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Peptidoglycolipid Nature of the Superficial Cell Wall Sheath of Smooth-Colony-Forming Mycobacteria

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The most superficial cell wall layer present in smooth-colony-forming mycobacteria was isolated from serovar 20 of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* (MAIS) serocomplex and examined chemically and by electron microscopy. Most (70 to 80%) of the fibrillar material consisted of an array of serologically active, acetylated C-mycosidic peptidoglycolipids with the basic structure

fatty acyl-D-Phe-D-*a*Thr-D- Ala-L-alaninol-*O*-(3-4-di-*O*-Me-rhamnose)
|
O
|
6-d-talose-[rhamnose-2-*O*-Me-fucose-2-*O*-Me-rhamnose]

but in which the location of acetyl groups and the arrangement of monosaccharides have not been defined. Apparently, all serovars within the MAIS complex are characterized by structurally related superficies in which the monoglycosyllipopeptide portion is invariable but the oligosaccharide attachment is peculiar to each serovar. These unique inert structures may be an important factor in shielding the pathogen within phagolysosomes from lysosomal enzymes.

The ability of certain pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, *M. lepraemurium*, and *M. leprae*, to persist in granulomatous lesions appears to be directly related to their capacity to survive in the host's macrophages (21). *M. lepraemurium*, in particular, is able to endure and multiply within the macrophage, even after fusion of the phagosome has taken place (7). This attribute has been correlated to the presence of a protective sheath, which apparently surrounds these mycobacteria in vivo. Electron microscopic examination of tissue infected with *M. lepraemurium* (6, 8, 11, 12) and *M. leprae* (14, 26, 27) produced evidence of an electron-transparent zone surrounding the bacillus in situ. The suggestion that these zones represent a protective capsule was made originally by Chapman et al. (8) and subsequently by other researchers (11, 12). Later, Draper and Rees isolated the material constituting this zone from in vivo-cultivated *M. lepraemurium* and found it to be a collection of "parallel fibrils wreathed longitudinally around the bacteria" (12). These fibrils were found to contain key amino acids and 6-deoxyhexoses previously found in "C-mycosides" (12). Draper later isolated similar fibrillar material from an in vitro-cultivated strain of *Mycobacterium avium* and presented evidence that it too consisted of C-mycosides (10). Thereafter, Kim and colleagues demonstrated the presence of these fibrillar structures surrounding another strain of *Mycobacterium*, *Mycobacterium* sp. NQ (16).

Until recently the basic structure of C-mycosides had been given as (18, 25):

fatty acyl-D-Phe-(D-*allo*Thr-D-Ala)$_{1,2}$L-alaninol-*O*-6-deoxysugar
|
O
|
6-deoxysugar

However, studies by Brennan and Goren (4) and Brennan et al. (5) have demonstrated that individual serovars of the *M. avium-M. intracellulare-M. scrofulaceum* (MAIS) complex contain two classes of C-mycosides, which were called apolar C-mycosidic peptidoglycolipids (PGLs) and polar C-mycosidic PGLs. The apolar class of C-mycosides is essentially similar to the above structure; however, the second class differs in

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that it has an oligosaccharide attached to the D-*allo*-threonine instead of the single 6-deoxyhexose. Furthermore, it has been suggested that the oligosaccharides in the polar PGLs from serovars differ in composition and contain the antigenic determinants which allow for serological specificity within the MAIS complex (4).

With the knowledge that these type-specific surface antigens are basically C-mycoside in composition, it was important to see whether they were synonymous with the fibrils reported by Draper (10, 12). If this were the case, then the determination of their structure could prove to be a valuable tool in the understanding of mycobacterial survival within phagolysosomes. Knowledge of the PGL structure would also be beneficial for devising a method of interrupting their synthesis, thus depleting the mycobacteria of their outer sheath.

This report is concerned with the isolation and structural analysis of the superficial fibrillar material which is produced by certain smooth-colony mycobacteria.

MATERIALS AND METHODS

Growth of mycobacteria and isolation of the superficial cell wall. *Mycobacterium* sp. NQ (3) was grown in 7H11 medium as static cultures or by shaking (4). L-[*methyl*-^{3}H]methionine (13.11 Ci/mol; 0.5 μCi/ml of medium) was added to shaking flasks in early log phase (6 days). Shaken cells were harvested in early stationary phase (3 weeks), whereas static cultures were allowed to grow for 7 weeks before harvesting.

