Further Specific Extracellular Phenolic Glycolipid Antigens and a Related Diacylphthiocerol from *Mycobacterium leprae*

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*Mycobacterium leprae* in infected armadillo tissue produces extracellular phthiocerol-containing lipids in amounts well in excess of the bacterial mass. The principal component (1.38 mg in 1 g of liver, wet weight, containing 3.7 \( \times 10^{10} \) *M. leprae* bacilli) consists of a mixture of two phthiocerol homologs, 3-methoxyl-4-methyl-9,11-dihydroxystearo compound and 3-methoxyl-4-methyl-9,11-dihydroxystearo compound, assuming certain enantiomeric configurations for the sugar substituents; the \( R \)-acyl functions are identical with those in the diacylphthiocerol. Phenolic Glycolipid-III reacts in enzyme-linked immunosorbent assays with sera from patients with leprosy and with rabbit antiserum raised against whole *M. leprae*. The phthiocerol-containing lipids may be synonymous with the electron transparent capsules of *M. leprae*, and their unreactive state may confer on them the role of passive protectors of the bacillus.

\[
\begin{align*}
\text{OCH}_3 \\
\text{CH}_3 \text{-(CH}_3)_n \text{-CH-CH-CH-CH-(CH}_3)_n \text{-CH-CH-CH-CH-CH}_3 \\
\text{OH} \text{ OH} \text{ CH}_3 \\
\end{align*}
\]

\( n = 16 \) or 18

in which the hydroxyl functions are acylated by a mixture of three "mycoseric acids": 2,4,6,8-tetramethylhexacosanoate, 2,4,6,8-tetramethylheptacosanoate, and 2,4,6,8-tetramethyltriacontanoate. The structures were established by saponification of the native lipid, direct probe electron impact- or chemical ionization-mass spectrometry of the phthiocerol or its permethylated derivative, and gas-liquid chromatography-electron impact-mass spectrometry of the m at the fatty acids. In addition to the previously reported *M. leprae*-specific triglycosylphenolacidyl phthiocerol (Hunter, S. W., Fujiwara, T., and Brennan, P. J. (1982) J. Biol. Chem. 257, 15072-15078), the extracellular products contain small amounts (about 60 \( \mu \)g/g of infected liver, wet weight) of two other phenolic glycolipids, one of which (Phenolglycopipid-III) has been structurally elucidated,

\[
6-O-Me-Glcp(\beta1\rightarrow4)2,3-di-O-Me-Rhap(a1\rightarrow)
\rightarrow2)3-O-Me-Rhap a1\rightarrow
\text{phenol-CH}_2-(\text{CH}_3)_n,
\]

\[
\text{OCH}_3 \\
\text{CH}-\text{CH}_2-\text{CH-(CH}_3)_n-\text{CH-CH-CH}_2-\text{CH}_3 \\
\text{OR} \text{ OR} \text{ CH}_3
\]

For some time, we have been interested in the typing antigens of *Mycobacterium*, their structures, physiological and pathogenic roles, and use as specific tools in the serodiagnosis of infections (1-4). The discovery in *Mycobacterium leprae* of a phenolic glycolipid (Phenic Grolipid-I) with an oligosaccharide entity unique to that species was a landmark event in that it allowed a facile chemical-based delineation of *M. leprae* from all other mycobacteria (5-7). Moreover, its activity against lepromatous leprosy sera promised an antigen with the capability of sensing leprosy prior to the onset of overt symptoms. Furthermore, the fact that quite large quantities of the glycolipid were secreted into the tissue milieu surrounding the foci of infection suggested an involvement in the pathogenesis of *M. leprae*. We now report the presence amid these secretions of even greater quantities of a diacylphthiocerol with a structure akin to that which is part of Phenolic Glycolipid-I. Moreover, small quantities of other antigenic phenolic glycolipids have been isolated from the *M. leprae* secretions, characterized to varying extents, shown to differ from Phenolic Glycolipid-I in the nature of the oligosaccharide substituents, and demonstrated to be reactive against anti-*M. leprae* rabbit serum and that from humans with symptoms of disease.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Isolation of Diacylphthiocerol**—Total CHCl_3/CH_3OH soluble lipid from infected tissue was extracted with ether, and ether-soluble lipids were fractionated on columns of silicic acid.

