Earlier we reported on the presence of a specific phenolic glycolipid (Phenicolic Glycolipid-I) in Mycobacterium leprae, and in infected armadillo tissues (Hunter, S. W., and Brennan, P. J. (1981) J. Bacteriol. 147, 728–735). It had an inherent oligosaccharide, composed of 3-O-Me-rhamnose, 2,3-di-O-Me-rhamnose, and 3,6-di-O-Me-glucose, glycosidically linked to the phenol substituent. The structure of the oligosaccharide has now been determined, by partial acid hydrolysis, per methylation, ‘H NMR, and 13C NMR as:

\[
\text{OCH}_3
\]

\[
\text{H}_3\text{O}^+ - \text{CH}_3 - (\text{CH}_2)_{11} - \text{CH} - \text{CH} - \text{CH} - (\text{CH}_2)_{11} - \text{CH} - \text{CH} - \text{CH}_2 - \text{CH}_3
\]

OH

OH

CH_3

Combined gas-liquid chromatography-mass spectrometry showed three tetra-methyl branched "mycocerosic" acids, Cs0, Cs2 and Cs4, with molecular weights (as methyl esters) of 466, 494, and 522, respectively. These are esterified to the hydroxyl functions of the branched glycolic chain. Evidence is also presented that the glycolipid is immunologically active, reacting with rabbit antisera to *M. leprae* and with sera from lepromatous leprosy patients.

Species-specific antigens have long been sought in *Mycobacterium leprae*, to be used for subclinical diagnosis of leprosy and to differentiate the disease from tuberculosis and other infections caused by a plethora of environmental mycobacteria. Some investigators have concentrated on protein antigens (1, 2) in the belief that *Mycobacterium*, in a manner analogous to many Gram-positive and Gram-negative bacteria, produce characteristic cell wall proteins. Impressed by the array of mycobacterial cell wall lipids of myriad biological activities (3), we have looked among them and found evidence of species-specific antigenicity (4). Our recent evidence of a phenolic glycolipid in *M. leprae* with a unique sugar arrangement (5) offered the promise of a chemically defined antigen that could become an important diagnostic tool for leprosy. We now report on the structural elucidation and serological activity of this glycolipid, thereby further substantiating its distinctiveness and antigenicity. A brief report on some of this work has been published (6).

**EXPERIMENTAL PROCEDURES**

*M. leprae* Infected Armadillo Tissue—Livers and spleens from infected *Dasypus novemcinctus* were obtained from E. E. Storrs, Florida Institute of Technology, Melbourne, FL, and W. F. Kirchheimer, United States Public Health Service Hospital, Carville, LA, through the auspices of D. D. Gwinn, Leprosy Program Officer, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Annuals had been inoculated with *M. leprae* (usually 10^6 acid fast bacilli intravenously) and sacrificed, usually two years later, with the onset of disseminated leprosy (7, 8). Livers used in the present work contained on average about 4 X 10^8 acid fast bacilli/g, wet weight, of tissue, and spleen contained about 8 X 10^6 bacilli/g. Organs (30 g) were usually irradiated (2.5 Mrads; 137Cs), homogenized on ice for 3 min in 120 ml of buffer composed of 0.135 M NaCl, 0.2 M Tris, and 0.01 M MgSO4 (9) using the Sorvall Omnimixer 17106 (DuPont Instruments, Newton, CT). The homogenate was centrifuged at 10,000 X g for 10 min. The *M. leprae*-free supernatant was the major source of the *M. leprae*-specific phenolic glycolipids. "Live" or dead *M. leprae*, depending on whether or not the organ had been irradiated, were obtained from the pellet, as described by Draper (9).

