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Mycobacterium leprae—The outer lipoidal surface

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Abstract. There is now a considerable body of evidence to suggest that the phthiocerol-containing lipids, including the phenolic glycolipids, comprise the so-called "peribacillary substance", "spherical droplets", "foamy structures" and "capsular materials" of *Mycobacterium leprae.* Thus, the phthiocerol-containing lipid capsule may be directly responsible for the intracellular survival of *Mycobacterium leprae.*

Keywords. *Mycobacterium leprae*; phenolic glycolipids; diacylphthiocerol; dimycocerosylphthiocerol; peribacillary nature; monoclonal antibody; enzyme linked immunosorbent assay; diagnostic tool.

The outer surface of *Mycobacterium lepraemurium* and related species such as *M. avium, M scrofulaceum, M. intracellulare*, and *M. cheloneii* have been identified chemically and characterized ultrastructurally. The subject matter has been reviewed in depth (Goren and Brennan, 1979; Draper, 1982) and succinctly (Draper, 1983; Brennan, 1983) in recent times. It is composed of a fibrillar or crystalline capsule which in the main consists of haptenic polar mycoside *C* glycopeptidolipids (GPL) with the general structure.

Fatty Acyl-CO-NH-D-Phe-D-aThr-D-Ala-L-Alaninol-O-(3,4-Me_2-α-L-Rhap)
|
O
|
Oligosaccharide

Apparently, all of the GPL-producing strains of mycobacteria are intracellular parasites and it has been suggested that the glycolipid protects bacteria from intracellular degradation (Draper and Rees, 1973). In general, the GPL-producing mycobacteria are found within phagolysosomes, and it has been proposed that GPL, due to a preponderance of D-amino acids and methoxyl substituents, is metabolically inert and accordingly provides passive protection, but which, because of its fibrillar construction, could still be permeable to nutrients (Goren, 1977; Hunter and Brennan, 1981).

M. leprae does not produce the kind of fibrillar capsule evident in *M. lepraemurium* and *M. avium* (Nishiura, 1960; Fukunishi *et al*., 1982); electron microscopy has clearly established the unrelatedness between the human and murine leprosy bacillus in this respect.

Studies of the ultrastructure of *M. leprae,* mostly *in situ* in human or armadillo tissue,

show cells about 2·5 μm long and about 0·2 μm wide, surrounded by a continuous cell wall composed of an inner dense layer, about 11 nm, and an extensive electron transparent outer layer (Imeada *et al*., 1968; David *et al*., 1978). Early investigators of the microscopic properties of *M. leprae* noted material (capsular matrices, transparent halos, sheaths) which bound the organism into clumps or globi (Hanks *et al*., 1961). The relationship of these structures to the 8 mm diameter quasi-crystalline particles seen in sectioned *M. leprae* (David *et al*., 1978) is not clear. However, in a recent series of elegant ultrastructural analyses, Fukunishi and others redefined the electron transparent zone and inferred that the thing and the material described in the older literature as peribacillary substance, small spherical droplets, foamy structures, and capsular materials were synonymous of substances surrounding *M. leprae* within the phagolysosomes of human leprae cells, macrophages of nude mice, or *M. leprae*-infected armadillos (Fukunishi *et al*., 1980, 1982; Nishiura *et al*., 1980). They further inferred that the electron transparent zones of individual bacilli coalesced with each other to form distinct intracytoplasmic foamy structures when the lesion became old, and that these were mycobacterial in origin.

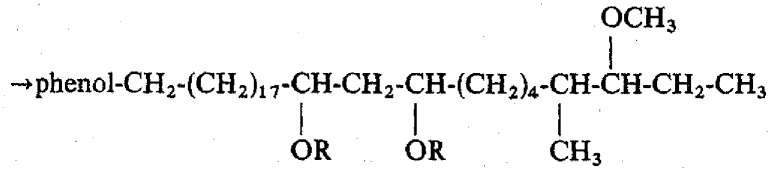
For a time it was hotly debated if these bodies were bacterial in origin. Hanks (1961), from cytological evidence and the fact that such materials were confined to the leprosy bacillus and disappeared during sulphone therapy, persuasively reasoned that they originated in *M. leprae.* Moreover, since 5–10 % chloroform in aqueous systems declumped and dispersed *M. leprae,* he concluded that mycobacterial lipids were the major bonding substances in the electron transparent material. Since the material of the capsule can be stained with Sudan Black B, Fisher and Barksdale (1971) and Nishiura (1960) had concluded that the electron-transparent zone which surround *M. leprae in vivo* is lipid.

