REVIEW ARTICLE

The wolf at the door

Some thoughts on the biochemistry of the tubercle bacillus

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> It was coughing, obviously, a man coughing; but coughing like to no other [he] had ever heard, and compared with which any other had been a magnificent and healthy manifestation of life: a coughing that had no conviction and gave no relief, that did not even come out in paroxysms, but was just a feeble, dreadful welling up of the juices of organic dissolution.
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> Thomas Mann, *The Magic Mountain*

Mycobacterium tuberculosis infects one-third of the world's population. Every year 8 million new cases of tuberculosis are reported, with 3 million deaths world-wide. Globally, it is the leading cause of death from a single infectious agent, surpassing even malaria. These grim statistics emerged from the recent World Congress on Tuberculosis, held in Bethesda, USA, on 16 - 19 November 1992. Even more sobering is the fact that despite its discovery by Robert Koch more than 100 years ago, the biochemistry and physiology of *M. tuberculosis* are poorly understood in molecular terms, as are the means by which it so successfully enters and multiplies within its host cell, the macrophage.[1] Bluntly stated, the virulence determinants that dramatically distinguish *M. tuberculosis* from its free-living, saprophytic cousins are completely unknown. Without fundamental advances in our understanding in these areas we are unlikely to develop effective therapeutic regimens, which are becoming ever more urgent as existing strategies are being blunted and even rendered useless by the growing problem of dual *M. tuberculosis*/HIV infections and emergent multi-drug-resistant tuberculosis (MDR-TB).

M. tuberculosis is a sinister organism. Although descended from a family of saprophytes (organisms living on dead or decaying organic matter),[2] it is a facultative intracellular pathogen that effortlessly invades what should be a hostile and aggressive host cell, the macrophage. This strategy appears to be central to the *modus operandi* of *M. tuberculosis* since without efficient colonisation of macrophages an infection cannot be established; in this regard *M. tuberculosis* resembles other facultative and obligate intracellular pathogens, such as *Leishmania*, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Legionella pneumophila* and *Bordetella pertussis*. Moreover, in common with these organisms *M. tuberculosis* effectively escapes macrophage killing mechanisms (especially *human* macrophages) and replicates intracellularly.[3,4] The infectiousness of this pathogen is so great as to border on the bizarre, as there appears to be no threshold of organisms required to produce infection: in animal studies between 1 and 10 bacilli in airborne droplet nuclei can cause infection.[1] Unusual also is the remarkable capacity for dormancy (or latency) of tubercle bacilli in old, walled-off primary foci: some investigators believe, although there are no hard data, that these bacilli are never eradicated during the lifetime of the infected individual, even with no evidence of active disease (the lifetime risk of developing active disease is 10%[1]).

However, *M. tuberculosis* is not limited to an intracellular environment. As infection progresses to active disease, bacilli burst out of macrophages and grow extracellularly within a solid, necrotic (caseous) lesion. If not contained, the caseous lesion can break into a bronchus allowing formation of a large cavity, or the solid necrosis liquefies, in either case creating a rich medium for the extracellular proliferation of bacilli, which may reach 10^9/ml.[1] Thus the cunning and victorious intracellular parasite returns to the practices of its saprophytic ancestors, feasting on dead and decaying human tissues.

How, in molecular terms, are these properties to be explained? It is difficult to reconcile the notion that the tubercle bacillus is essentially inert — and that all the manifestations of tuberculosis are solely the result of the host immune response – with the fact that there are numerous strains within the *M. tuberculosis* complex — and other closely related mycobacterial species — that differ profoundly in the type and severity of disease caused. It is more likely that there are strain- and species-specific virulence factors that are central to the pathogenicity of *M. tuberculosis* and to the process of dormancy.

Over the years, a great deal of significance has been attached to two peculiar characteristics of the tubercle bacillus: its prodigiously thick and waxy cell wall, and its slow growth.[2] In this article a brief review is presented of these characteristics and their significance. At the outset, however, it should be noted that despite intensive investigation these two hallmarks have not been consistently linked to virulence; their role in dormancy is speculative.