1 Purification of Phen GL-I—The present procedure is a modification of one described previously (5). On this occasion, *M. leprae* was not used as a source of glycolipids. The supernatants from homogenized infected organs (10 g) were extracted by the Bligh-Dyer monophasic extractant (10), the residue being then re-extracted with 100 ml of CHCl3/CH3OH (2:1) at 50 °C overnight. The pooled extracts were washed (11), evaporated to dryness, and tributylated with 10 ml of diethyl ether. Dried ether-soluble lipids were dissolved in CHCl3 and applied to a column (1.1 X 14 cm) which was successively irrigated with two bed volumes each of CHCl3 and 2% CH3OH in CHCl3. The lipid in the latter eluate was re-applied to a similar column, eluted likewise, and 1-ml fractions analyzed by TLC. The glycolipid was finally purified by preparative TLC in ether/acetone (8:2). Supernatant was centrifuged at 10,000 X g, for 10 min. The *M. leprae*-free supernatant was the major source of the *M. leprae*-specific phenolic glycolipids. "Live" or dead *M. leprae*, depending on whether or not the organ had been irradiated, were obtained from the pellet, as described by Draper (9).

1 The abbreviations used are: Ac, Acetyl; AFB, acid fast bacilli; Phen GL, phenolic glycolipid; Me, methyl; Rhap, rhamnopyranosyl; Glcp, glucopyranose; GLC, gas-liquid chromatography; MS, mass spectrometry; TLC, thin layer chromatography; AFB, acid fast bacilli; PBS, phosphate-buffered saline; pm, parts per million; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay.

1 Irrigation of the column with 5% methanol in CHCl3 removed two sparse more polar phenolic glycolipids (Phen GL-II and -III).
tants from homogenates were also lyophilized, in which case the Bligh-Dyer procedure was by-passed, and extraction with CHCL/CHOH (2:1; 100 ml/10 g of tissue) was applied, followed by a Folch biphasic wash (11).

Preparation of Deacylated Phen GL-I, Fatty Acids, and the Phenolic Phthiocerol Core—The procedures were based on those described by Leclercq and Gantambodi (22) and Sarda (13). Phen GL-I was hydrolyzed with 10% NaOH in benzene/methanol (1:2; 1.5 ml) in a sealed tube under N₂ at 100 °C for 18 h. Lipids were extracted with CHCl₃ from the water-diluted, acidified (pH 4) mixture. The washed (11) CHCl₃ phase was applied to a column of silicic acid/Celite (2:1) which was washed with 20 ml to elute fatty acids, and secondly, with 5% CH₂Cl₂ in CHCl₃ to remove the deacylated Phen GL-I. Methanolysis (1 N methanolic/HCl, 100 °C, 5 h) of the deacylated Phen GL-I removed sugar residues, and the phenolic phthiocerol core was recovered in the CHCl₃ phase after partitioning (11).

Methylations—Deacylated Phen GL-I (3.5 mg) was dissolved in 0.5 ml of dimethylsulfoxide (Pierce Chemical Co., Rockford, IL), followed by the addition of 0.7 ml of 2.5 N methylsulfinyl carbamion (14). The reaction mixture was stirred at room temperature under N₂ for 1 h, followed by the addition of 0.7 ml of CH₂Cl (Pierce Chemical Co.) at 0 °C, again stirred for 4 h. H₂O (0.5 ml) was added and the mixture extracted with 2 × 5 ml of CH₂Cl₂. The combined CH₂Cl₂ phases were washed with water, evaporated to a syrup, and applied to a column (1 × 65 mm) of Sephadex LH-20 (15).Fractions (0.5 ml) were examined by TLC in ether/acetone (9:1) for the detection of two. Such two fractions (9:1; 0.35), in fractions 12–17, were observed. Both were isolated by preparative TLC. The faster one was the fully methylated Phen GL-I and was subjected to further analyses. Infrared evidence of hydroxyl group absorption indicated that the slower product was incompletely methylated. The phenolic phthiocerol core of Phen GL-I was permethylated in similar fashion, except that the reaction mixture was extracted with hexane, and final purification by preparative TLC was accomplished with ether/hexane (8:2).