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\( ^1 \) Portions of this paper (including "Experimental Procedures," part of "Results," Figs 5-12, and Footnote 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-3053, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

\( ^2 \) The abbreviations used are: Phen GL, phenolic glycolipid (meant to replace the nebulous term "mycoside"); Me, methyl; GLC, gas-liquid chromatography; ELISA, enzyme-linked immunosorbent assay; MS, mass spectrometry; EI, electron impact; CI, chemical ionization; AF, acid fast bacilli; PBS, phosphate-buffered saline; Gelp, glucopyranose; Rhap, rhamnopranose.

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acid (7). TLC of proceeds from the CHCl₃ eluate, in CHCl₃ as solvent, showed a major lipid not obvious in the more polar solvent ether/aceton (8:2) used previously (7); this lipid was not present in tissues from uninfected armadillos. The CHCl₃-eluted lipids were applied to a second column of silicic acid in hexane, and irrigation with 2 bed volumes of hexane removed all of the major lipid. It was purified to homogeneity by preparative TLC in CHCl₃. 123 mg of the lipid were present in 89 g of infected liver, wet weight (3.7 × 10¹⁰ acid fast bacilli/g of tissue), i.e. 1.38 mg/g of liver, wet weight, or 4.98 mg/g of liver, dry weight, assuming 27.7% of the liver is dry weight (6). These figures are to be compared to 0.25 mg of Phen GL-1/g of liver, wet weight, or 0.90 mg/g of liver, dry weight (6), when corrected for equal acid fast bacilli content.

**Structure of the Diacylphthiocerol—**The chromatographic properties of the apolar lipid in CHCl₃ suggested a phthiocerol dimycocerosate or a phenolic phthiocerol dimycocerosate (13). However, the infrared spectrum (Fig. 1A), marked by deep carboxy absorption at 1740 cm⁻¹, a strong band at 1380 cm⁻¹ due to several methyl branches, ether absorption at 1100 cm⁻¹, and the absence of hydroxyl group absorption in the region of 3600 cm⁻¹, was in accordance with a diacylphthiocerol, and indeed was similar to published spectra of phthiocerol dimycocerosate (13–15). In particular, there was an absence of specific peaks at 1515, 1588, and 1618 cm⁻¹, and of ultraviolet absorbing maxima which, if present, would suggest a phenolic ether (13, 14, 16). According, a diacylphenolicphthiocerol was ruled out.

The putative diacylphthiocerol (24 mg) was dissolved in 500 μl of benzene and refluxed with 1 ml of 15% KOH in methanol. The washed (9) organic phase was subjected to TLC in CHCl₃. Two products were obtained and both were more polar than the original (Fig. 2A). The slower moving product (Rₓ = 0.06), the presumed phthiocerol, was detected by orcinol/H₂SO₄, and a faster moving product (Rₓ = 0.18), the fatty acids, could be detected by H₂SO₄/dichromate with charring. Both of these products were isolated by preparative TLC; yields from 24 mg of native lipid were 7.2 mg of fatty acids and 8.2 mg of phthiocerol. Infrared spectroscopy of the phthiocerol (Fig. 1B) showed the emergence of hydroxyl group absorption at about 3400 cm⁻¹ and the loss of ester group absorption.

Electron impact-mass spectrometry of the phthiocerol (Fig. 3A) yielded the most useful information in the lower middle mass range. The position of the diol system with respect to the methyl branch and methoxyl group was determined and shown to be as expected based on the phthiocerols from other mycobacterial species (17, 18). Cleavage α to the hydroxyl groups yielded the expected ions (m/z 187 and m/z 231), and most of the remaining fragments were of secondary origin resulting from loss of methanol and water from the primary fragments. For example, m/z 199 is m/z 231-CH₃OH, and m/z 181 and 158 are due to further loss of 1 and 2 mol of H₂O, respectively. The m/z 187 fragment gives rise to m/z 155 and m/z 137 on loss of CH₂OH followed by dehydration. The upper middle mass range yielded less explicit information, a problem shared by others in different contexts (18). In particular, the length of the phthiocerol backbone could not be unequivocally ascertained.