Members of the *Mycobacterium* genus characteristically produce lipids of extraordinary structure (Goren and Brennan, 1979; Minnikin, 1982) and *M. leprae* is no exception. Of those, the phthiocerol-containing types are the most species-specific and of the most fundamental and applied interest. To date, we have recognized and fully characterized three phenol-phthiocerol triglycosides in *M. leprae* (Hunter and Brennan, 1981, 1983; Hunter *et al.,* 1982; Fujiwara *et al*., 1984):

3,6-Me_2-β-D-Glcp(1 → 4)2,3-Me_2-α-L-Rhap(1 → 2)3-Me-α-L-Rhap-α-1 →
Phen GL-I

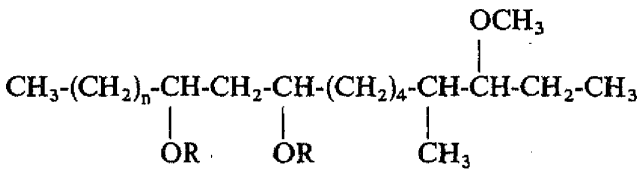
3,6-Me_2-β-D-Glcp(1 → 4)3-Me-α-L-Rhap(1 → 2)3-Me-α-L-Rhap-α-1 →
Phen GL-II

6-Me-β-D-Glcp(1 → 4)2,3-Me_2-α-L-Rhap(1 → 2)3-Me-α-L-Rhap-α-1 →
Phen GL-III



R = a mixture of three mycocerosic acids; 2,4,6,8-tetramethylhexacosanoate; 2,4,6,8-tetramethyloctacosanoate; 2,4,6,8-tetramethyltriacontanoate.

In addition, the non-phenylated, non-glycosylated dimycocerosylphthiocerol has been isolated in large quantities from infected armadillo tissue and characterized completely (Hunter *et al.,* 1982; Draper *et al*., 1983).



n = 16, 18

Phenolic glycolipid I is also present in human lepromas (Young, 1981).

Young (1981) had reported chromatographic evidence for a non-glycosylated phenol-dimycocerosylphthiocerol in *M. leprae* from a human source although as yet we have been unable to confirm this (Hunter *et al*., 1982).

The practical implication of these glycolipids is that the trisaccharides, 3,6-di-*O*-methyl-*ß*-D-glucopyranosyl-(1 → 4)-2,3-di-*O*-methyl-α-*L*-rhamnopyranosyl-(1 → 2)-3-*O*-methyl-α-*L*-rhamnopyranose in phenolic glycolipid I, and 3,6-di-*O*-methyl–*ß*-D-glucopyranosyl-(1 → 4)-3-*O*-methyl-α-*L*-rhamnopyranosyl-(1 → 2)-3-*O*-methyl-α-*L*-rhamnopyranosyl in phenolic glycolipid III are highly specific for the serodiagnosis of leprosy (Cho *et al.,* 1983; Brett *et al*., 1983; Young and Bachanan, 1983). Moreover, these oligosaccharides have been the basis of artificial antigens highly suitable for the serodiagnosis of leprosy (Fujiwara *et al*., 1984; Cho *et al*., 1984). For instance, coupling 3,6-di-*O*-methyl -*ß*-D-glucopyranosyl-(1 → 4)-2,3-di-*O*-methyl-α-L-rhamnopyranose to protein by reductive amination produced the product, ε-*N*-l-[l-deoxy-2,3-di-*O*-methy-4-0(3',6'-dimethyl-*ß*-D-glucopyranosyl-rhamnitol]-lysyl-bovine serum albumin which was highly sensitive in enzyme linked immunosorbant assay and showed excellent concordance with the native glycolipid in analysis of 223 sera from patients throughout the granulomatous spectrum of leprosy; the correlation coefficient, by random sampling, was 0·842.

