Characterization of the Specific Antigenicity of *Mycobacterium fortuitum*†

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ABSTRACT: *Mycobacterium fortuitum*, biovar. *fortuitum*, the cause of serious skin and soft-tissue infections, can be differentiated from *M. fortuitum*, biovar. *peregrinum*, and other rapidly growing opportunistic mycobacteria by the presence of a unique antigenic glycolipid. The glycolipid is among the simplest of the acyltrehalose-containing lipooligosaccharide class. The application of ^1H and ^{13}C NMR, methylation analysis, FAB/MS, and other procedures demonstrated the structure, β-D-Glc*p*-(1→6)-2-*O*-acyl-α-D-Glc*p*-(1↔1)-3,4,6-tri-*O*-acyl-α-D-Glc*p*. Thus, practically all environmental mycobacteria, many of them opportunistic pathogens, can be differentiated serologically and chemically on the basis of unique sugar arrangements within a few classes of glycolipids. The simplicity of the structure in *M. fortuitum fortuitum* combined with the distinct roughness of the parent strain raises the intriguing possibility that it is a spontaneous rough variant of the other mycobacteria with more elaborate glycolipids.

The rapidly growing nontuberculous mycobacteria [the original group IV of the atypical mycobacteria, i.e., the rapidly growing potential pathogens (Wolinsky, 1979)], are now recognized as the cause of serious cutaneous or soft-tissue infections, usually arising from prior contaminated injections, trauma, or surgery (Fonseca et al., 1987; Hendrick et al., 1987; Woods et al., 1986). They are also recognized as the source of infections in previously damaged lungs or corneas and are responsible for bacillemia due to contaminated prostheses, catheters, or underlying immune deficiency (Wallace et al., 1983, 1985). These skin, soft-tissue, and organ infections are largely due to *Mycobacterium fortuitum* and *Mycobacterium chelonei*. The identification and differentiation of these species is based primarily on growth and colony characteristics and a number of "biochemical reactions" (Silcox et al., 1981); satisfactory genetic probes are not yet available for the identification of these important opportunistic pathogens. We previously demonstrated that the dominant surface antigens of all strains of *M. cheloneae*, both subspecies *cheloneae* and subspecies *abscessus*, were of the glycopeptidolipid class related to those of the *Mycobacterium avium* complex (Tsang et al., 1984). However, the surface antigens of *Mycobacterium fortuitum* biovar. *fortuitum* and *Mycobacterium fortuitum* biovar. *peregrinum* differed (Tsang et al., 1984). The latter were also of the glycopeptidolipid class but were of a distinctive structure (Lopez-Marin et al., 1991), apparently unique to *M. fortuitum* biovar. *peregrinum*. On the other hand, strains of *M. fortuitum* biovar. *fortuitum* showed other characteristic glycolipids, the structures of which were not determined. We have returned to the question of these structures and find that some but not all isolates of *M. fortuitum* biovar. *fortuitum* are distinguished by a novel triglucosyl-containing glycolipid based on acylated trehalose. The structural elucidation of this product and the realization of its relationship to the simple acyltrehaloses on the one hand, and the multiglycosylated acyltrehaloses on the other, raised some intriguing notions on the relationship between different mycobacteria.

Experimental Procedures

Growth of M. fortuitum. *M. fortuitum* isolates (1-59, 5-54, 2-74, 2-53, 2-46, 10-31, 10-18, 10-11, 11-5, 10-17, 10-14, 10-22, 11-1) obtained from the collection once maintained at the National Jewish Center, Denver, Colorado (Tsang et al., 1984), and now maintained here, were grown in 1-L Fernbach flasks for 4–5 days in glycerol, alanine, and salts medium and were then autoclaved. Both cells and medium were evaporated to dryness at 50 °C, and the resulting solid was extracted with $CHCl_3$–CH_3OH (2:1). Dried extracts were dissolved in the biphasic mixture $CHCl_3$–CH_3OH–H_2O (8:4:2) (Folch et al., 1957); the contents of the lower organic phase were used as a source of lipid. For a more detailed lipid analysis, *M. fortuitum* 10-22, one of the isolates which yielded the characteristic glycolipid, was chosen as the prototype strain and grown on a large scale to yield approximately 7.9 g of washed lipid from 72 g of dried cells.