Superficial cell wall was released from cells by a gentler variation of previously described procedures (16), designed to cause minimal cell rupture. Cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and suspended, without washing, in phosphate-buffered saline. The suspension (1 ml of packed cells per 30 ml of phosphate-buffered saline) was blended in a Vortex mixer with intermittent chilling in ice for 3 min and centrifuged at 6,000 × *g* for 10 min to remove whole cells. The supernatant was again centrifuged at 10,000 × *g* for 15 min and finally at 30,000 × *g* for 1 h. This 30,000 × *g* pellet was then washed with 0.05% Tween 80 in phosphate-buffered saline buffer using a combination of low-speed centrifugation (10,000 × *g*, 10 min) to remove cells and high-speed centrifugation (30,000 × *g*, 1 h) to collect the outer cell wall fraction. The washing procedure was repeated with distilled water. Resulting material was then lyophilized for further analysis. Spent medium was also examined as a source of extracellular fibrillar structures. It was subjected to the same centrifugation steps described for the cells blended in a Vortex mixer.

Cellular fractions were directly mounted on Formvar carbon-coated grids, treated with 2% glutaraldehyde for 30 min, washed with double distilled water, and stained with 1.5% phosphotungstic acid. Grids were examined in a Hitachi model HU 12 transmission electron microscope operating at 75 kV.

Extraction and purification of lipids from cells and superficial cell wall. Lipids were extracted from lyophilized whole cells with $CHCl_3$-CH_3OH (2:1) at 50°C for 18 h (4). To extract lipids from the superficial cell wall structures, 3.5 mg of the 30,000 × *g* pellet was dissolved in 6 ml of $CHCl_3$-CH_3OH (2:1), and water (1 ml) was added. The suspension was mixed, and the lipids in the lower chloroform phase were analyzed.

Pure preparations of the native polar PGLs were obtained by fractionating total lipid extracts on a column of DEAE-cellulose (acetate) (4). Columns were successively irrigated with $CHCl_3$-CH_3OH-H_2O (9:1:0.1; 8:2:0.2; 7:3:0.3; etc). Pure polar PGLs appeared in the 7:3:0.3 and 6:4:0.4 eluates. Alternatively, to obtain a fraction containing both polar and apolar deacetylated PGLs, total lipid was treated with 0.2 N NaOH in $CHCl_3$-CH_3OH (4) and applied to a silicic acid column which was eluted with $CHCl_3$ to remove fatty acids followed by 30% CH_3OH in $CHCl_3$ to obtain a preparation containing PGLs. The PGLs from the 30,000 × *g* pellet after deacetylation were also subjected to similar elution steps with a small column (6 by 0.5 cm) of silicic acid-Celite.

Chromatography. Solvents used for silica gel and cellulose chromatography were: A, $CHCl_3$-CH_3OH-H_2O (60:12:1); B, $CHCl_3$-CH_3OH-H_2O (90:10:0.6); C, $CHCl_3$-CH_3OH (98:2); D, 1-butanol–ethanol–water (4:1:5, upper phase); E, 1-butanol–pyridine–water (6:4:3), F, 1-butanol–pyridine–water (10:3:3); G, 2-propanol–NH_4OH (2:1); H, hexane-ether-acetic acid (90:10:3).

PGLs on silica gel thin-layer chromatographic (TLC) plates were located with orcinol-H_2SO_4; sugars won cellulose TLC plates were located with aniline-oxalate; sugar alcohols and oligosaccharides on paper chromatograms were visualized with a $AgNO_3$-NaOH dip reagent; acetohydroxamate on cellulose TLC plates was located with acidic $FeCl_3$. Use of these reagents has been described (4).

Gas-liquid chromatography (GLC) was conducted on a Hewlett Packard 5710A gas chromatograph coupled to a Hewlett Packard integrator, model 3380A. Alditol acetates were separated on 6-ft (ca. 1.8 m) columns of 3% SP-2340 or 10% SP-2401 on 100–120 Supelcoport, or on 3% OV-225 or 3% ECNSS-M on 100–120 Gas-Chrom Q. Operating conditions are described in the figure legends.

Analytical procedures. Lipid preparations containing native PGLs were dissolved in $CHCl_3$-CH_3OH and treated with an equal volume of 0.2 N methanolic NaOH at 37°C for 30 min (4). This treatment served to deacetylate the PGLs and saponify orthodox glycerides. That acetyl groups only were released from the PGLs by this treatment was demonstrated as described previously (4), i.e., by treating the pure polar PGLs with alkaline hydroxylamine and recognizing acetohydroxamate as the sole product by TLC in solvent G. *Allo*-threonine-linked carbohydrates were released from the purified polar PGLs by the following modification of our previously described reductive alkali-catalyzed β-elimination reaction (4). Deacetylated polar PGLs were dispersed by sonication, dissolved by gentle heating in 50% aqueous ethanol containing 0.4 M NaOH and 1.2 M $NaBH_4$, and maintained at 60°C for 24 h (for analysis of the linkage sugar, NaB^3H_4 was employed). Excess borohydride was destroyed with 2 M CH_3COOH, and boric acid was removed as methyl borate. The residue was partitioned between $CHCl_3$-

CH_3OH and water. The bottom chloroform phase contained the residual alkali-degraded PGLs (4). The dried aqueous phase, containing the reduced oligosaccharide, was applied to a column of Sephadex G-25 followed by a column of Sephadex G-15. Carbohydrate in the eluted fractions was monitored by the phenol-H_2SO_4 assay (13).

