Structure and Relevance of the Oligosaccharide Hapten of *Mycobacterium avium* Serotype 2

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The type-specific antigen of *Mycobacterium avium* serotype 2, the most ubiquitous of serotypes within the *M. avium* complex and a major cause of disseminated and localized infections, was isolated and purified. It is of the glycopeptidolipid (mycoside C) class with a characteristic oligosaccharide hapten. This was released as the oligosaccharide alditol by base-catalyzed reductive β -elimination, and the structure was established by a combination of gas chromatography-mass spectrometry, methylation analysis, fast atom bombardment mass spectrometry, and ^{13}C and ^1H nuclear magnetic resonance as 2,3-di-*O*-methyl-L-fucopyranosyl-($\alpha 1 \rightarrow 3$)-L-rhamnopyranosyl-($\alpha 1 \rightarrow 2$)-6-deoxytalitol. A feature of the work was the elucidation of the absolute (enantiomeric) configuration of the sugars. This same structure, in much less detail, was previously reported as the species-specific hapten of strains of *Mycobacterium paratuberculosis*. Thus, the work raises the intriguing possibility that some *M. avium* serotypes are synonymous, at least in outer cell wall anatomy, with the agent(s) of paratuberculosis (Johne's disease), an insidious disease of vast proportions in ruminants. In addition, recognition of a specific determinant will allow a precise study of the epidemiology of *M. avium* infections in humans and animals.

Mycobacterium avium, particularly serotype 2, long recognized as a pathogen for birds (33) and other animals (27), has also been identified as a ubiquitous organism capable of causing chronic infections in humans (35). In certain instances, such as those described by Wolinsky (39), such infections may develop into disease. The startling observation in recent years that a variety of serotypes of *M. avium* and *Mycobacterium intracellulare* are responsible for disseminated infections in many of the patients with acquired immunodeficiency syndrome (2, 16) has resulted in renewed interest in these environmental mycobacteria. *M. avium* and *M. intracellulare* have slow growth rates (29) and may cause disease clinically similar to that caused by *Mycobacterium tuberculosis* (39). However, they differ from *M. tuberculosis* in several respects, particularly in resistance to most antituberculosis drugs (18, 39). Accordingly, there is a need to distinguish such agents of "atypical mycobacterioses" from those responsible for mammalian tuberculosis. We conform to the principle that a study of the physiology of *Mycobacterium* spp., notably that of the cell wall, will provide information pertinent to drug resistance, persistence, and differentiation; the last is particularly important in tracing the epidemiology of infections. Previously, we had shown that the type-specific antigens of serotypes of *M. avium* and *M. intracellulare* (the so-called *M. avium* complex) (3-5) and a few other mycobacteria (37), including strains of *Mycobacterium paratuberculosis* (8), are of the mycoside C, glycopeptidolipid (GPL) class, composed of an invariant core, fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-alaninol-O-(3,4-di-*O*-methyl-rhamnose), with a type-specific oligosaccharide hapten attached to the hydroxyamino acid, threonine. In the present communication, the structure of the characteristic oligosaccharide hapten of serotype 2, one of the most ubiquitous of the *M. avium* complex, is described in full detail, and the application of this information to the study of the etiology of a form of mycobacteriosis, paratuberculosis, is demonstrated.

MATERIALS AND METHODS

Mycobacteria. Authenticated (38) strains of *M. avium* serotype 2, other *M. avium* complex serotypes, and *M. paratuberculosis* strains 18 and ATCC 19698 (8) were grown in 7H9 medium as described previously (5).

