still limited, it is already clear that *M. tuberculosis* employs novel pathogenic strategies for both replication and persistence in vivo, many of which involve novel lipid mediators in its cell envelope. Many of the bacterial genes discussed above are attractive targets for therapeutic intervention, either through drug development or through incorporation into vaccine strains. It is also already clear that a more complete understanding of the pathogenic strategies of this highly successful intracellular pathogen will elucidate novel features of macrophage defenses and the host immune response (Stenger et al., 1998; Moody et al., 2000). In addition to this anticipated scientific dividend, the dividend of greatest immediate importance is the development of new antibiotics and vaccines against this deadly disease. With the entry of *M. tuberculosis* into the multidisciplinary scientific discipline of microbial pathogenesis, it is our hope that these dividends will soon be realized.

Acknowledgments

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