Intact and deacetylated polar PGLs were hydrolyzed with Kiliani solution (10 ml of concentrated HCl, 55 ml of water, 35 ml of glacial CH_3COOH [15]) for 7 h in a steam bath. The hydrolysate was extracted with hexane, neutralized with a stream of N_2, and passed through a small column of Dowex 50 H^+ in methanol. Alternatively, the PGLs were methanolyzed with 2.2 M HCl in CH_3OH at 84°C for 48 h. The reduced oligosaccharide was hydrolyzed with 1.3 or 2.6 M CF_3COOH at steam temperature (94°C) for 2 to 6 h. Preparation of 6-deoxyhexitol acetates was carried out according to Stellner et al. (24). The intact reduced oligosaccharide was acetylated with acetic anhydride-pyridine (1:1) at room temperature overnight.

Serology was conducted by previously described procedures (4). The rabbit antiserum to *M. intracellulare* serovar 20 had a titer of 1:160. All other procedures have been described (4).

Isolation of the sugar component of the "lipid core." The deacetylated polar PGL from cells labeled with [*methyl*-^{3}H]methionine (19,000 cpm/mg of lipid) was subjected to alkali-catalyzed β-elimination. The alkali-degraded PGL remaining after elimination of the oligosaccharide was hydrolyzed with Kiliani reagent. The neutral hydrolysate was applied to sheets of Whatman no. 1 filter paper and chromatographed in solvent D. Only one reducing sugar (R_{rha} = 1.90) was obvious. This was eluted from the paper and applied to a column of Sephadex G-10 (105 by 1 cm). A single peak was obtained in which carbohydrate and radioactivity coincided. Recovery of the pure monosaccharide was 2.4 mg (33,900 cpm) from about 5.6 mg of carbohydrate in the alkali-degraded PGL.

Mycobacterium sp. NQ was a gift from L. Barksdale, New York University School of Medicine. Reagents for electron microscopy were obtained from Electron Microscopy Sciences, Fort Washington, Pa. L-[*methyl*-^{3}H]methionine was purchased from ICN Pharmaceuticals Inc., Irvine, Calif. NaB^3H_4 (specific activity, 5 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. The synthesis of 2-*O*-Me-fucose will be described at a later date. The origin of the other 6-deoxyhexoses has been described (4). The SP products for GLC were obtained from Supelco Inc., Bellefonte, Pa., whereas ECNSS-M and OV-225 were purchased from Applied Science Laboratories, State College, Pa.

RESULTS

Characterization of *Mycobacterium* sp. NQ. The particular organism used was chosen because Barksdale and colleagues had extensively used it to demonstrate the existence in certain mycobacteria of an outermost cell wall layer composed of long parallel filaments (2, 16). Seroagglutination (22) of *Mycobacterium* sp. NQ, employing antisera raised against the 31 serovars, showed a 4+ response against serovar 20 antisera, a 2+ response against serovar 41 antisera, and no activity against any of the other antisera. The type-specific PGLs from *Mycobacterium* sp. NQ (see below) were identical to those from a stock culture of *M. intracellulare* MAIS serovar 20 in solvent A and in the other TLC solvent systems described previously (5). On this basis we conclude that *Mycobacterium* sp. NQ is serovar 20.

Electron microscopy of the external fibrillar layer. Examination of the washed 30,000 × *g* pellet by electron microscopy (Fig. 1) revealed strands of parallel fibers. Individual fibers measured about 4 nm in diameter and apparently were transparent since underlying fibers were visible. Although fibrillar material was found in both static subsurface and shaken cultures, the best source was static cultures. However, surface (pellicle)-grown cells, which have a predilection for rough morphology, are devoid of fibrils.

As discerned by transmission electron microscopy, the 30,000 × *g* pellet contained mostly cell-free fibrillar material. Occasionally, cells were also observed in this pellet with fibrils lying parallel to the long axis or arranged diagonally or transversally, much as described by Draper (10). Treatment of the 30,000 × *g* pellets with 0.05% Tween and subsequent centrifugation were successful in removing visible cells with only cell-free fibrillar material remaining. Although recovery of fibrils (0.57% of cell mass) was comparable to that reported by others (0.66%, reference 10), it hardly represents the full cellular complement since fibrils were seen in earlier centrifugal fractions. It appears that, during its isolation, the superficial cell wall spontaneously disaggregated into small fibrils and to its monomeric PGLs, which then appeared in all centrifugal fractions. Evidence for this phenomenon came from the observation that when 30,000 × *g* fractions were allowed to stand in buffer for prolonged periods, the yield of fibrillar material was markedly diminished. Moreover, we have successfully regenerated microscopically discernible fibrils by mounting emulsions of pure polar PGLs on Formvar carbon-coated grids.