TLC of lipid extracts. For analysis of isolates based on the characteristic deacetylated GPL (dGPL) profiles, cells (ca. 50 mg) dried to crispness in vacuo over P_2O_5 , were extracted with 3 ml of CHCl_3 - CH_3OH (2:1) at 50°C for 18 h. After centrifugation at $1,400 \times g$ for 15 min, the supernatant was recovered and washed with 0.5 ml of water. The lipid-rich CHCl_3 phase was evaporated under a stream of N_2 , and the contents were saponified with NaOH (0.1 M) in 1.5 ml of CHCl_3 - CH_3OH (1:2) at 37°C for 30 min. The mixture was extracted with 1.5 ml of CHCl_3 and 0.5 ml of H_2O , and the lower organic phase was dried under N_2 . The resultant lipids were dissolved in CHCl_3 - CH_3OH (2:1) before thin-layer chromatography (TLC). Routine TLC was conducted on silica gel 60-precoated sheets (E. Merck AG, Darmstadt, Federal Republic of Germany) in CHCl_3 - CH_3OH - H_2O (30:8:1) or CHCl_3 - CH_3OH - H_2O (65:25:4) and sprayed with 10% H_2SO_4 , seeking the alkali-stable, yellow-gold spots typical of the dGPLs (6). The type-specific dGPL antigens from serotypes of the *M. avium* complex were included on such plates for comparison.

Isolation and purification of specific GPLs. Large stocks of isolates were grown in 2.8-liter Fernback flasks. Flasks were autoclaved, and cells were harvested, or the entire suspension consisting of both medium plus bacilli was evaporated to dryness in crystallizing dishes, for convenience. The dried residue was twice extracted with CHCl_3 - CH_3OH (2:1, 40 ml/g) at 50°C for 18 h, and the dried lipid extracts were suspended in CHCl_3 - CH_3OH - H_2O (4:2:1) to yield a biphasic mixture. The lower CHCl_3 phase was used as the source of lipid.

To isolate alkali-stable dGPL, approximately 1-g lots of washed lipid were suspended in 90 ml of 0.1 N NaOH in CHCl_3 - CH_3OH (1:2) and kept at 37°C for 30 min. CHCl_3 (90

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ml) and 30 ml of H₂O were added, the suspension was mixed, the aqueous phase was discarded, and the CHCl₃ phase was washed with 0.2 volume of CH₃OH-H₂O (1:1). The dried alkali-stable lipids were dissolved in the minimal volume of CHCl₃ and applied to columns (25 by 2 cm) of Florisil in CHCl₃ and eluted initially with 3 bed volumes of 15% acetone in CHCl₃ followed by an equal volume of CHCl₃-CH₃OH (2:1) to remove total (i.e., polar and apolar) GPLs.

For isolation of the type-specific polar dGPL, the total dGPL was suspended in the minimum volume of CHCl₃ and applied to columns (20 by 1.2 cm) of silicic acid-Celite (2:1) equilibrated in CHCl₃. Columns were eluted with 4 bed volumes of 15% acetone in CHCl₃ followed by 5, 10, and finally 33% CH₃OH in CHCl₃. The characteristic polar dGPL of serotype 2 appeared in the 10% CH₃OH eluates. Final purification was accomplished by preparative TLC on 20- by 20-cm silica gel G plates (Fisher Scientific Co., Pittsburgh, Pa.) in CHCl₃-CH₃OH-H₂O (30:8:1). A second such purification was often required for acceptable purity.

Preparation of the reduced oligosaccharide (r-Ose). Pure dGPL was subjected to alkaline borohydride reductive β -elimination (7) to release the specific oligosaccharide hapten as the oligosaccharide alditol (r-Ose). dGPL (200 mg) was dissolved in 6 ml of C₂H₅OH-H₂O (5:1) followed by 4 ml of 1 M NaOH and 16 mmol of NaBH₄ and heated for 24 h at 60°C. The reaction mixture was neutralized with acetic acid and flash evaporated in the presence of CH₃OH to remove boric acid. The dried residue was partitioned between 60 ml of CHCl₃-CH₃OH (2:1) and 10 ml of H₂O and centrifuged at $1,400 \times g$ for 10 min. The upper aqueous phase was recovered and evaporated. The residue was suspended in 1 ml H₂O and fractionated on a column (150 by 2.5 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.). Final purification was achieved on a column (220 by 1 cm) of Sephadex G-15. Column eluates were assayed for carbohydrate with phenol-H₂SO₄ (12).