Purification of Glycolipid. Washed lipids (7.9 g) were applied to a column (2.5 × 30 cm) of Florisil (100–200 mesh) (Fisher Scientific Co., Pittsburgh, PA) which was irrigated with 250 mL of $CHCl_3$ followed by 5%, 10%, 15%, 20%, 25%, and 80% CH_3OH in $CHCl_3$. Eluates were collected in bulk and examined by TLC[1] in $CHCl_3$–CH_3OH–H_2O (30:8:1) for glycolipid content after spraying with 10% sulfuric acid in ethanol and heating at 110 °C for 5 min. The 10% CH_3OH in $CHCl_3$ eluate, rich in the characteristic glycolipid (the component with a black response on spraying), was rechromatographed on a column (1 × 30 cm) of Florisil (100–200 mesh) (Fisher Scientific Co.) and irrigated with more gradual gradations of solvents. Fractions (10 mL) were collected and monitored for glycolipid purity by TLC.

Deacylation of Glycolipid. Glycolipid (30 mg) in 3 mL of $CHCl_3$–CH_3OH (2:1) was treated with 0.1 N NaOH in CH_3OH (3 mL) at 37 °C for 1 h (Hunter et al., 1983), neutralized with acetic acid, evaporated to dryness, and partitioned between $CHCl_3$ and H_2O, and the aqueous phase, which contained the neutral oligosaccharide, was applied to a column (1 × 100 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA) which was irrigated with H_2O. Fractions (1

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; 2D COSY, two-dimensional chemical shift correlated spectroscopy; GC/MS, gas chromatography–mass spectrometry; GC, gas chromatography; FAB/MS, fast atom bombardment–mass spectrometry; EI/MS, electron impact–mass spectrometry; NMR, nuclear magnetic resonance; Glc, glucose; *p*, pyranosyl; R_F, retardation factor; R_T, retention time; TLC, thin-layer chromatography; TFA, trifluoroacetic acid.

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mL) were assayed for carbohydrate (Dubois et al., 1966). The lower organic phase from the above separation served as a source of fatty acids. These were methylated and the fatty acid methyl esters were examined by GC/MS (Ryhage & Stenhagen, 1963) using program 3 (see below).

Glycosyl Composition by Alditol Acetates. Purified glycolipid was hydrolyzed in 250 μL of 2 M TFA at 120 °C for 2 h as described (McNeil et al., 1987a). Glycosyl residues were reduced with NaB^2H_4 and the resultant alditols were per-*O*-acetylated and examined by GC and GC/MS (McNeil et al., 1987a) using program 1.

Absolute Configuration of Glycosyl Residues. The purified glycolipid was hydrolyzed in 1 M HCl in (*R*)-(−)-2-butanol (Aldrich Chemical Co., Milwaukee, WI), trimethylsilylated with TRI-SIL (Pierce Chemical Co., Rockford, IL), and the trimethylsilyl (*R*)-(−)-2-butylglycosides were examined by GC/MS (Gerwig et al., 1978). Glucose was also hydrolyzed in 1 M HCl in (*R*)-(−)-2-butanol and (*S*)-(+)-2-butanol (Aldrich Chemical Co.), and the resulting trimethylsilylated products were compared to those from the purified glycolipid by GC/MS (Gerwig et al., 1978).

Methylation of Glycolipid and Glycosyl Linkage Analysis. The glycolipid (5 mg) was suspended in 0.5 mL of dimethyl sulfoxide (Pierce Chemical Co., Rockford, IL), and 100 μL of 4.8 M dimethyl sulfinyl carbanion was added (Stellner et al., 1973; Hakomori, 1964). The reaction mixture was stirred for 1 h. CH_3I (Aldrich Chemical Co.) (50 μL) was slowly added and the suspension stirred overnight (Hakomori, 1964). The reaction mixture was then diluted with 0.5 mL of water and the resulting product applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA), as described (York et al., 1986). The per-*O*-methylated glycolipid appeared in the acetonitrile eluant as determined by TLC in $CHCl_3$-CH_3OH (98:2). The per-*O*-methylated glycolipid was hydrolyzed using 250 μL of 2 M TFA at 120 °C for 2 h (McNeil et al., 1987a). The resulting hydrolysate was reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC/MS.

Location of Acyl Functions or Glycolipid. In order to establish the location of acyl functions on the oligosaccharide backbone, the native glycolipid was subjected to the neutral alkylating conditions of Prehm (1980), as follows. To the pure glycolipid (2 mg), under N_2, was added 30 μL of 2,6-di-*tert*-butylpyridine, 20 μL of methyltrifluoromethanesulfonate, and 200 μL of trimethyl phosphate. The reaction mixture was stirred at room temperature for 5 h, following which 1 mL of water was added. The mixture was applied to a C_{18} Sep-Pak cartridge (Waters) as described (York, 1986). The ethanol eluant was dried to yield the naturally acylated, per-*O*-methylated glycolipid, which was further methylated with C^2H_3I using the Hakomori methylation procedure (Hakomori, 1964) and purified on a C_{18} Sep-Pak cartridge (Waters), as described (York et al., 1986). The per-*O*-trideuteriomethylated, per-*O*-methylated oligosaccharide was recovered in the acetonitrile eluant and subjected to acid hydrolysis using 250 μL of 2 M TFA at 120 °C for 2 h (McNeil et al., 1987a). The resulting hydrolysate was reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC/MS.