Composition of the fibrillar layer. Analyses on a preparation of the fibrils are summarized in Table 1. Of this material, 95% is lipid. Of this, about 12% is neutral lipid, some of which may be residual Tween 80 from the washing step. The majority (88%) of the lipids were eluted from silicic acid with 30% CH_3OH in $CHCl_3$. Mild alkali treatment of this eluate, to destroy possible phosphoglycerides, followed by further silicic acid chromatography, showed that

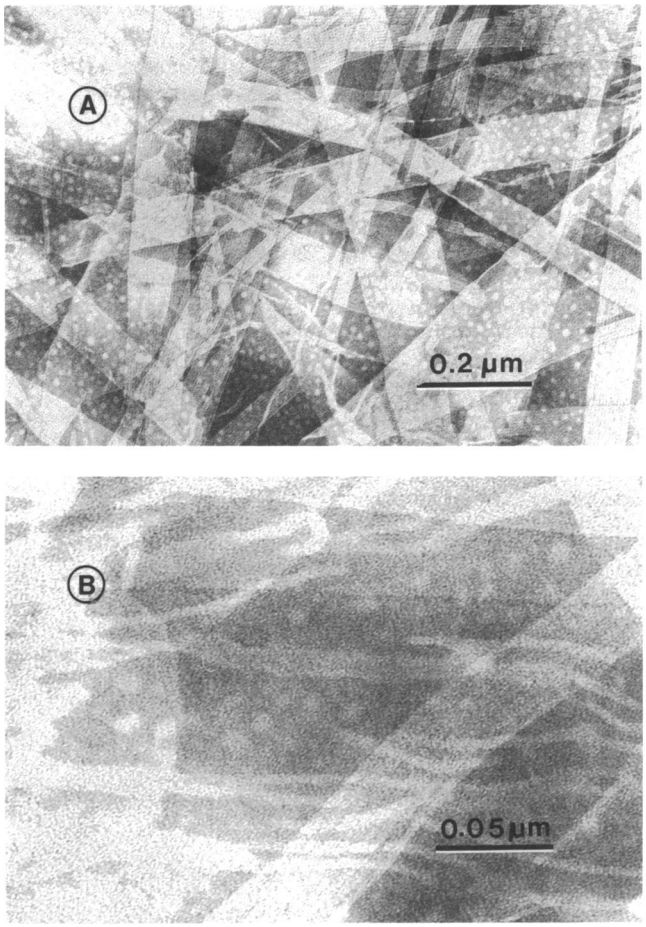

FIG. 1. *Electron micrograph of the Tween 80-washed 30,000 × g pellet, negatively stained with 1.5% phosphotungstic acid. (A) Lower magnification. (B) Higher magnification.*

about 78% of the eluted lipids were alkali-stable, deacetylated PGLs.

TLC in solvent A (Fig. 2) showed that the patterns of PGLs from both the fibrillar fraction and whole cells were similar; both contained four identical major polar PGLs (I–IV) and an undetermined number of barely discernible apolar PGLs at the top of the plate. Moreover, when the PGL-containing preparations from both sources were treated with 0.2 N NaOH, one identical deacetylated polar PGL was obtained (Fig. 2), indicating that the four polar PGLs differed one from the other simply in the number of acetyl groups at the hydroxyl positions. From carbohydrate determinations on polar and apolar PGLs eluted from preparative plates, it was determined that about 70% of the total PGLs are of the polar variety.

Structures of polar PGLs. The polar PGLs are the predominant lipid class in the isolated fibrils and hence were the objects of more detailed chemical and serological analyses; the deacetylated polar PGL, obtained from the pure intact PGLs of whole cells, was used. Carbohydrate content was the same whether estimated as total carbohydrate (13) or 6-deoxyhexose (9) and amounted to 2.60 μmol of rhamnose equivalents per mg of PGL. Fatty acids were estimated gravimetrically at 1.11 μmol of palmitic acid equivalent per mg of PGL. Colorimetric estimation of the peptide was unsatisfactory; however, GLC of the amino compounds as their *N*-trifluoroacetyl-*n*-butyl esters (4) showed only phenylalanine, threonine, alanine, and alaninol in equimolar quantities. These data are similar to those obtained previously and suggest a tetrapeptide-based polar PGL (4). The percent figures for carbohydrate, (43%), lipid (28.6%), and peptide (28.5% by difference) are reasonably close to the theoretical figures (49%, 27%, 24%,

TABLE 1. *Analysis of the fibrillar fraction (30,000 × g pellet) from serovar 20*

Component	μg/mg of dry fraction[a]	μg/mg of lipid[b]	μg/mg of polar lipid[c]
Protein	40		
Total lipid	950		
Neutral lipids		120	
Polar lipids		880	
PGLs			770
Phosphoglycerides			220

[a] Protein was estimated by a modified Lowry procedure (19). Total lipid was estimated gravimetrically as the material in the lower phase of a biphasic extractant.