Analytical procedures. Purified dGPL was hydrolyzed with 2 M CF₃COOH for 3 h at 100°C to obtain constituent sugars. Alditol acetates were prepared and chromatographed on a 1.8-m column of 3% SP-2340 on 100-120-mesh Supelcoport (Supelco, Bellefonte, Pa.) at a N₂ flow rate of 45 ml/min as described previously (21). Other details are given in figure legends.

r-Ose was perdeuteriomethylated by the procedure of Stellner et al. (36) and purified by preparative TLC in ether-acetone (5:1). Demethylation of r-Ose was performed by a modification of the procedure of Mort and Bauer (30) as follows. r-Ose (1 mg) suspended in 1 ml of ethylenediamine (distilled over KOH) in a 100- by 13-mm test tube was agitated vigorously on a Vortex mixer together with three 1-cm lengths of Li wire (0.25-cm diameter). A deep blue color indicative of electron solvation was maintained for 1 h by supplementing (every 10 to 15 min) the reaction mixture with an additional length of Li wire. The mixture was chilled in an ice bath and slowly reacted with 5 ml of H₂O by alternately thawing and freezing. Ethylenediamine was removed by flash evaporation with toluene. The residue was suspended in 1 ml of H₂O, neutralized with acetic acid, and passed through a 5- by 1-cm column of Dowex 50 X-8 H⁺ (100-200 mesh; Bio-Rad). The eluate containing the demethylated but glycosidically intact r-Ose was dried on a flash evaporator.

Constituent sugars of the r-Ose, the perdeuteriomethylated r-Ose, and the demethylated r-Ose were determined by gas chromatography (GC) or GC-mass spectrometry (MS) of alditol acetates. Samples were hydrolyzed with 2 M

CF₃COOH for 3 h at 100°C (21); however, NaB[²H]₄ was used as the reducing agent. GC of such alditol acetates was performed on a 30-m by 0.25-mm, 0.2- μ m film thickness SP2340 fused silica capillary column (Supelco) installed on a Varian model 3700 gas chromatograph equipped with a flame ionization detector (250°C) and a universal capillary injector (250°C). Analyses were recorded on a Hewlett-Packard model 3380A integrator. Alditol acetates derived from the r-Ose and demethylated r-Ose were chromatographed at 210°C isothermally; those derived from the perdeuteriomethylated r-Ose were chromatographed with a temperature program of 170°C for 3 min followed by an increase of 4°C/min to 235°C. GC-MS was conducted on an identical SP2340 capillary column with similar temperature programs; the fused silica column was installed in a Perkin-Elmer model Sigma 3 gas chromatograph equipped with a split injector (1:30) and maintained at 250°C. Mass spectra (70 eV) were recorded on a VG Instruments (Winford, England) model MM16F low-resolution mass spectrometer and data system with a capillary GC inlet, direct probe, and both electron and chemical ionization capabilities.

(+)-2-Butyl glycosides from demethylated r-Ose, L-fucose, and L-rhamnose were prepared and trimethylsilylated for GC by the procedure of Gerwig et al. (15). Trimethylsilyl derivatives were chromatographed on a 30 m- by 0.32-mm, 0.25- μ m film thickness Durabond-1 fused silica column (J&W Scientific, Rancho Cordova, Calif.). The column was installed in a Hewlett-Packard model 5710 gas chromatograph equipped with a dropping needle injector (R. C. Allen Glass, Boulder, Colo.) and a flame ionization detector; both the injector and detector were maintained at 250°C. Analyses were recorded on a Hewlett-Packard model HP3390A integrator. The trimethylsilyl (+)-2-butylglycosides were chromatographed with a temperature program of 150°C for 2 min followed by an increase of 2°C/min to 210°C.

¹H nuclear magnetic resonance (¹H-NMR) and ¹³C-NMR were recorded on a Nicolet NT-360 and Nicolet 1180 computer operating in the Fourier-transform mode. Proton-coupled ¹³C spectra were obtained by using gated decoupling. ¹³C signals for the C-1 atoms of the rhamnosyl and 2,3-di-O-Me-fucosyl residues were determined by selective proton decoupling experiments. Chemical shifts are reported with respect to internal acetone (82.225 for ¹H-NMR; 829.8 for ¹³C-NMR).