The wall

On the face of it, the mycobacterial cell wall appears to be unique among prokaryotes. Attention to it was drawn early on by the unusual property of acid-fast staining, which placed the mycobacteria outside of the broad classification scheme based on Gram staining. Subsequent chemical analysis revealed it to contain an unusually high proportion of lipids — over 60% of the weight of the cell wall — which, moreover, are of considerable complexity.[5] The mystique of the mycobacterial cell wall was further reinforced by the bewildering intricacy evident on ultrastructural (electron microscopy) analysis, which generated the impression that it was unlike any other.[6] However, careful review of the ultrastructural data by Draper[6] indicated that some of this confusion was the result of technical artifacts and that the cell wall structure was considerably simpler than initially thought when it was appreciated that essentially the mycobacterial envelope consists of three components: the plasma membrane, the cell wall proper, and an outer, loosely attached capsule; the latter is highly variable depending on species and culture conditions. Rigorous chemical dissection of the cell wall proper has established that none of the core components is unique to mycobacteria, but that the sum of its parts forms an entity that is unusual and characteristic for this genus.[6,7] Thus, the differences between the walls of mycobacteria and other prokaryotes are mostly quantitative rather than qualitative.

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As is evident from Fig. 1, the mycobacterial cell wall skeleton consists of three covalently linked macromolecules, peptidoglycan, arabinogalactan, and mycolic acid. The peptidoglycan layer forms the backbone of the cell wall skeleton, as is the case for virtually all bacteria (Gram-positive and Gram-negative), differing only in detail. Typically, peptidoglycans are composed of chains of a polysaccharide — formed from alternating units of glucosamine and muramic acid — cross-linked by tetrapeptide side-chains.[5] The next layer is a complex polysaccharide called arabinogalactan, which is covalently linked to muramic acid residues of the peptidoglycan. The arabinogalactan consists of a homopolysaccharide backbone of galactose units, from which chains of arabinose units branch off.[7] A wide variety of other bacterial cell walls have side-chain polysaccharides attached to the peptidoglycans, such as, for example, the teichoic acid chains in Gram-positives[8] (see Fig. 1); in at least two groups — corynebacteria and nocardias — the polysaccharide is immunologically similar to the arabinogalactan of mycobacteria.[6]

The third layer of the cell wall skeleton consists of mycolic acids, which are attached as esters to terminal arabinose residues of arabinogalactan (Fig. 1). Mycolic acids are known to be high-molecular-weight β-hydroxy fatty acids with a long α-side-chain (the terms α and β refer to the carbons adjacent to the carboxylate function). The main carbon (or alkyl) chain in each mycolic acid contains 50 - 60 carbon atoms, whereas the α-branched side-chain contains another 24 carbon atoms. Considering that conventional fatty acids in animals contain between 16 and 20 carbons, the considerable complexity of these lipids can be appreciated; also, these carbon chain lengths transform the properties of mycolic acids from those of fats or oils into those of *waxes*. Indeed, it seems likely that the mycolic acids are primarily responsible for producing a continuous outer waxy coat in these organisms.[5] Once again, though, it is notable that these molecules are not unique to mycobacteria; they are also found in corynebacteria and nocardias, albeit with shorter chain lengths.[6]

In addition to the covalently linked macromolecules described above that together constitute the cell wall skeleton, the *M. tuberculosis* cell wall contains considerable quantities of non-covalently-associated glycolipids and proteins. Of these, the most interesting glycolipid is lipoarabinomannan (LAM, not to be confused with arabinogalactan discussed above). This moiety consists of a polysaccharide, containing arabinose and mannose units, covalently linked to a phosphatidylinositol group that anchors the molecule in the mycobacterial cell membrane. The polysaccharide extends through the peptidoglycan layer to the external side of the cell wall, analogous to lipoteichoic acids of Gram-positive organisms.[7,8] LAM has elicited considerable interest because it has broad immunological activities analogous to lipopolysaccharide (or endotoxin) of Gram-negative rods.[9] However, claims that it constitutes a primary virulence factor for *M. tuberculosis* are difficult to accept in light of the fact that LAM is found in all mycobacteria irrespective of virulence.[7]

Other loosely bound, complex, free lipids and glycolipids include trehalose-based glycolipids in which long-chain fatty acids, including mycolic acids, are esterified to the disaccharide trehalose (the dimycolyltrehaloses are also called 'cord factor'); sulphated acyl trehaloses, also called sulphatides; phenolic glycosides and peptidoglycolipids (mycosides); and true waxes or cerides (e.g. phenolic phthiocerol).[5] Most of these free wall-lipids are exceedingly complex and many remain structurally uncharacterised. These lipids are thought to interdigitate with the structural mycolic acids covalently bound to the arabinogalactan layer (Fig. 1) and contribute con-