Previously, we had found that the permethylated diacylphenolic phthiocerol derived from Phen GL-I yielded a simple mass fragmentation pattern with relatively little secondary fragmentation (7). Hence, the phthiocerol (8.0 mg) was methylated, purified by preparative TLC (recovery, 3.6 mg) (Fig. 2B), and examined by chemical ionization-mass spectrometry (Fig. 3B) with NH₃. The derivatized phthiocerol did accept a proton from the ammonium ion, but apparently did not form M+ 18 ions. As in the case of the undervatized phthiocerol, an intense base peak at m/z = 73 was evident, arising from cleavage between carbons 3 and 4, and is due to the CH₃-CH₃.CH(OH)Me⁺ ion (17). The high mass range (m/z > 400) was also informative; two M+ 1 ions, at m/z 499 (11%) and m/z 527 (7.1%), were evident. Large primary fragments were also observed in the upper middle mass range at m/z 311, 283,
Phthiocerol-containing Lipids of M. leprae

Fig. 3. Mass spectra of phthiocerol by electron impact-mass spectrometry (A) and permethylated phthiocerol by chemical ionization-mass spectrometry with NH₃ (B).

201, and 137. One can with confidence assign m/z 311 and m/z 283 to the ions CH₃(CH₂)₉-OCH₃ and CH₂(CH₂)₈-OCH(OCH₃)₂, respectively, and therefore define the chain length of the two phthiocerol homologs. Indeed, the data are consistent with the presence of a mixture of two phthiocerols, 3-methoxy-4-methyl-9,11-dihydroxyoctacosane (9,11-octacosadiol,3-methoxyl-4-methyl) and 3-methoxy-4-methyl-9,11-dihydroxytriacontane (9,11-triacontadiol,3-methoxyl-4-methyl).

The fatty acids of the acylated phthiocerol were examined by GLC (Fig. 4) and gas-liquid chromatography-electron impact-mass spectrometry of the methyl esters. The composition was qualitatively identical with that of Phen GL-I (7), although there were slight quantitative differences. Accordingly, the acyl functions of the diacylphthiocerol are composed of a mixture of the C₉₀ 2,4,6,8-tetramethylhexacosanoate, the C₉₀ 2,4,6,8-tetramethyloctacosanoate, and the C₉₄ 2,4,6,8-tetramethyltriacontanoate.

DISCUSSION

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 (21, 22). Early investigators of the microscopic properties of M. leprae noted material ("capsular matrices," "transparent halos," and "sheaths") which bound the organism into clumps and "globi" (23–25), and debated whether they were of true bacterial origin. Hanks (26), from cytological evidence and the fact that such materials were confined to the leprosy bacillus and disappeared during sulfone 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." Recently, in a series of elegant ultrastructural analyses, Fukunishi et al. (27) redefined the electron transparent zone and inferred that it and 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 (28), macrophages of nude mice (27), or M. leprae-infected armadillos (29). 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 probably lipid in nature and of mycobacterial
Whether the phthiocerol-containing lipids define the full contribution of mycobacterial lipids to the electron transparent zone is a moot question. It is quite likely that mobilized host neutral lipid (cholesterol and cholesteryl esters) should also contribute. Perhaps one will also encounter in the zonal region phosphomannosinosides, Wax D, cord factor, and perhaps sulfolipids (for a review see Ref. 33). Mycobacteria are akin to Gram-positive bacteria in that the basic cell wall architecture is of a peptidoglycan covalently linked via phosphodiester bridges to a massive arabino-galactan (which replaces the more conventional teichoic acids). However, unlike Gram-positive bacteria, the carbohydrate polymer is extensively acylated with mycolic acid residues, creating an environment that is not a suitable haven for extramembranous proteins. Thus, in our opinion, a search for specific cell wall protein antigens in M. leprae and other mycobacterial species is futile in view of the unfavorable cell wall milieu.