NMR Analysis. Routine ^1H, ^{13}C, 2D $^1H/^{13}C$, and 2D COSY NMR spectra were recorded on a Bruker Ace-300 at the Colorado State University Department of Chemistry Central Instrument Facility. Spectra was obtained for the native glycolipid at a concentration of 70 mg/0.5 mL of C^2HCl_3–$C^2H_3O^2H$ (2:1) before and after exchanging protons with C^2HCl_3–$C^2H_3O^2H$ (2:1). Spectra for the oligosaccharide were obtained in ^2H_2O at a concentration of 10 mg/0.5 mL of ^2H_2O, before and after exchanging protons with ^2H_2O.

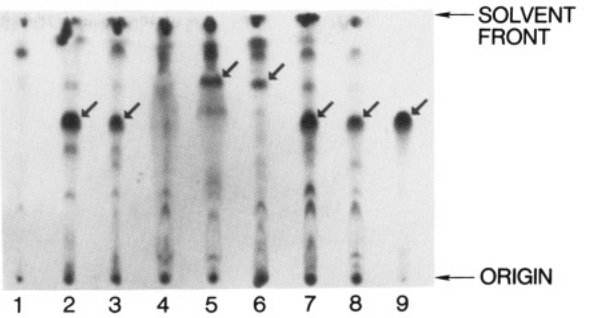

FIGURE 1: TLC of the total extracted lipids of isolates of *M. fortuitum fortuitum*. Lane 1, isolate 10-31; lane 2, 10-18; lane 3, 10-11; lane 4, 11-5; lane 5, 10-17; lane 6, 10-14; lane 7, 11-1; lane 8, 10-22; lane 9, purified glycolipid from isolate 10-22. The TLC solvent was composed of $CHCl_3$–CH_3OH–H_2O (30:8:1). The plate was sprayed with 10% H_2SO_4 in ethanol and heated at 110 °C for 5 min.

Gas Chromatography–Mass Spectrometry Analysis. GC/MS of alditol acetates was performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5790 mass selective detector. Samples were injected in the splitless mode. The injection port temperature was 290 °C and the transfer line 280 °C. The column used was a 12-m HP-1 (Hewlett-Packard, Avondale, PA). The oven was programmed to hold at 50 °C for 1 min, followed by a 30 °C/min rise to 200 °C, and 8 °C/min rise to 320 °C, and an 8-min hold at this temperature (program 1). The temperature program that was applied to all trimethylsilyl derivatives of butyl glycosides involved a 50 °C hold for 1 min followed by a 30 °C/min rise to 140 °C, a 6 °C/min rise to 260 °C, a 30 °C/min rise to 280 °C, and a final 2-min hold at this final temperature (program 2). A modified version of program 2 was used for the analysis of trimethylsilylated fatty acid methyl esters (program 3), where the final temperature was 325 °C and was held at this temperature for 10 min. The mass spectrometer was set to scan from 50 to 800 amu with 0.81 scans per second.

Gas Chromatography. Gas chromatography was routinely conducted on a fused silica capillary column of Durabond-1 (J&W Scientific, Rancho Cordova, CA) as described (McNeil et al., 1987a).

Fast-Atom Bombardment–Mass Spectrometry. FAB/MS was performed on a VG 7070 EHF mass spectrometer with an 10A tech saddle field gun operating at 7–8 kV and 1 mA with xenon gas. Samples were applied in 50:50 glycerol–thioglycerol on a thioglycerol matrix. The mass spectrometer was controlled by a VG series 2000 data system. Scanning was from 2000 to 50 amu at 20 s per decade.