[b] Neutral lipid was that eluted by $CHCl_3$ when lipids from the 30,000 × *g* pellet were applied to a column (45 by 5 mm) of silicic acid-Celite (2:1). Polar lipids were subsequently eluted with $CHCl_3$-CH_3OH (7:3).

[c] To estimate the amounts of PGLs and phospholipids, the polar lipids from the previous column were treated with 0.2 N NaOH and applied to a new column. The fatty acids, eluted with $CHCl_3$, were an indicator of phosphoglyceride content. The subsequent $CHCl_3$-CH_3OH (7:3) eluate contained the deacetylated PGLs.

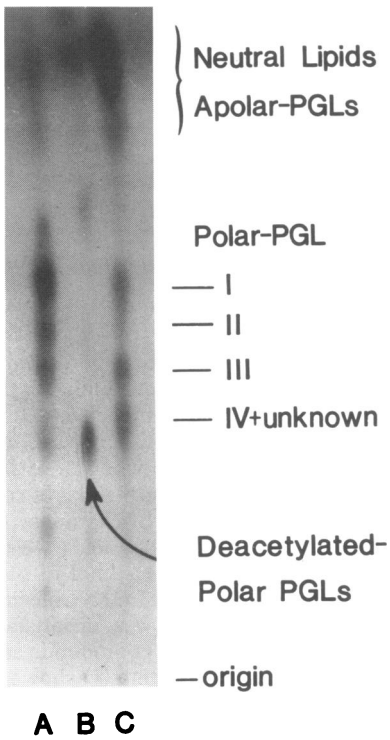

FIG. 2. *TLC in solvent A of: (A) intact polar PGL preparation from MAIS serovar 20; (B) deacetylated polar PGLs from serovar 20; (C) lipid extract from 30,000 × g pellet. Plate was sprayed with 0.1% orcinol in 40% H_2SO_4 and heated (4).*

respectively) for a 3-hydroxy, mono-unsaturated, C_{32} fatty-acylated, tetrapeptide-based PGL with a tetrasaccharide attachment (see below). The fatty acids of the deacetylated PGL from serovar 20 were identified only insofar as TLC in solvent H and GLC-mass spectrometry as described previously (4) showed an assortment of C_{32}, C_{34}, and C_{36}, 3-hydroxy unsaturated fatty acids, as well as orthodox C_{16} and C_{18} saturated fatty acids.

The constituent sugars in the polar PGL from serovar 20 were analyzed. Five sugars were present in the polar PGL hydrolysate, as shown by GLC of the alditol acetates (Fig. 3) and cellulose TLC (solvent D) of the reducing sugars. Three of the sugars, 3,4-di-*O*-Me-rhamnose (R_{rha} = 1.8), 6-deoxytalose (R_{rha} = 1.2), and rhamnose, were readily identified on cellulose thin-layer plates by comparison with the authentic standards. The mobilities of the two remaining sugars (R_{rha} = 1.3 and 1.5) were indicative of mono-*O*-Me-6-deoxyhexoses (4). They were identified as 2-*O*-Me-rhamnose and 2-*O*-Me-fucose by cochromatography of their alditol acetates with those of authentic sugars on SP-2340 and ECNSS-M and by cellulose TLC in solvent D.

To determine which of these sugars were associated with the lipid core and which constituted the oligosaccharide, the acetyl-free polar

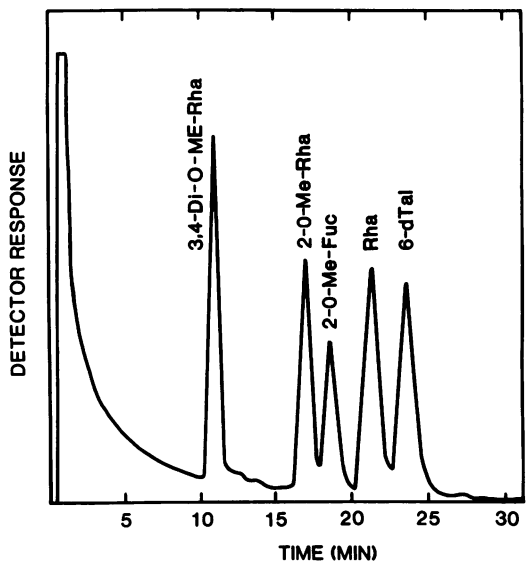

FIG. 3. *GLC of the alditol acetates derived from the polar PGLs. The deacetylated polar PGLs (200 μg) were hydrolyzed with Kiliani reagent for 7 h at 94°C. Preparation and acetylation of the 6-deoxyhexitols are described in the text. GLC was conducted on a 6-ft (ca. 1.8-m) coiled column of 3% SP-2340 on 100-120 Supelcoport at 175°C with N_2 flow rate of 60 ml/min.*