Analytical and Other Chemical Procedures—TLC was conducted on commercial Silica Gel G plates (Redplates, Fischer Scientific Co., Pittsburg, PA). Ether/acetone (8:2) was the routine TLC solvent for the native Phen GL-I. Lipids on preparative plates were located through their opacity under intense light or after gentle spraying with water. Bands were excised with a razor blade, and lipids recovered by elution with CHCl₃/CH₂Cl₂ (2:1). Orcinol/H₂SO₄ (5) was used to locate glycolipids on analytical plates, and CH₂Cl₂/dichromate was used for sugar-free lipid products. Except where indicated, intact Phen GL-I and the deacylated and permethylated products were hydrolyzed by 2 M CF₃COOH at 100 °C for 3 h. Acid was removed by evaporation with N₂. Prior to cellulose TLC of sugars, hydrolysates were mixed with an equal volume of methanol, and the aqueous phase passed through a small column (1 × 6 cm) of mixed cation and anion exchange resins (MB-3, Mallinkrodt Chemical Works, St. Louis, MO). Partial acid hydrolysis of Phen GL-I was accomplished by suspending the glycolipid by sonication in 0.3 N HCl and hydrolyzing at 100 °C for 2 h. Acid was removed at room temperature by evaporation with N₂. Reducing sugars were resolved by cellulose TLC (E. Merck, Darmstadt, W. Germany) in 1-butanol/pyridine/water (6:4:3) and stained with aniline-oxalate. Alditol acetates were prepared and separated by GLC, as described before (5). The sources or synthesis of the authentic O-methyl sugars used for comparative purposes have been described previously (16, 17). GLC-MS was routinely performed using compounds cited in the text and a Finnigan Model 3200E quadrupal system coupled to a Finnigan Model 6000 data and graphic output, operating with an inlet temperature of 250 °C, an ionization potential of 70 eV and an ion source temperature of 250 °C. Mass spectra by direct insertion probe were obtained with an Associated Electrical Industries MS-12 instrument with a probe temperature gradient of 100–250 °C. "H NMR and "C NMR spectra were recorded with a Nicolet NT-360 spectrometer at a concentration of 4–5 mg/ml for "H NMR and 15–16 mg/ml for "C NMR.

Immunological Procedures—M. leprae (10 mg), purified from armodiobol tissue (No. 222, 9.6 × 10⁹ AFB/g), as described above, was sonicated in 1.5 ml of saline using a Savant (Hicksville, NY) Micerator Model 1000 with an Ultrasonics Heat Systems (Plainview, NY) bath and then emulsified in 1.5 ml of incomplete Freund's adjuvant in a Vortex (15) homogenizer for 5 min. The water-in-oil emulsion was injected intramuscularly or intradermally over a wide anatomical area (18) into young (4-month-old) California Satin rabbits. The intramuscular injection was followed by two similar boosters at weekly intervals. Rabbits were bled before the initial inoculation and then at intervals of 3, 5, and 7 weeks after the last inoculation. Serum was separated and stored at −20 °C without preservative.

ELISA was conducted on polycytoplasmide plates (Dynatech Laboratories, Alexandria, VA) using a distillation of techniques derived from Volker et al. (19) and Reggiardo et al. (20). CHCl₃/CH₂Cl₂ (1:1; 1 ml) of the lipid was added (b.p. 60 °C) (1 mg/ml; 5 ml) were applied to wells and dried at 37 °C for 10 min. Wells were washed with 0.1% Tween 80 in PBS, followed by the addition of serum (50 μl) at the appropriate dilution. After further washings, horse-seradish peroxidase (30 μl) conjugated to goat anti-rabbit IgG (Sigma) diluted 1:1000 in PBS/Tween was added, followed by a 45-min incubation and additional washings. Finally, 50 μl of H₂O₂-O-phenylenediamine substrate in citrate-phosphate (19) buffer was added, followed by a 60-min incubation. The reaction was terminated with 2.5 N H₂SO₄ (50 μl) and the absorbance read at 498 nm. Sera from normal and M. leprae-infected armadillos were obtained from E. E. Storrs. Serum from a lepromatous patient was kindly provided by R. H. Gelber, Mary's Help Hospital and United States Public Health Service Hanson's Disease Project, San Francisco, CA, and mixed lepromatous sera was kindly provided by J. T. Douglas, University of Hawaii, Honolulu. Immunodiffusion of liposomes containing the authentic phen GL-I against rabbit serum was conducted as described by Payne et al. (21). Liposomes contained sphingomyelin (Supelco, Inc., Bellefonte, PA), cholesterol, dictetyl sulfate (Sigma), and Phen GL-I in the following amounts 2.0, 0.73, 0.065, and 0.23 mg in 125 μl of Tris-buffered saline (0.15 M NaCl, 20 mm Tris, pH 8.0). Liposomes were sonicated (about 1 h) until the vesicles were uniformly small as shown by light microscopy. Agarose (Indubiose A45, L'Industrie Biologique, Société Anonyme, 92-Gennevilliers, France) gels were composed of 0.6% in Tris-buffered saline.