Results

Isolation of a Major Characteristic Glycolipid from Some Strains of M. fortuitum biovar fortuitum. The results of TLC of a few isolates of *M. fortuitum* are shown in Figure 1 (lanes 1–8). The results reported previously were corroborated, with evidence of three distinctive patterns: a nondescript profile (isolates 10-31 and 11-5; lanes 1 and 4), one marked by a glycolipid with a pink-brown coloration (isolates 10-17 and 10-14; lanes 5 and 6), and the most distinctive of all, those (isolates 10-18, 10-11, 11-1, and 10-22; lanes 2, 3, 7, and 8) with a lipid which produced a black color response, indicative of lipids containing hexose. Alkaline treatment of the total lipid from isolates 10-11, 11-5, 11-1, and 10-22 resulted in the loss of this glycolipid, a feature of the alkali-labile lipooligosaccharides of *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium malmoense*, and *Mycobacterium*

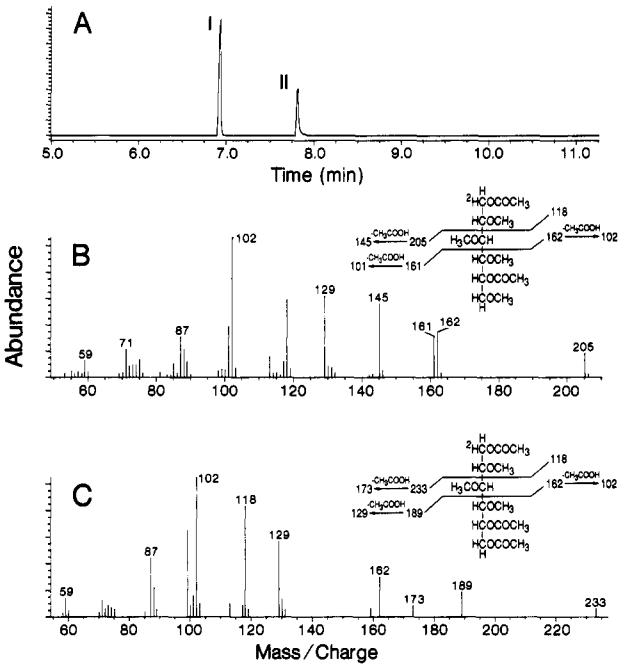

FIGURE 2: GC/MS profile of the alditol acetates derived from the per-*O*-methylated oligosaccharide. (A) Total ion chromatogram; I, 1,5-di-*O*-CH_3O-2,3,4,6-tetra-*O*-CH_3-glucitol (R_T 6.93 min); II, 1,5,6-tri-*O*-CH_3O-2,3,4-tri-*O*-CH_3-glucitol (R_T 7.82 min); (B) mass spectrum of (I) showing characteristic fragment ions; (C) mass spectrum of (II) showing characteristic fragment ions.

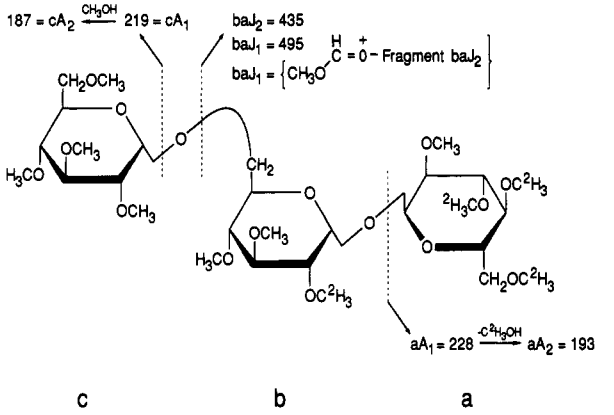

FIGURE 3: Structure of the per-*O*-deuteriomethylated, per-*O*-methylated trisaccharide: 2,3,4,6-tetra-*O*-CH_3-β-D-Glc*p*-(1→6)-2-*O*-C^2H_3-3,4-di-*O*-CH_3-α-D-Glc*p*-(1↔1)-3,4,6-tri-*O*-C^2H_3-2-*O*-CH_3-α-D-Glc*p*, showing characteristic electron-impact ions.

tuberculosis Canetti (Hunter et al., 1983, 1988; McNeil et al., 1987b; Daffe et al., 1991b). *M. fortuitum fortuitum* 10-22 was chosen for detailed analysis, and a large amount of the bacteria was grown. The total lipid extract was purified by low-pressure absorption chromatography on columns of Florisil irrigated with $CHCl_3$ and gradual increments of CH_3OH in $CHCl_3$. The purified glycolipid (Figure 1, lane 9) amounted to 70 mg, from 72 g of dried cells.