Although the search for lipid-based immunodeterminants in Mycobacterium is of recent inception (1–4), others have pursued species-specific protein antigens for a considerably longer period. Harboe and Closs (34), in recently reviewing these efforts, precisely defined the criteria to be met in order to accept an antigen as M. leprae-specific and concluded that no one had “so far been able to demonstrate any M. leprae-specific protein antigen.” Clearly, mycobacteria in reactions with its external environment favor lipids of myriad structures, such as cord factors as agents of toxicity, sulfolipids as inhibitors of phagosome-lysosome fusion, and Wax D as an immunopotentiator (33). In anatomical terms, one can envisage all of these substances as soluble in “a sea” of arabinomycoly residues and thus their appearance among the outer integuments of M. leprae would not be surprising. In macrophages, M. leprae has been observed not alone in phagolysosomes but also free in the cytoplasm outside the vacuolar system (35), and the bacillus may also actually inhibit phagosome-lysosome fusion (36), thus providing an alternative means for self-protection.

Most of the considerations presented above on the nature of M. leprae secretions are probably also directly applicable to human leprosy. Young (37) has recently examined by chromatographic means the lipid composition of small portions of human skin biopsy from lepromatous leprosy patients, and, in the light of the retrospective advantage of the present and earlier (7) work, one can conclude that diacylphthiocerol and a glycolipid (presumably Phen GL-I) are present in amounts far in excess of that to be found in the bacilli alone.

The varieties of diacylphthiocerols now recognized in M. leprae are different from those previously isolated from M. tuberculosis and Mycobacterium bovis in that the cluster of 16 and 18 methylene groups in the phthiocerol substituent is less than the 20–22 typically encountered in the other pathogens; in the typical example of M. tuberculosis, these are 9,11-dotriacontanediol and 9,11-tetracontanediol-3-methoxy-4-methyl, (17, 18, 33) compared to the 9,11-octacosanediol-3-methoxy-4-methyl and 9,11-triacontanediol-3-methoxy-4-methyl in M. leprae.

Despite the advent of the armadillo as a source of M. leprae, supplies are limited and must be carefully husbanded. Hence, the recognition of an M. leprae-specific determinant in Phen GL-III, simpler in structure than its predecessor, Phen GL-I, presents the opportunity of a more facile synthesis of an artificial antigen which would be a valuable diagnostic and

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* Currently being synthesized in conjunction with T. Fujiwara (Institute for Natural Science, Nara University, Japan) and G. O. Aspinall (York University, Downsview, Ontario, Canada).
We thank Dr. Draper for his generosity through Dr. Philip Draper from the United Nations Disease Program/World Bank/World Health Organization Special Program (Immunology of Leprosy) for Research and Training in Tropical Diseases. Our stocks of “supernatant lipids” were supplemented by material obtained by material obtained through Dr. Philip Draper from the United Nations Disease Program/World Bank/World Health Organization Special Program (Immunology of Leprosy) for Research and Training in Tropical Diseases. We thank Dr. Draper for his generosity.

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SUPPLEMENT

To: Further Specific Extracellular Phenolic Glycolipid Antigens and a Related Dialkyldiphosphatidylglycerol from Mycobacterial Cells

By: Shirley W. Hunter and Patrick J. Brennan

EXPERIMENTAL PROCEDURES

Materials - The source and other details of M. leprae-infected amoeba liver s (6-7) were described previously. Lipids were dissolved in chloroform, a mixture of methanol in chloroform, or methanol (8). Gas chromatography and derivatization materials were obtained from Supelco, Inc., Bellefonte, PA. Phthiocerol plates were obtained from Millipore. St. Louis, MO and Johnson-Matthey, Demer, Co., respectively. Silica gel 60, 63 to 200 mesh (tachypyl) was obtained from E. Merck, Darmstadt, W. Germany. Gas chromatographic and derivatization materials were obtained from Snyder, Inc. Bellfonte, PA. Phthiocerol plates were obtained from Millipore, St. Louis, MO.

Chromatography and Spectroscopy - Initial separation of ethereal-soluble lipids derived from total lipid (7) was performed on columns of silica acid/Cellite (8) in chloroform, proceding to mixtures of methanol in chloroform, methanol, and water. The polar lipids were isolated and described described previously (8). Lipids were further resolved on silica gel thin layer plates coated with chloroform (7), or with water in the case of preparative chromatograms. Supernats were resolved on cellulose thin layer plates in 1-lactic acid/95:5 aqueous (3:1:3) as described by Stellner &. (10).

RESULTS

Early Stages of the 10% 6-step, followed by a mixture of -11 and -III in the later stages. The three phenolic glycolipids are compared by TLC in Figure 5.