The phthiocerol-containing lipids of *M. leprae* are extracellular and comprise the capsular electron-transparent zone. That they are extracellular was obvious from the time of their early discovery; the bulk of them were found in supernatants of homogenized leprosy-infected tissue after the bacteria had been centrifuged down (Hunter and Brennan, 1981). Further evidence that the capsular material of *M. leprae* is composed in large measure of the phthiocerol-containing lipids is indirect although persuasive. The quantities of diacylphthiocerol and phenolic glycolipid recovered from infected tissue is far in excess of that to be expected from the bacillary load, as shown by the following considerations. In recent work which is an extension and amplification of earlier work (Hunter *et al*., 1982), we extracted 10·1 g (wet wt) of an infected armadillo liver containing 10^{10} acid fast bacilli/g. The total lipid obtained was 564·8 mg. Exactly 400 mg of this total lipid fraction was injected onto a high pressure Altech silica column (25 cm × 4–6 mm, 5 micron) in $CHCl_3$-CH_3OH (98:2) and the peak height of phenolic glycolipid I was estimated by comparing with standard preparations. In 10·1g of the tissue there were 1·675 ± 0·05 mg of phenolic glycolipid I *i.e.* 165·8 ± 5 $\mu g/10^{10}$ acid fast

bacilli. This figure is 2·5 times greater than that reported previously (Hunter *et al*., 1982). One individual cell of *M. leprae* is $3{\cdot}5 \pm 1{\cdot}0\times10^{-14}$ g (Draper and Misell, 1977). Accordingly, $3{\cdot}7 \times 10^{10}$ acid fast bacilli amount to 1·44 mg and, in effect, according to data presented above, and previously (Hunter *et al*., 1982), 1·44 mg of *M. leprae* produce 2mg of cell-dissociated phthiocerol-containing lipids in the form of the diacylphthiocerol and phenolic glycolipid I.

To address directly the question of the cellular location of the phthiocerol-containing lipids, the following investigation was conducted (Hooper, L. C., Barrow, W.W., Cho, S-N. and Brennan, P. J., unpublished results). Rabbits were inoculated with an emulsion containing 5 mg of glycolipid-methylated bovine serum albumin of which about 25 % weight consisted of the glycolipid. Rabbits were bled at 3, 5 and 7 weeks postinoculation. Enzyme linked immunosorbent assay (Cho *et al*., 1983) showed that the antiserum was directed to the phenolic glycolipid and not to any other bacterial product. The presence of *M. leprae* on tissue slides of infected armadillo tissue, prepared by pressing an impression onto glass slides (Kawamura, 1977), was then observed using the rabbit anticomplex antiserum by means of an indirect immunofluorscent antibody procedure (Barrow and Brennan, 1982). The highest dilution for which + 3 to +4 fluorescence could be observed was 1:64 and peak titre was at 3 weeks postinoculation. In the infected armadillo tissue, not alone did rod-shaped bacilli flouresce but also large patches of the surrounding tissue. The results clearly indicate that the phenolic glycolipid occupies a superficial location on the *M. leprae* cell and also appears in adjoining regions. Young *et al.* (1984) in an elegant study using anti-glycolipid monoclonal IgM have recently arrived at similar conclusions.

The anti-glycolipid rabbit antiserum and the indirect immunofluorscent procedure have also been used as an immunochemical probe to observe *M. leprae* following phagocytosis (Hooper, L. C, Barrow, W. W., Cho, S-N. and Brennan, P. J., unpublished results). Adherent cells were allowed to phagocytose irradiated *M. leprae* and then treated with antiglycolipid antiserum followed by goat antirabbit IgG fluorescein conjugate. Examination using incident-light excitation with exciter-barrier filter and reflection combination showed that the injected bacilli had retained their glycolipid capsule.

M. leprae as an obligate intracellular pathogen is capable of surviving and replicating within the cells of the monocyte-macrophage system, and the mechanism by which it resists destruction by phagocytes is not at all understood. Possibilities in the context of oxidative killing are: an ability to combat phagocytic microbicidal activity by inhibiting the respiratory burst; an inability to stimulate the production of reactive oxygen metabolism; prevention of the interaction of the oxygen metabolites with the bacillus following phagocytosis; or resistance to the oxygen metabolites. A disarrangement of non-oxidative antimicrobial systems is also possible. The evidence summarized here of a capsule composed of a surfeit of chemically inert phthiocerol-containing lipid highly suited for the role of passive protectors of the resident bacterium, clearly implicate those substances in resistance to intracellular degradation. Hence, the phthiocerol-containing lipids may be regarded as virulence factors in leprosy; their chemical comportment with a considerable degree of natural methyl substitution in sugar, phthiocerol and acyl substituents alike should neutralize the attention of toxic oxidative metabolites or host degradative enzymes. A possible explanation of the vast

quantities of this material surrounding globi of bacilli is that they represent the skeletal remains of past bacilli, the sole survivors of lysosomal degradation of the cell wall from earlier cells which help perpetualize the remaining persistant bacilli.

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