RESULTS

Purification of Phen GL-I—Earlier studies (5) indicated the presence in M. leprae and infected armadillo tissue of a novel glycolipid, related to phenolic "mycoside A" (13) but unique in its carbohydrate composition. Isolation and preliminary characterization was greatly facilitated by the surprisingly large quantities of the glycolipid in tissue, even after removal of all visible traces of the bacteria (2.2 mg/g, dry weight, from liver with 9 × 10⁹ AFB/g). Organs with a high bacterial load (10⁶-10⁷ AFB/g) contained a much greater abundance of the M. leprae-specific lipid than those of lower titer and, moreover, host lipids did not substantially interfere with the isolation. However, in livers with a load of 10⁶-10⁷ AFB/g, partially purified preparations of Phen GL-I were contaminated with a white substance of armadillo origin (probably cholesterol). The glycolipid could be preferentially removed with hexane, due to its solubility as opposed to the relative insolubility of the contaminant. Accordingly, the partially purified Phen GL-I, present in the 2% methanol eluate from silicic acid, was extracted with a small quantity (1–2 ml) of hexanes, before the final TLC step. Recovery of glycolipid from whole infected liver, from the supernatant arising from homogenized liver, and from the bacillus itself, is shown in Table I. Obviously, the supernatant is the most sensible and economical source, since the bacillus itself remains unaffected and may be used for other purposes.

Sequence of Sugars in Phen GL-I—Based on GLC-MS of the native sugars of Phen GL-I, and the demethylated parental sugars, we had concluded that Phen GL-I contained a 2,3-di-O-Me-rhamnose, a 3,6-di-O-Me-rhamnose, and a 3,6-di-O-Me-glucosamine (5). To establish the sequence of sugars in Phen GL-I, 10 mg of the lipid was suspended in 0.5 ml of 0.3 N HCl and hydrolyzed at 100 °C for 2 h. TLC of the products in ether/acetone (9:1), and spraying with orcinol/H₂SO₄ showed a simple pattern of two major glycolipids (Rₖ 0.7 and 0.4), in addition to the unhydrolyzed Phen GL-I (Rₖ 0.15), with few minor products. The two major degradation products and the unhydrolyzed glycolipid were recovered by preparative TLC and examined by analytical TLC (Fig. 1, lanes 1, 2, and 3). All
were pure. The identity of the constituent sugars of each was established by cellulose TLC of the aldose, and GLC of the alditol acetates (Fig. 1, panels 1, 2, and 3) after hydrolysis with 2 M CF,COOH. The residual unhydrolyzed glycolipid (lane 1, panel 1) contained 2,3-di-O-Me-rhamnose, 3-O-Me-rhamnose and 3,6-O-Me-glucose, as established previously (5). The degraded glycolipid of intermediate polarity (lane 2, panel 2) contained 2,3-di-O-Me-rhamnose and 3-O-Me-rhamnose. The least polar glycolipid (lane 3, panel 3) contained 3,6-di-O-Me-glucose → 2,3-di-O-Me-rhamnose → 3-O-Me-rhamnose was suggested.