Structural Characterization of Oligosaccharide Backbone. The nature of the individual glycosyl units in the glycolipid was explored through hydrolysis, reduction with NaB^2H_4, per-*O*-acetylation, GC, and GC/MS of the alditol acetates. GC of the alditol acetates showed the presence of only one sugar. Cochromatography with 1,2,3,4,5,6-hexa-*O*-Ac-glucitol and GC/MS (R_T 9.72 min; *m/z* 115, 103, 145, 187, 128, 139, 170, 218, 260, 289, 362) established that this sugar was glucose. Proton NMR of the deacylated glycolipid revealed the presence of three anomeric proton signals at 4.45 ppm ($J_{1,2}$ = 7.62 Hz), 5.20 ppm ($J_{1,2}$ < 1.5 Hz), and 5.20 ppm ($J_{1,2}$ < 1.5 Hz). The ^{13}C NMR of the deacylated glycolipid showed distinctive resonances at 96.1 and 105.5 ppm in the ratio of 2:1 for the anomeric carbons. The signals at 4.45 and 5.20 ppm were assigned to one β- and two α-glucosidic linkages, respectively (Hunter et al., 1983). The overlapping α-glucose signals (2 × 5.20 ppm) were suggestive of an α,α'-trehalose (Usui et al., 1974) and further implied a structural similarity to the nonreducing trehalose-containing glycolipids of *M. kansasii* and other species (Hunter et al., 1983). The enantiomeric configurations of the glucose residues were determined by comparative GC/MS analyses of standard D-glucose trimethylsilyl (*S*)-(+)-2-butyl glycosides and (*R*)-(−)-2-butyl glycosides (Gerwig et al., 1978). The latter derivatives cochromatographed exclusively with the trimethylsilyl (*R*)-(−)-2-butyl glycosides prepared from the glycolipid, indicating that the glucosyl residues are in the D absolute configuration. GC/MS of the alditol acetates produced by the per-*O*-methylated, deacylated glycolipid (Figure 2) showed 1,5-di-*O*-CH_3CO-2,3,4,6-tetra-*O*-CH_3-glucitol (R_T 6.93 min; *m/z* 101, 102, 118, 145, 161, 162, 205) and 1,5,6-tri-*O*-CH_3CO-2,3,4-tri-*O*-CH_3-glucitol (R_T 7.82 min; *m/z* 102, 118, 129, 162, 189, 233) in the approximate ratio of 2:1. Accordingly, the existence of two terminal glucose and one 6-linked glucose residues was established from the glycosyl linkage data. The structure of the oligosaccharide was thus established as β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1↔1)-α-D-Glc*p*, since the ^1H and ^{13}C NMR data pointed to the presence of an α,α'-trehalose unit, and the glycosyl linkage data demonstrated that the remaining β-D-glucose residue was located at C-6 position of the α,α'-trehalose unit.

Location of Acyl Functions. The fatty acids derived from the deacylation of the lipooligosaccharide were methylated, trimethylsilylated, and examined by GC/MS (Ryhage & Stenhagen, 1963). The results indicated that four classes of fatty acid methyl esters were present in the native glycolipid. Each class was characterized by strong McLafferty rearrangement ions at *m/z* 88 and 101 [indicative of α-methyl branched (mycocerosate and mycolipenic) fatty acid methyl esters], *m/z* 55 (indicative of unsaturated fatty acid methyl esters), and *m/z* 74 (indicative of saturated fatty acid methyl esters) (Ryhage & Stenhagen, 1963). Cochromatography (Ryhage & Stenhagen, 1963) helped identify the fatty acids as follows: $C_{14:0}$, 11%; $C_{16:1}$, 14%; C_{17} mycocerosate, 8%; $C_{18:0}$, 7%; C_{19} mycocerosate, 15%; C_{19} mycolipenic, 23% and $C_{19:0}$, 23%.

The location of these acyl functions on the oligosaccharide backbone was established using the procedure of Prehm (1980), a modification of that of Arnarp et al. (1975). The glycolipid was suspended in trimethyl phosphate and reacted with methyl trifluoromethanesulfonate and 2,6-di-*tert*-butylpyridine. The per-*O*-methylated lipooligosaccharide was purified on a C_{18} Sep-Pak cartridge (York et al., 1986) and treated with methylsulfinyl carbanion and C^2H_3I (Hakomori, 1964) to replace the *O*-acyl functions with *O*-C^2H_3 groups. The resulting per-*O*-trideuteriomethylated, per-*O*-methylated oligosaccharide, which showed characteristic cleavage ions (Figure 3) by EI/MS, was hydrolyzed with 2 M TFA, reduced with NaB^2H_4, and per-*O*-acetylated. GC/MS of the resulting alditol acetates revealed the presence of I, 1,5-di-*O*-CH_3CO-3,4,6-tri-*O*-C^2H_3-2-*O*-CH_3-glucitol (R_T 6.76 min; *m/z* 105, 118, 132, 154, 165, 167, 214); II, 1,5-di-*O*-CH_3CO-2,3,4,6-tetra-*O*-CH_3-glucitol (R_T 6.77 min; *m/z* 101, 102, 118, 145, 161, 162, 205); and III, 1,5,6-tri-*O*-CH_3CO-2-O-C^2H_3-3,4-di-*O*-CH_3-glucitol (R_T 7.60 min; *m/z* 105, 121, 129, 165, 177, 189, 233). These results established that the acyl functions