PGL was subjected to alkali-catalyzed reductive cleavage, and the products were partitioned between $CHCl_3$-CH_3OH and water. The alkali-degraded PGL in the $CHCl_3$ phase was shown to be chromatographically similar to the lipid likewise derived from serovar 9 (Fig. 4). Previously we concluded that the alkali-degraded PGL from serovar 9 had the structure

fatty acyl-CO-NH-Phe-CO-NH-2-butenoic-CO-

NH-Ala-CO-NH-alaninol-*O*-(3,4-di-*O*-Me-Rha)

in which the *allo*-threonine residue was converted to the 2-amino-2-butenoic acid substituent during the β-elimination reaction. The chromatographic resemblance between the alkali-degraded PGLs from serovars 9 and 20 suggests similar structures. To determine whether the constituent sugar in the product from serovar 20 was in fact 3,4-di-*O*-Me-rhamnose, the ^3H-sugar associated with the alkali-degraded PGL grown in the presence of [*methyl*-^3H]methionine was isolated as described above. Cellulose TLC and paper chromatography (solvent D) showed that radioactivity and reactivity to aniline oxalate coincided with and corresponded exactly to authentic 3,4-di-*O*-Me-rhamnose. Moreover, the sugar in question, as the alditol acetate, cochromatographed with the diacetate of 3,4-di-*O*-Me-rhamnitol on SP-2340, SP-2401, ECNSS-M, and OV-225. Finally, a portion of the sugar was demethylated with BCl_3 (1), and GLC of the reduced acetylated product showed that rhamnose was the only unmethylated 6-deoxyhexose produced.

The water-soluble products from the β-elimination reaction were applied to a column of Sephadex G-25, followed by Sephadex G-15 (Fig. 5). Chromatography of the resulting pure reduced oligosaccharide is shown in Fig. 6. Oddly, on paper chromatography (Fig. 6A), it had mobility similar to that of rhamnitol and ran much faster than the disaccharides, maltose and trehalose. On TLC the chromatographic properties were somewhat more typical of an oligosaccharide (Fig. 6B). Moreover, when the reduced oligosaccharide was fully acetylated and compared with octaacetyl trehalose, it displayed chromatographic properties highly indicative of an oligosaccharide (Fig. 6C). Presumably, in partition chromatography the lipophilic nature of the inherent sugars lends to the reduced oligosaccharide a mobilty far in excess of conventional hexose-containing ogligosaccharides. Only when the

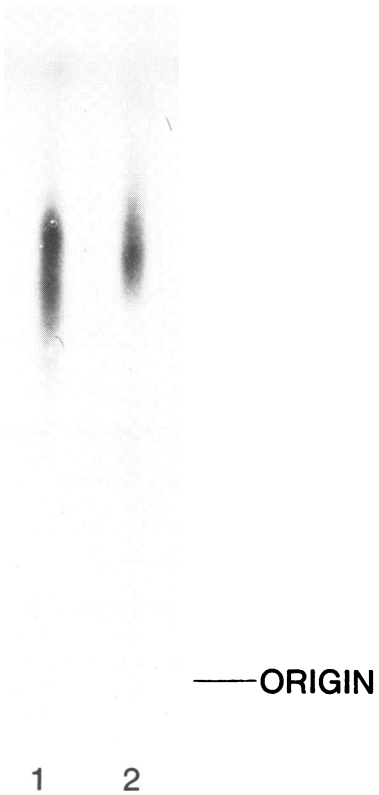

FIG. 4. *TLC of the alkali-degraded lipid remaining after alkaline borohydride cleavage of the pure deacetylated polar PGL from serovar 9 (lane 1) and serovar 20 (lane 2). Plate was chromatographed in solvent B, sprayed with 0.1% orcinol in 40% H_2SO_4, and heated.*

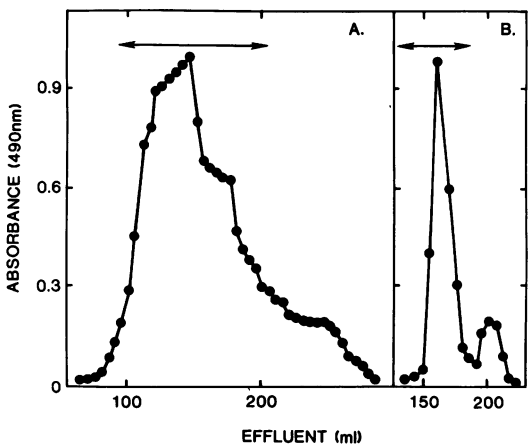

FIG. 5. *Purification of the reduced oligosaccharide. The aqueous phase resulting from alkaline reductive cleavage of the pure acetyl-free polar PGL (205 mg) was applied to Sephadex G-25 (105 by 1 cm) in water (A). Mixed fractions were applied to Sephadex G-15 (105 by 1 cm) (B). About 15 mg of pure reduced oligosaccharide was recovered.*