To confirm the sequence of sugars and establish the intersugar linkages, Phen GL-I (15 mg) was methylated by the Hakomori procedure (13), 10 mg of the pure permethylated product being recovered after Sephadex LH-20 gel filtration and preparative TLC in ether/acetone (9:1). The permethylated glycolipid was hydrolyzed with 2 M trifluoroacetic acid and the partially methylated alditol acetates examined by GLC (Fig. 2). Three products were observed, corresponding to the acetates of authentic 3,4-di-O-Me-rhamnitol, 2,3-di-O-Me-rhamnitol, and 2,3,4,6-tetra-O-Me-glucitol. The results of electron impact MS on these peaks is shown in Fig. 3. The fragmentation pattern of Peak A was near perfect for a 1,2,5-tri-O-acetyl-6-deoxy-3,4-di-O-methyl hexitol (15) with characteristic primary fragments at \( m/e \) 189, 131, and 87. Peak B showed an excellent fit for 1,4,5-tri-O-acetyl-6-deoxy-2,3-di-O-methylhexitol (15) with typical primary fragments at \( m/e \) 203, 170, and 117, and 87. Peak C was perfect for 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl hexitol (15) with primary fragments at \( m/e \) 205, 161, and 117. The secondary fragments in each case support these designations. Thus, the following structure for the trisaccharide constituent of Phen GL-I was suggested: 3,6-di-O-Me-glucosopyranosyl(1→3)-2,3-di-O-Me-rhamnosopyranosyl(1→3)-3-O-Me-rhamnopyranosyl (1→phenol).

Anomeric Configurations-Previously, we assigned the 'H NMR anomeric signals of intact Phen GL-I to 4.24, about 4.42 and 5.45 ppm (5). Based on the 'H NMR spectra of the deacylated Phen GL-I and that of the phenolic phthiocerol core (see Fig. 7), these have now been reassigned to 4.42, 5.10, and 5.45 ppm (Fig. 4). The 4.42-ppm peak is assigned to the \( H_1 \) of 3,6-di-O-Me-glucose because of its large coupling constant (7.6 Hz). The 5.45-ppm signal is assigned to the 3-O-Me-rhamnose since the deshielding effect of the phenol substituent should ensure for the 3-O-Me-rhamnose the most downfield position of all of the anomeric protons. The remaining anomeric signal (5.10 ppm) is therefore assigned to the 2,3-di-O-Me-rhamnose. The chemical shifts of the \( H_2 \) and \( C_1 \) of 3,6-di-O-Me-glucose (4.42 and 105.5 ppm, respectively) are in good agreement with those of Me-\( \beta \)-glucoside (4.64 and 105.5 ppm) (22). The large \( H_2-H_3 \) coupling constant \( (J_{2,3} = 7.6 \text{ Hz}) \) indicates the \( \beta \) configuration, if the glucose is in the \( \beta \) enantiomeric configuration. For 3-O-Me-rhamnose, the chemical shift of \( H_1 \) (5.45 ppm) is closer to that of synthetic phenyl-\( \alpha \)-L-rhamnoside (5.52 ppm) than to that of synthetic phenyl-(2,3,4-tri-O-acetyl)-\( \beta \)-L-rhamnoside (4.74 ppm) (the \( \beta \)-acetyl groups have little effect on the chemical shift of the \( H_1 \)). Also, the chemical shift of the \( C_1 \) of 3-O-Me-rhamnose (97.4 ppm) is close to that for synthetic phenyl-\( \alpha \)-L-rhamnoside (97.8 ppm). Most importantly, the large \( C_1-H_1 \) coupling constant \( (J_{C_1, H_1} = 173.1 \text{ Hz}) \) of the 3-O-Me-rhamnose indicates an \( \alpha \)-configuration (22). As for the 2,3-di-O-methyl-rhamnose, the chemical shift of the \( H_1 \) (5.11 ppm) agrees with that of Me-\( \alpha \)-L-rhamnoside (5.04 ppm) (22), and the \( C_1 \) chemical shift (98.8 ppm) agrees with that of methyl 2,3-di-O-Me-\( \alpha \)-L-rhamnoside (99.5 ppm, calculated from Refs. 22 and 23). Again, the high \( C_1-H_1 \) coupling constant \( (J_{C_1, H_1} = 168.6 \text{ Hz}) \) suggests the \( \alpha \)-configuration, if the sugar is in the \( \alpha \) form (22). Thus, assuming that the 3,6-di-O-Me-glucopyranosyl is in the \( \beta \) configuration and the 3-O-Me-rhamnosyl and 2,3-di-O-Me-rhamnosyl are in the \( \alpha \) configurations, one can assign

![Fig. 1. Sequencing of Phen GL-I trisaccharide by partial acid hydrolysis and GLC of alditol acetates.](image)

![Fig. 2. GLC of the alditol acetates derived from permethylated Phen GL-I.](image)

*3 E. Tarelli and P. Draper have independently arrived at similar conclusions on the anomeric configurations. Details of the synthesis of the model compounds and further 'H NMR and 'C NMR data will be published in conjunction with these workers.
Glycolipid Antigen of *M. leprae*

15075

Fig. 3. Mass spectra of the alditol acetates obtained from permethylated Phen GL-I (Fig. 2). Spectra were recorded at an ionization potential of 70 eV. A, B, and C refer to the peaks in Fig. 2, in order of elution.