Table I: Chemical Shift of Ring Protons of Native Glycolipid Established through ^1H, ^{13}C, 2D ^1H COSY, and 2D $^1H/^{13}C$ NMR Spectroscopy

	t-β-D-Glc*p*	6-α-D-Glc*p*	*t*-α-D-Glc*p*
H-1	4.31	5.21	5.17
H-2	3.25	4.73	3.73
H-3		3.99	5.28
H-4		3.48	4.95
H-5		3.94	3.91
H-6a		4.08	4.11
H-6b		3.83	4.39

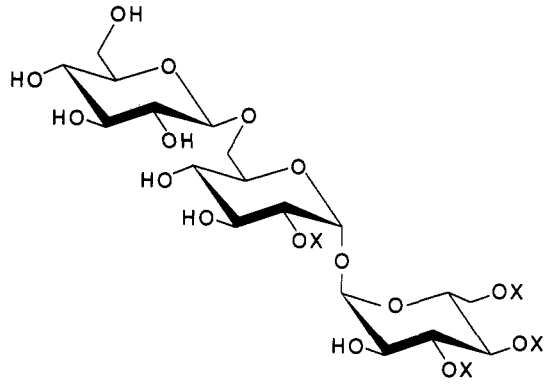

FIGURE 4: Complete structure of the trehalose-containing lipooligosaccharide, where X = acyl function (Table I).

were located at the 3-, 4-, and 6-OH positions of one of the two terminal glucoses and the 2-OH position of the internal 6-linked glucose residue. A close inspection of the ^1H, ^{13}C, 2D ^1H COSY, and 2D $^1H/^{13}C$ NMR spectra of the native glycolipid (Table I) allowed a resolution of the question of whether the acyl functions were located on the right-hand (β-D-Glc*p*) or left-hand (α-D-Glc*p*) terminus.

Connectivities were found for *t*-α-D-Glc*p* from H-1 to H-6_b, which suggested, from the chemical shift of the ring protons, that this residue was acylated at the 3-, 4-, and 6-hydroxyl positions. Hence, from the MS and NMR data, the *O*-acyl groups on the native glycolipid must be positioned on the 3-, 4-, and 6-hydroxyl of the right-hand terminus (α-D-Glc*p*). Therefore, the complete structure of the native glycolipid of *M. fortuitum, fortuitum* is shown in Figure 4, where X = acyl function. The native oligosaccharide and the per-*O*-methylated oligosaccharide were analyzed by positive-ion FAB/MS. The $(M + H)^+$ ion at m/z 505 was observed for the native oligosaccharide and $(M + NH_4)^+$ ion at m/z 676 for the per-*O*-methylated oligosaccharide, respectively. These results gave a clear indication of the molecular weights of the native oligosaccharide and were in accord with the glycosyl composition analysis.

Antigenicity of the Characteristic Glycolipid of M. fortuitum. Rabbit antibodies were raised to the whole bacterium and to the glycolipid itself. The results of the application of these in ELISA demonstrated (Table II), as expected, vigorous reactivity between the anti-whole bacterium and whole cells, but also between these antibodies and the glycolipid. Thus, the glycolipid in its native state as part of the bacterium is highly immunogenic. However, as an immunogen, the glycolipid is poor; weak reactions were observed between the bacterium and the antiglycolipid antibodies and less still between the glycolipid and the antiglycolipid antibodies.

Discussion

A significant outcome of the present study is the identification of a characteristic glycolipid, a β-D-Glc*p*-(1→6)-2-*O*-acyl-α-D-Glc*p*-(1↔1)-3,4,6-tri-*O*-acyl-α-D-Glc*p*, in isolates of

Table II. Antigenicity of β-D-Glc*p*-(1→6)-2-*O*-acyl-α-D-Glc*p*-(1↔1)-3,4,6-tri-*O*-acyl-α-D-Glc*p* of *M. fortuitum fortuitum*

	antigen ELISA value (A_{490})[a]	
antibody	whole cells	glycolipid
anti-whole bacteria		
1:1600	1.100	0.726
1:3200	0.983	0.526
antiglycolipid		
1:400	0.279	0.063

[a] In the case of whole cells, 100 μg of *M. fortuitum fortuitum* was loaded onto wells. When the purified glycolipid was the solid-phase antigen, 50 μg was used. The anti-whole bacterium antibodies were tested at a titer of 1:1600 and 1:3200, whereas the antiglycolipid antibody was used at a dilution of 1:400.