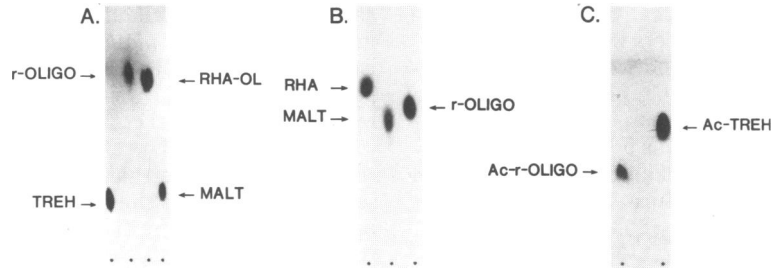

FIG. 6. *Chromatography of the reduced oligosaccharide. (A) Paper chromatography of reduced oligosaccharide in solvent E. Spots were detected with the $AgNO_3$-NaOH dip reagent. (B) Silica gel TLC of reduced oligosaccharide in solvent F. Plate was sprayed with 0.1% orcinol in 40% H_2SO_4. (C) Silica gel TLC of the acetylated reduced oligosaccharide in solvent C. Plate was sprayed with orcinol-H_2SO_4. MALT, maltose; RHA, rhamnose; RHA-OL, rhamnitol; TREH, trehalose; Ac-TREH, octa-acetyltrehalose; Ac-r-OLIGO, acetylated reduced oligosaccharide.*

oligosaccharide was fully acetylated and subjected to absorption chromatography did the effect of its size manifest itself.

Figure 7 shows the sugar composition of the pure reduced oligosaccharide obtained from the G-15 Sephadex column. The individual sugars, 2-*O*-Me-fucose, 2-*O*-Me-rhamnose, rhamnose, and 6-deoxytalose, were present in the relative proportions of 1.28:0.83:1.05:1, respectively, suggesting a tetrasaccharide. Incidentally, the absence of 3,4-di-*O*-Me-rhamnose in the oligosaccharide further substantiates the evidence that this sugar is inherent to the core lipopeptide region.

To determine which of the four sugars present occupied the alditol terminus, the reduced oligosaccharide was hydrolyzed with CF_3COOH, and the products were chromatographed on cellulose TLC in solvent D and stained with aniline oxalate. Three reducing sugars were thus shown, 2-*O*-Me-fucose, rhamnose, and 2-*O*-Me-rhamnose. 6-Deoxytalose was absent, evidently because it was present as its alcohol. Some of the hydrolysate was also acetylated without further reduction, and the retention time of the products was compared with those of the fully reduced and acetylated sugars by GLC on OV-225 (160°C). The only peak from the two preparations which cochromatographed was due to 6-deoxytalitol. Finally, ^3H-reduced oligosaccharide, obtained from an alkaline reductive cleavage reaction containing NaB^3H_4, was hydrolyzed and subjected to radiochromatography in solvent D. When the products were scanned for radioactivity, only one radioactive peak was evident which corresponded to 6-deoxytalitol.

Serology on the polar PGLs. The four native polar PGLs from cells of serovar 20 were purified free of one another by a combination of column chromatography and TLC and reacted against rabbit antisera raised against the parent organism. The activity of polar PGL II is shown in Fig. 8. Likewise, polar PGL I, III, IV, a mixture of the native polar PGLs from the 30,000 × *g* superficial cell wall fraction, and the fully deacetylated polar PGL were active, and all gave a single line of identity on Ouchterlony gels. Thus, it was shown that the polar PGLs, besides being the major constituents of the external sheath, are also the typing antigens of serotype 20, and the presence of acetyl functions seems to have little bearing on serological activity.

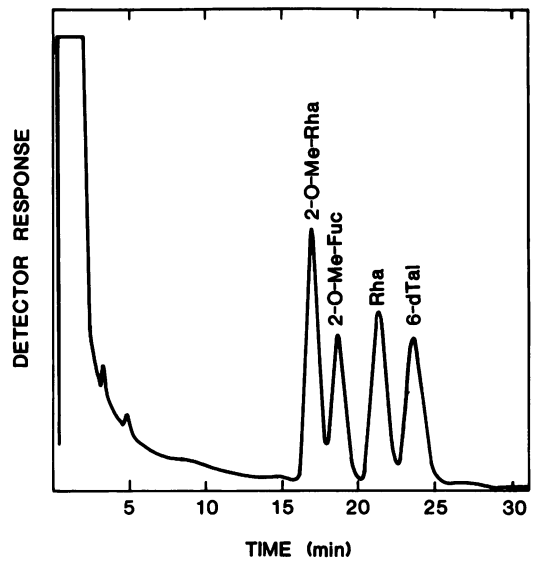

FIG. 7. *GLC of the alditol acetates derived from the reduced oligosaccharide. The pure preparation (150 μg of carbohydrate) from Sephadex G-15 was hydrolyzed for 2 h at 94°C with 2.6 M CF_3COOH, reduced, and acetylated. GLC operating conditions are described in the legend to Fig. 3.*