Fig. 4. Proton NMR of Phen GL-I at 360 MHz in CDCl₃.

the following anomeric configurations to the trisaccharide:

3,6-di-O-Me-GlcP(1→4)2,3-di-O-Me-Rhap(1→2)3-O-Me-
Rhap(1→α)phenol.

Structures of the Fatty Acids and Phenolic Phthiocerol Core of Phen GL-I—Alkali treatment of intact Phen GL-I (30 mg) yielded the deacylated Phen GL-I and fatty acids. These were separated on a column of silicic acid; the acids (9 mg)

Fig. 5. GLC-MS of the acyl methyl esters from Phen GL-I.

Saponification conditions, 10% NaOH in benzene/methanol (1:2), 160 °C for 18 h. Fatty acids were methylated with diazomethane.

GLC conditions, 1.8-m column of 3% OV-1 on 80/100 Supelcoport, temperature gradient 240-300 °C at 4 °C/min.
being removed with CHCl₃ and the deacylated Phen GL-I (12 mg) appearing in a subsequent 5% CH₂OH in CHCl₃ eluate. The fatty acid mixture was esterified with diazomethane prior to analysis. The GLC pattern (Fig. 5), log retention time, and comparison with a variety of standard fatty acid methyl esters suggested three homologs of methyl mycocerosate (see Ref. 3 for review). GLC-MS confirmed this impression (Fig. 5). The three fatty acid methyl esters gave molecular ion peaks, M⁺ 466, 494, and 522, corresponding to a homologous series of C₃₀, C₃₂, and C₃₄ mycocerosate carboxylic methyl esters. The mass spectrum of peak 2 (M⁺ 494) showed high peaks at m/e 88 and 101 (indicated in Fig. 5) and very small peaks at m/e 74, 115, and 157 (not shown) indicative of methyl side chains at positions 2, 4, 6, and 8 (24). In fact, the spectrum of peak 2 was well nigh identical to that described by Asselineau et al. (24) for the major (65%) methyl mycocerosate in the mixture isolated originally by R. J. Anderson from tubercle bacillus. From a comparison of the mass fragmentation of peaks 2 with those reported by both Asselineau et al. (24) and Gastambide-Odier and Sarda (13), it is clear that peak 2 is the methyl ester of 2,4,6,8-tetramethyl hexacosanoate and 2,4,6,8-tetramethyl triacontanoate esters. Such acids have been isolated in small amounts from various strains of mycobacteria (3, 24).

The phenolic phthiocerol core (4.9 mg) was recovered after acid hydrolysis of the deacylated Phen GL-I (9.6 mg). It was permethylated and the fully methylated lipid separated from the partially methylated product by preparative TLC in ether/hexane (8:2). Mass spectrometry of the directly inserted sample (Fig. 6) showed peaks at mass numbers 73 and 121 which are characteristic of the two termini of the methyl ether of a phenolic phthiocerol (12, 13, 25, 26). The strong fragment ion at m/e 403 indicates the presence of 17 methylene groups on one segment of the backbone, and the m/e 201 fragment ion confirms the presence of four methylene groups on the other segment of the phthiocirol backbone (25, 26). A weak parent molecular ion peak, M⁺ 618, without evidence of homologous peaks, suggests a single molecular species of molecular weight 618. This information allows us to propose a structure (Fig. 6) based on the β-glycol phthiocerol. It is now also possible to assign, with some authority, protons to many of the signals of the ¹H NMR spectrum of the phenolic phthiocerol core of Phen GL-I (Fig. 7).