M. fortuitum biovar. *fortuitum*. The presence of a glycolipid with the overt characteristics of this one was first observed by Jenkins et al. (1971) and was confirmed by Pattyn et al. (1974) and Tsang et al. (1984); clearly, from its characteristic color on staining and its TLC mobility, the same glycolipid was being noted throughout. Until now, this glycolipid has defied characterization, perhaps because inappropriate isolates were used; it apparently is not expressed in full quantity by all isolates of *M. fortuitum*, biovar. *fortuitum*.

The antigenic distinction between *M. fortuitum fortuitum* and *M. fortuitum peregrinum* has already been mentioned; *M. fortuitum peregrinum* isolates contain a characteristic member of the glycopeptidolipid class of antigens (Lopez-Marin, 1991). The fact that not all isolates of *M. fortuitum fortuitum* contain the characteristic β-D-Glc*p*-(1→6)-2-*O*-acyl-α-D-Glc*p*-(1↔1)-3,4,6-tri-*O*-acyl-α-D-Glc*p* described here may be a matter of some significance. Levy-Frebault et al. (1983) recognized six environmental isolates of *M. fortuitum* that did not fit the biochemical patterns of the two recognized biovariants of *M. fortuitum*, i.e., *M. fortuitum fortuitum* and *M. fortuitum peregrinum*. Likewise, Wallace et al. (1991) collected a much larger assemblage of clinical strains that did not conform to the specification of *fortuitum* or *peregrinum* and which the authors referred to as the third biovariant complex. The collection of *M. fortuitum* isolates used in the present study are clearly devoid of *M. fortuitum* biovar. *peregrinum* isolates; these, with their characteristic, easy-to-identify GPL antigens, were removed from the collection (Tsang, 1984). However, the collection was not further screened for members of the third biovariant. Accordingly, the possibility remains that the characteristic β-D-Glc*p*-(1→6)-2-*O*-acyl-α-D-Glc*p*-(1↔1)-3,4,6-tri-*O*-acyl-α-D-Glc*p* is selective for members of either the third biovariant or of *M. fortuitum* biovar. *fortuitum*.

This is an important point. The nature of the clinical disease usually associated with *M. fortuitum fortuitum*—nonpulmonary, primary skin, and soft tissue infections—has been mentioned above. Apparently, infections caused by the third biovariant do not differ significantly; again, most of them involve skin, soft tissue, or bone and occurred after puncture wounds or open fracture. On the other hand, isolates of the third biovariant differed from biovar. *fortuitum* in resistance to pipemidic acid and in their use of mannitol and inositol as carbon sources (Silcox et al., 1981; Wallace et al., 1991). Most importantly, isolates were resistant to doxycycline and cefoxitin. Thus, the possibility that such opportunistic pathogens can be differentiated on the basis of a major surface antigen is important and is being further pursued.

The results of the analysis conducted to date (Hunter et al., 1983, 1988; McNeil et al., 1987b; Daffe et al., 1991b), of the surface glycolipids of the acyltrehalose-containing lipooligo-

saccharide class, present hints of other intriguing biochemical principles. Glycolipids of this class are now known to extend from the most elemental kind, i.e., the basic acylated trehalose, to those with an extended linear oligosaccharide chain, which, invariably, in their most complex and antigenic form, contain a complex acylamido sugar. Thus, we[2] and colleagues (Minnikin et al., 1985) have described the elemental acyltrehaloses of the appropriate class (i.e., those containing medium-chain-length, methyl-branched fatty acids such as mycolipenic acid) as distinct from those containing mycolic acids (Minnikin & Goodfellow, 1980). We and others have also described the glycosylated versions of these, such as a diglycosyl acyltrehalose (Camphausen et al., 1987) with and without additional pyruvyl groups (Saadat & Ballou, 1983) and a large range of multiglycosylated acyltrehaloses, all species-specific, from such as *M. kansasii* (Hunter et al., 1983), *M. szulgai* (Hunter et al., 1988), *M. malmoense* (McNeil et al., 1987b), and an atypical strain of *M. tuberculosis* (Daffe et al., 1991b). In an effort to correlate these individual acyltrehalose-containing glycolipids with other phenotypic traits—with a view to understanding mycobacterial pathogenesis—we have noticed a relationship between colony morphology and the nature of the inherent acyltrehalose-containing glycolipid. This relationship is best seen in the case of *M. kansasii* in which smooth variants contain the full range of multiglycosylated acyltrehaloses and the rough variants are completely devoid of these (Belisle & Brennan, 1989); it did not occur to us at the time to search for elemental acyltrehalose in the rough variants. Likewise, an unclassified strain of *Mycobacterium*, with a monoglycosyl acyltrehalose, presents a consistently rough appearance (Camphausen et al., 1987). But above all, the majority of field isolates of *M. tuberculosis*, those which yield the elemental acyltrehalose, are consistently of classically rough morphology, and, to further accentuate the point, a minority of both field and laboratory isolates of *M. tuberculosis* are of distinct smooth appearance, and they do express a multiglycosylated acyltrehalose (Daffe et al., 1991a).