DISCUSSION

Results from this investigation substantiate evidence for the presence of a superficial sheath

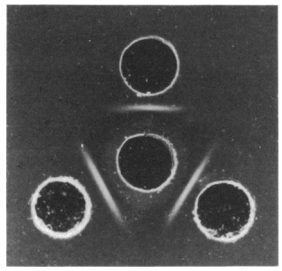

FIG. 8. *Agar gel immunodiffusion (Ouchterlony) of pure polar PGL II isolated from serovar 20. The pure lipid was sonicated in phosphate-buffered saline (1 mg/50 μl) and added in amounts of 100, 200, and 400 μg to wells 1, 2, and 3, respectively. To the center well was added 20 μl of rabbit antiserum to serovar 20 with a titer of 1:160. Gels were developed in a moist sealed atmosphere at room temperature.*

surrounding certain nontuberculous (atypical) mycobacteria. Moreover, analysis of this superficial material has revealed that the structural matrix consists largely of polar PGLs, related to those reported earlier by Brennan and Goren (4) for another member of the MAIS serocomplex. Also, the PGLs from serovar 20 were demonstrated to have activity towards rabbit antiserum raised against the homologous organism. On the basis of previous work (4, 5), it seems most likely that this activity and the polar PGLs responsible for it are entirely specific for serovar 20.

At this time it is difficult to define the exact architecture of this extracellular component; the apparent instability of the filamentous structures accentuates the difficulties. It appears from the results reported herein and those revealed by others (10, 12, 16) that the fibrillar network is superficial and covers the entire cell. It almost certainly corresponds to the most superficial, L_1, layer, succinctly described by Barksdale and Kim (2) as "filaments, tapes, or ribbons" when revealed by negative-staining techniques. However, as substantial amounts of PGLs can be obtained from shaken cultures which contain very few observable filaments, it appears that at least some of this component is more rigidly attached to the cell. This view is enhanced by the fact that ample quantities of the PGLs can be chemically extracted from cells which have been stripped of the outer sheath by mechanical means. It is entirely possible that the PGLs constitute at least two major surface regions: an inner, more rigidly attached, "micro" capsule and an outer, less firmly attached, "macro" capsule. The outer region is probably the one which constitutes the electron-transparent zone described in earlier reports (6, 11, 14). Thus, it is likely that as the cell grows in a static mode, it produces more of these antigenic PGLs which combine to form a superficial sheath. An interesting possibility, which has not been examined, is that the outer sheath is composed of the antigenic polar PGLs, whereas the inactive apolar PGLs are mostly confined to the inner region.

The overall structures of the antigenic PGLs from serovar 20 are consistent with those already identified in serovar 9 (4) and other serovars (P. J. Brennan, H. Mayer, G. O. Aspinall, J. E. Nam Shin, unpublished data). Differences are based on the number and types of sugars attached to the threonine in the peptide portion. In serovar 20 these sugars are apparently present as a tetrasaccharide with 6-deoxytalose as the linkage sugar. The other three sugars contained within the oligosaccharide have been identified as 2-*O*-Me-fucose, 2-*O*-Me-rhamnose, and rhamnose, but their order is not yet known. However, in the PGLs from serovars 8, 9, and 25, rhamnose is always penultimate to the reducing end of the oligosaccharide (P. J. Brennan, G. O. Aspinall, and G. R. Gray, unpublished data). In addition, a characteristic of all of these oligosaccharides is that some or all of the free hydroxyl groups are acetylated.

Since *M. intracellulare* serovar 20 is a member of the MAIS serocomplex, it is reasonable to propose that all serovars are endowed with a sheath of exquisitely specific polar PGLs. Indeed, this principle may apply to all smooth-colony mycobacteria, since we have recently shown that species (e.g., *Mycobacterium kansasii*) devoid of the C-mycosidic PGL antigens contain yet other undefined antigenic peptidoglycolipids (P. J. Brennan, unpublished data). Moreover, MAIS organisms and *M. lepraemurium* are antigenically related (17, 23), and Draper and Rees (12) have presented evidence showing that the capsular material isolated from *M. lepraemurium* contains C-mycosides. Although the presence of C-mycosides in *M. leprae* has not been examined, it has been suggested that the electron-transparent zone which surrounds *M. leprae* in vivo is lipid (20). Thus, the findings in this report may be inferentially im-

portant in understanding the pathogenicity of not only all nontuberculous smooth-colony mycobacteria, but also of *M. leprae*.

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