**Antigenicity of Phen GL-I—**To show immunological activity in extremely apolar lipids, such as the phenolic glycolipids, is technically difficult since they are effectively insoluble in the aqueous milieu used in the usual assays. This problem has plagued us in previous efforts to relate antigenicity to Phen GL-I (5). However, ELISA, as described under “Experimental Procedures,” seemed more amenable to the presumed requirement for an oligosaccharide with an exposed orientation. Sera raised in rabbits vaccinated either subcutaneously or intramuscularly with killed whole M. leprae contain high titer

**TABLE II**

<table>
<thead>
<tr>
<th>Serum Antigen</th>
<th>ELISA reading (458 nm)</th>
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<tbody>
<tr>
<td>Rabbit, I.D., pre-immunization</td>
<td>Whole M. leprae lipid (6 µg)</td>
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<tr>
<td>Rabbit, I.D., anti-M. leprae</td>
<td>Whole M. leprae lipid (5 µg)</td>
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<tr>
<td>Rabbit, I.M., pre-immunization</td>
<td>Whole M. leprae lipid (5 µg)</td>
</tr>
<tr>
<td>Rabbit, I.M., anti-M. leprae</td>
<td>Pure Phen GL-I (5 µg)</td>
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</tr>
<tr>
<td>Human, lepromatous patient†</td>
<td>Pure Phen GL-I (5 µg)</td>
</tr>
</tbody>
</table>

* Pooled normal sera from Dr. J. T. Douglas.
† Pooled lepromatous sera from Dr. J. T. Douglas.
‡ Sera from a single lepromatous patient from Dr. R. H. Gelber.
antibodies to total lipid extracts from *M. leprae* and to pure Phen GL-I, as demonstrated by ELISA (Table II). Presumably, Phen GL-I accounts for the majority of the species-specific antigenicity associated with the lipids of *M. leprae*, since, previously, only phenolic glycolipid-containing lipid fractions were found to be serologically active (4). Moreover, ELISA demonstrated that sera from leprosy patients have antibodies to Phen GL-I. We have also shown (results not included) that liposomes containing Phen GL-I form strong precipitates in agarose gels with the rabbit antisera to whole *M. leprae*, serum from infected armadillos, and serum from lepromatous leprosy patients.

**DISCUSSION**

A chemically characterized, readily available *M. leprae* specific antigen has long been sought by leprologists to be used for diagnostic and taxonomic purposes. Not until the advent of the nine-banded armadillo as a rich source of *M. leprae* had this aspiration become realistic. In parallel work on the “atypical” nontuberculous mycobacteria, we had found that species- and type-specific antigenicity were glycolipid in essence (27) and, in the context of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* (MAIS) complex, consisted of an invariant fatty acyl peptide, of the “C-mycoside” disposition, with a species- or type-specific oligosaccharide appendage (15, 16, 17, 28). A search for C-mycoside based antigens in *M. leprae* came to naught (4) but exposed in the bacillus itself, and notably, in the surrounding tissue milieu, another class of glycolipids of the phenolic “mycoside A” class (5). The structural elucidation of the C-mycoside antigens had been greatly facilitated by the elegant work of D. W. Smith et al. (21, 29), Lancee and Asselineau (30), and Voland et al. (31); our contribution was to demonstrate that the invariant C-mycoside core was tempered by a variable oligosaccharide antigenic determinant. Likewise, as this paper indicates, in the phenolic glycolipids of *M. leprae*, an analogous narrative is unfolding. A rather common diacyl phenolic phthiocerol core, the basic elements of which were first noted by Smith et al. (29) and finally precisely characterized by Demertau-Ginsburg and Lederer (12) and Gastambide-Odier and Sarda (13), is affixed to a unique *M. leprae*-specific oligosaccharide. The core of Phen GL-I conforms in broad structural principles to the “aromatic analogue of phthiocerol” and its attached mycocerosic acids, described by the French workers (32), but is distinctive in the absence of homologs in the phthiocerol function and in the presence of shorter chain fatty acyl esters. The distinctiveness, uniqueness, and species specificity of Phen GL-I lies in the trisaccharide appendage. The only other closely related known phenolic glycolipid is the “mycoside A” from *M. kansasii* (13), and its oligosaccharide composition and TLC mobility are distinctly different (4). Accordingly, Phen GL-I appears to be a perfect chemical marker for *M. leprae*. With the exception of the enantiomeric configuration of the sugar constituents, the structure of Phen GL-I has been fully defined. The key experiment in its structural elucidation was partial acid hydrolysis. The usual approach to sequencing oligosaccharides, involving use of glycosidases and NaIO4/NaBH4, was precluded by natural O-methyl groups or recalcitrant sugars. Partial acid hydrolysis was the only amenable, simple approach and the products of it were pleasingly clear-cut and easy to analyze. In addition, the availability of surprisingly large quantities of the glycolipid in infected tissue, free of *M. leprae*, greatly facilitated structural elucidation. There are other *M. leprae* specific glycolipids in infected armadillo tissue present in sparse quantities.1 Of these (Phen GL-III) has been partially characterized and appears identical to Phen GL-I but for the presence of 6-O-methylglucopyranose in place of the 3,6-di-O-methylglucopyranose at the nonreducing terminus.2 Complete structural elucidation of the phenolic glycolipids is important in that it now allows chemical synthesis of the trisaccharide and perhaps production of an *M. leprae*-specific artificial antigen.