Thus, in this work, we have begun to realize the existence of a relationship between the simple, basic acyltrehaloses and the multiglycosylated forms, and this realization leads us to propose that many of the known mycobacterial strains may be spontaneous mutants of one another, perhaps, as is the case among the rough and smooth variants of *M. avium*,[3] due to spontaneous genetic deletions resulting in loss of sizable amounts of carbohydrate substituents.

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[2] G. S. Besra, R. C. Bolton, M. McNeil, M. Ridell, P. J. Brennan, and D. E. Minnikin, unpublished results. Analysis of the lipids of *M. tuberculosis* H37Rv revealed a series of glycolipids based on 2,3-di-*O*-acyltrehalose. These acylated trehaloses differed only in their fatty acyl population and were mainly comprised of three types, saturated straight chain C_{16}–C_{19} acids, C_{21}–C_{25} mycocerosic acids, and C_{24}–C_{28} mycolipanolic acids. It was apparent that at least six molecular species differing by their fatty acyl substituents comprised this family of acylated trehaloses.

[3] J. T. Belisle, J. M. Inamine, and P. J. Brennan, unpublished results.

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Structural Characterization of x_2 Glycosphingolipid, Its Extended Form, and Its Sialosyl Derivatives: Accumulation Associated with the Rare Blood Group p Phenotype†

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ABSTRACT: It has been suggested that the x_2 glycosphingolipid (GSL) could offer a structural basis for a P-like antigen activity found in blood group p individuals [Kannagi R., Fukuda, M. N., Hakomori, S. (1982) *J. Biol. Chem. 257*, 4438]. The structures of the x_2 and sialosyl-x_2 GSLs have been confirmed unequivocally as shown below by $^+$FAB-MS, methylation analysis by GC-MS, and ^{1}H-NMR. We have established a

x_2 GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer

sialosyl-x_2 NeuAcα2→3GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer

monoclonal antibody (TH2) specific for the GalNAcβ1→3Galβ1→4GlcNAc epitope, the terminal trisaccharide of x_2 GSL. Application of MAb TH2 on TLC immunoblotting together with chemical analysis indicates the following points of interest: (i) the existence of extended type GSLs having the same x_2 terminal structure; (ii) the chemical quantities of x_2, sialosyl-x_2, and extended x_2 found in blood cells and in various tissues including carcinomas being nearly the same; (iii) considerably larger quantities of x_2 and x_2-derived structures found in blood samples of rare blood group p individuals. The accumulation of x_2 and its derivatives in blood cells of p individuals is in contrast to the occurrence of these GSLs as extreme minor components in normal human red blood cells and tissues, and they may be responsible for the reported P-like activity in blood group p individuals [Naiki, M., & Marcus, D. M. (1977) *J. Immunol. 119*, 537].

First described by Landsteiner and Levine (1927), the P blood group system consists of three antigens, P, P_1, and P^k, and five phenotypes, the common P_1 and P_2 and the rare p, P_1^k, and P_2^k [for reviews, see Prokop and Uhlenbruck (1969) and Race and Sanger (1975)]. The P_1 and P^k antigens, while structurally related by virtue of having the identical immunodominant terminal sugar, are products of divergent pathways and possess distinct serological activities. The P^k and P antigens, on the other hand, are directly biosynthetically related (see Figure 1) [for reviews on immunochemistry of the P system, see Watkins (1980) and Marcus et al. (1981)].

Although blood group p individuals do not express the P antigen identified as Gb_4, p RBCs[1] show reactivity with anti-P serum (Naiki & Marcus, 1977). One candidate for this P-like

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[1] Abbreviations: BSA, bovine serum albumin; CM, chloroform–methanol; CMW, chloroform–methanol–water; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunostaining assay; $^+$FAB-MS, positive ion fast atom bombardment mass spectrometry; FITC, fluorescein isothiocyanate; GC–MS, gas chromatography–mass spectrometry; GSL, glycosphingolipid; ^{1}H-NMR, proton nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; Ig, immunoglobulin; IHW, isopropanol–hexane–water; MAb, monoclonal antibody; PBS, phosphate-buffered saline; PG, paragloboside; PMAA, partially methylated alditol acetate; RBC, red blood cell; RT, room temperature; SPG, sialosylparagloboside; WBC, white blood cell.

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