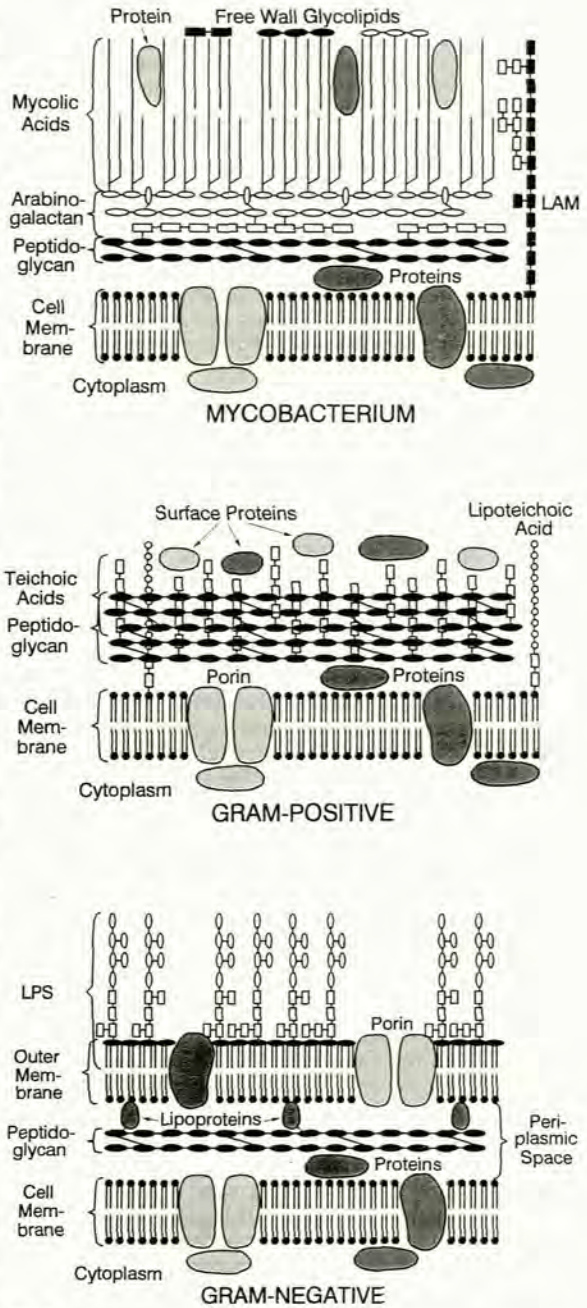

FIG. 1.

Schematic representations of mycobacterial, Gram-positive and Gram-negative cell walls; not drawn to scale. Ovals and rectangles depict sugar residues. Circles represent the polar headgroups of phospholipids. Long straight lines represent fatty acyl chains (mycolic acids and membrane phospholipids). Note that the backbone of the mycobacterial wall is based on peptidoglycan, as is the case for almost all bacteria; in addition, it shares features with both Gram-positive and Gram-negative walls. In common with the former are: (*i*) polysaccharide side-chains – arabinogalactan in mycobacteria, teichoic acids in Gram-positives – linked to the peptidoglycan via phosphodiester bonds between N-acetylglucosamine and muramic acid (1→ 6 linkage);[5,7,8] and (*ii*) lipoarabinomannan (LAM), which is analogous to lipoteichoic acid in that both are polysaccharides anchored in the cell membrane via diacylglycerol units.[8,9] In common with Gram-negatives are: (*i*) presence of lipids external to the peptidoglycan layer; and (*ii*) LAM, although analogous to lipoteichoic acid (see above), also resembles lipopolysaccharide in physical and biological terms.[7] This scheme is based on references 5,7 and 8.

siderably to the extreme lipophilicity and waxiness of the mycobacterial cell wall.

For the most part the identity and functions of wall-associated proteins are unknown. Obscure also is how they are anchored or, indeed, the route through the cell wall for secreted proteins, which are abundant.[10] If the wall is uniformly dense and waxy, how do proteins penetrate? These are not esoteric questions. There is a growing awareness that the pathogenicity and virulence of invasive micro-organisms is dependent in part on cell-surface and secreted proteins that interact with host tissues and cells, enabling these organisms to invade, escape host defences, and replicate.[11] To date, virulence-determining cell-surface proteins analogous to, for instance, invasin of *Yersinia enterocolitica* or gp63 of *Leishmania*, have not been identified on *M. tuberculosis*. However, the search for such proteins is warranted, since despite intensive study none of the mycobacterial cell wall glycolipids has been convincingly and consistently linked to virulence.

In summary, the mycobacterial cell wall skeleton, though distinctly unusual, cannot be considered to be unique; rather, it differs only in detail from those of other bacteria. The composition and functions of wall-associated glycolipids and proteins are only partly understood and no single component has yet been demonstrated to be a primary virulence determinant in *M. tuberculosis*. It has been noted that the hydrophobic mycobacterial envelope constitutes a sophisticated barrier enabling survival under adverse conditions and obviating the need for specialised survival mechanisms such as sporulation.[5] Thus, the mycobacterial wall may play a central role in the maintenance of a long-lived dormant state, postulated by many to be responsible for latency and reactivation of tuberculosis.