For a time, the antigenicity of Phen GL-I was a moot question. It was the major component and the only truly specific lipid in fractions from *M. leprae*, which were aggre-gated by, and formed precipitates with, sera from leprosy patients and infected armadillos, and displayed no reaction with sera from patients with tuberculosis or an *M. avium* infection or with serum from a normal armadillo (4). Nevertheless, the pure Phen GL-I had no demonstrable activity as measured by immunodiffusion using sonicated suspensions of the glycolipid (5). To demonstrate antigenicity in lipids is technically difficult since the majority are effectively insoluble in the aqueous milieu of most serological assays; lipids of the type described here will form aggregates in which the oligosaccharide determinant may not be exposed. However, RIA and ELISA and the reaction substrate that they employ should ensure orientation of the glycolipid in a more favorable configuration. In conjoint studies, we have developed a solid phase radioimmunoassay, containing 125I-labeled *Staphylococcus aureus* Protein A, applicable to Phen GL-I, in which antibody levels are expressed as the ratio of uptake of labeled by leprosy serum to the mean uptake by normal serum. In a limited survey using sera from untreated patients from Thailand, we have found that 9/9 patients with polar lepromatous leprosy (33), 2/2 patients with borderline lepromatous leprosy, and 10/15 patients with borderline tuberculoid leprosy, and 7/8 patients with polar tuberculoid leprosy, were positive. Thus, the overall positive rate was 80%. The mean (±S.D.) antibody level of borderline lepromatous and polar leproma-tous leprosy patients was 10.8 ± 4.6, while that of borderline and polar tuberculoid leprosy patients was 2.2 ± 1.9. The antibody level of six patients with erythema nodosum lepro-sum was 3.7 ± 2.0. Additionally, in other collaborative studies, we have demonstrated that fluorescein-labeled anti-Phen GL-I antibodies, raised against complexed Phen GL-I-MBSA, fluoresced the surface of isolated *M. leprae* and that of bacilli in situ in infected tissue.3 Payne et al. (21) have also recently used the ploy, developed by Six et al. (34), of incorporating Phen GL-I into liposomes which were then used in immuno-diffusion. The test liposomes produced precipitates with undiluted sera from three patients with active polar lepromatous leprosy, a pool of serum from several lepromatous patients, and serum from an experimentally infected armadillo. However, no precipitates were formed with sera from patients with tuberculoid leprosy (borderline lepromatous and polar tuberculoid) and, thus, RIA may be more sensitive than liposome gel diffusion. Payne et al. (21) also found that no precipitates were formed with sera from mice heavily infected with *M. leprae* or with sera from two patients with active pulmonary tuberculosis. The limited experiments described above demonstrate that ELISA, which shows several technical advantages over RIA and immunodiffusion, should be amenable for the serodiagnosis of leprosy. In particular, ELISA was particularly suitable for demonstrating the immunogenicity of the Phen GL-I when presented in its natural state, as part of the outer cell wall architecture of the intact leprosy bacillus.
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