Phen-GL I

Phen-GL II

Phen-GL III

Footnote

* data were provided by Drs. M. H. Kaplan, R. M. Golner, J. T. Douglas, and T. N. Baer. A more extensive study of the correlation of anti-phenolic glycolipid activity with disease spectrum and drug regimen is now under way in conjunction with the above.

Figure 3. TLC of native Phen GL-I and a mixture of Phen GL-II and -III. Chromatography was developed in ether/acetone (8:1) and lipids isolated by 1N HCl in 80% BPO.

Figure 4. Proton NMR of Phen GL-I and -III at 360 MHz in CDCl3.
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A TLC of A. native Phen GL-I (1); decylated Phen GL-I (2); decylated Phen CL-I (3); and decylated Phen GL-II (4). Chromatogram was developed in CHCl₃/CH₃OH/H₂O (45:5:0.5) and i-2-pdms located with 1% orcmol in 40% H₂SO₄.

The TLC of A. native Phen CL-I (1); decylated Phen CL-I (2); deacylated CHcl₃/CH₃OH (45:5:0.5) and Itpads located with 1% orcmol in 40%

The TLC of A. native Phen GL-I (3); and deacylated Phen GL-II (4). Chromatogram was developed in diethyl ether.

The TLC of A. native Phen GL-I (3); and deacylated Phen GL-II (4). Chromatogram was developed in diethyl ether.

Figure 7. TLC of A. native Phen GL-I (1); decylated Phen GL-I (2); decylated Phen CL-I (3); and decylated Phen GL-II (4). Chromatogram was developed in CHCl₃/CH₃OH/H₂O (45:5:0.5) and i-2-pdms located with 1% orcmol in 40% H₂SO₄.

The TLC of A. native Phen GL-I (3); and deacylated Phen GL-II (4). Chromatogram was developed in diethyl ether.

Figure 8. TLC of the alditol acetates derived from Phen GL-I and Phen GL-II. TLC was conducted on a 1.8-m stainless steel column of 3% SP-2340 on 100/120 Superport at 200°C.

Figure 9. EI-MS of the alditol acetate of 4-O-Me-glucose derived from Phen GL-II. The sequence of sugars in Phen GL-II was established by grased acid hydrolysis, isolation of the partially degraded glycolipid by preparative TLC, and sugar analysis by GLC. The results (Figure 10) clearly suggest the sequence:

<table>
<thead>
<tr>
<th>m/z</th>
<th>Retention Time (min)</th>
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<tbody>
<tr>
<td>313</td>
<td>10</td>
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<tr>
<td>309</td>
<td>20</td>
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<tr>
<td>305</td>
<td>30</td>
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<tr>
<td>301</td>
<td>40</td>
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Figure 10. Sequencing of Phen GL-II oligosaccharide by partial hydrolysis and GC analysis. Phen GL-II was hydrolyzed in 3.0 M HCl at 105°C for 1 hr and the partially hydrolyzed glycolipids were isolated by preparative TLC. Alditol acetates were prepared from each glycolipid and analyzed by GLC (right) as described in Figure 8. 1. Obtained Phen GL-II; 2. diglycosyl phenolic lipid; 3. monoglycosyl phenolic lipid. TLC (left) was developed in diethyl ether/acetone (9:1) and sprayed with 1% orcmol in 40% H₂SO₄.

Figure 11. GLC/EI-MS of the alditol acetate derived from perdeuterioacetylated Phen GL-I. A 1.8-m stainless steel column of 3% SP-2340 on 100/120 Superport at 200°C, spectra were recorded at an ionization potential of 70 eV.

Figure 12. GLC/EI-MS as applied to Phen GL-II. Proprionate microtubes were coupled with the glycolipid dissolved in deoxycholate buffer. Rabbit sera were diluted 1/1000. Human sera 1/100 and conjugate 1/100. Other conditions are described under "Experimental Conditions." Left panel: A. pre-immune rabbit serum. B. serum collected 1 day after 1 week of intramuscular inoculation with whole irradiated M. leprae. Right panel: Numbers refer to random sera from individuals showing the various clinical forms of leprosy, except serum No. 1, which is from a non-symptomatic individual.
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