The metabolism

The second characteristic of *M. tuberculosis* that is distinctly unusual is its slow growth: the doubling time is in the order of 12 - 20 hours, which is remarkably slow in comparison with the 20 minutes of *Escherichia coli*.[12] However, whereas this is characteristic of members of the *M. tuberculosis* complex and other species such as *M. avium/intracellulare*, *M. scrofulaceum*, and *M. kansasii*, there are other mycobacteria — *M. fortuitum*, *M. chelonei*, and *M. smegmatis* — with considerably faster growth rates (doubling times of 1 - 2 hours) that approach those of most bacteria.[13] Thus, slow growth is not a defining characteristic of the entire genus, and moreover it does not correlate with virulence: for instance, whereas *M. tuberculosis* is a slow-growing species, the virulent H37Rv strain is faster growing *in vivo* in the mouse than the avirulent H37Ra strain;[14] the BCG strain of *M. bovis* is slow-growing but with minimal pathogenicity; and *M. fortuitum* is a fast-growing opportunistic pathogen. As frustrating as the inability to link slow growth with virulence and pathogenicity has been the failure, despite extensive study, to provide a metabolic explanation for this phenomenon.[12,15]

Arguments that attempt to explain slow growth on the basis of substrate limitation imposed by the thick lipoidal cell wall, or on the basis of the small number of ribosomal RNA (rRNA) genes (rRNA is required for protein synthesis) — *M. tuberculosis* contains one rRNA gene versus seven in *E. coli* — are not convincing in the face of large variations of growth rates among mycobacterial species that share these properties.[12] Further, it has not been possible to distinguish between inherent oddities of mycobacterial metabolism that can directly account for the slow growth as opposed to low metabolic activities that are a *consequence* of slow growth. It is pertinent that no fundamental difference between the metabolism of mycobacteria and other bacteria has yet been uncovered.[12,15]

The nutritional requirements of *M. tuberculosis* are simple and the organism can grow on minimal culture media containing a simple source of organic carbon (e.g. glycerol), inorganic nitrogen (NH_4^+), and the usual inorganic elements and trace elements;[12] no particular growth factor or vitamin is required.[15] However, the nutrition of *M. tuberculosis* must be considerably different when it grows within host tissue, particularly intracellularly, than when it is grown in an *in vitro* culture medium. It is likely to prefer to assimilate pre-assembled molecules — amino, nucleic and fatty acids, etc. — in order to reduce its synthetic metabolic load and thereby turn off (at the gene level) complex and energy-expensive synthetic machinery, allowing it instead to invest its metabolic energy in crucial virulence determinants required for survival within hostile macrophages. In this setting it is probable that *M. tuberculosis* is forced to compete with the metabolic processes of the host cell for nutrients,[12] which may become limiting with resultant starvation.

M. tuberculosis and mycobacteria in general appear to be singularly well suited to surviving starvation. Although they do not form spores, these organisms can be maintained in distilled water for 2 years (and probably much longer) with no loss of viability.[16] Curiously, this treatment produces a complete loss of acid-fastness and resistance to any form of staining (chromophobicity), which can be mimicked by chemical reduction, and this has been interpreted to mean that starvation results in a progressive shutting-off of oxidative metabolism and the onset of dormancy.[16,17] Similar changes appear in response to low oxygen tensions, and chromophobic tubercle bacilli have reportedly been isolated from chronic, caseous lesions.[16] Taken together, these data provide a picture of a highly adaptable organism, one that grows in a metabolically active, acid-fast form when conditions are favourable, and that can rapidly switch to an inactive chromophobic state under adverse conditions of starvation and hypoxia. These properties can explain how, after years and perhaps decades of dormancy, tuberculosis can be endogenously reactivated. It is conceivable that *M. tuberculosis* can never be completely eradicated by antimicrobial agents since dormant, non-replicating organisms are not likely to be susceptible to such agents.

Although the biochemical basis for the slow growth of *M. tuberculosis* remains to be elucidated, a teleological explanation for this phenomenon may be that the organism is thereby constantly in a semi-hibernating state with a minimal flow (or flux) through all metabolic pathways and is therefore well placed to survive the sudden onset of starvation or hypoxia. This, coupled with its unusually thick, waxy cell wall and facility for entering into a condition of profound dormancy, makes this organism almost invincible, and it is no surprise that the tubercle bacillus is one of the most formidable pathogens in the long-suffering history of mankind.

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