Analytical TLC was performed with the solvents described below. Compounds were visualized with a spray of 10% (vol/vol) concentrated H₂SO₄ in C₂H₅OH followed by heating at 110°C for 5 min.

Immunoassays. A TLC-enzyme-linked immunosorbent assay (ELISA) was conducted by applying two ca. 10- μ g portions of dGPL to parallel TLC strips 2 cm apart which were developed in CHCl₃-CH₃OH-H₂O (30:8:1). The dGPL was located on one strip with 10% H₂SO₄, and the other strip was employed in the TLC-ELISA as follows. The chromatogram was prewetted with 2% (wt/vol) polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) (pH 7.4) and blocked for 1 h at room temperature in 10% (wt/vol) polyvinylpyrrolidone in PBS. The strip was washed thoroughly with PBS, reacted with 500 μ l of a 1:2 dilution of rabbit anti-serotype 2 serum (38) in PBS for 1 h, washed, and treated further with 1 ml of a 1:500 dilution in PBS of goat anti-rabbit immunoglobulin G (IgG)-IgM-IgA horseradish peroxidase conjugate (Cappel Worthington, Malvern, Pa.). The strip was finally washed and reacted with substrate (6 mg of 4-chloro- α -naphthol [Bio-Rad] in 2 ml of

CH₃OH, 10 ml of PBS, 30 μ l of 30% H₂O₂) for 10 min in the dark, rinsed again with PBS, and dried in the dark.

The reaction of dGPL from *M. paratuberculosis* 18 with rabbit antiserum to the homologous organism was examined by plate ELISA. Purified dGPL was sonicated in ethanol (1 μ g/ml), and 50 μ l was dried overnight in wells of polystyrene microtiter plates (Dynatech Laboratories, Chantilly, Va.). Wells were blocked for 1 h with PBS containing 0.1% Tween 80 (PBS-Tween), rabbit antiserum prepared as described previously (38) and diluted serially in PBS-Tween was added to the plates, and the plates were incubated for 1 h at room temperature in a humid chamber. The plates were washed four times with PBS-Tween, and goat anti-rabbit IgG (Cappel Laboratories) diluted 1:1,000 in PBS-Tween was added. After incubation for 30 min, the plates were washed as described above. Substrate (50 μ l) was added, the plates were incubated in the dark for 30 min, the reaction was stopped by the addition of 2.5 N H₂SO₄, and the absorbance read at 490 nm on an automatic ELISA reader (Dynatech Laboratories). Serum dilutions at the point of decline from the plateaus of titration curves were chosen for the inhibition assays; the dilution of choice was 1:4,000.

For competitive inhibition ELISA, the serotype 2 polar dGPL was suspended in 1 ml of PBS by probe sonication, and 100 μ l of this preparation was added to 900 μ l of the diluted anti-strain 18 rabbit serum. The mixture was incubated at 37°C for 1 h. Portions of 50 μ l were added to wells of a microtiter plate precoated with *M. paratuberculosis* 18 polar dGPL and incubated at room temperature for 1 h. The remainder of the assay steps were identical to those described for the direct ELISA.

Materials. The origin of partially methylated rhamnitol and fucitol acetates used as GC standards has been described previously (7). CF₃COOH, NaB¹⁰H₄, (+)-2-butanol, and reagents for perdeuteriomethylation were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Materials for trimethylsilylation were purchased from Supelco. Analytical TLC was performed with silica gel 60 aluminum-backed

FIG. 1. GC of the alditol acetates derived from the pure polar dGPLs of *M. avium* serotype 2 and *M. paratuberculosis* 18. GC was conducted on a 1.8-m column of 3% SP-2340 on 100-120 mesh Supelcoport at 170°C isothermal temperature.

FIG. 2. A, TLC of the total alkali-treated lipids from several *M. avium* complex serotypes and *M. paratuberculosis* 18, demonstrating the relative mobilities of the specific polar dGPLs. The solvent was CHCl₃-CH₃OH-H₂O (30:8:1). B, TLC of the pure polar dGPL from strain 18 and serotype 2. Plates were sprayed with 10% H₂SO₄ and heated at 104°C for 10 min. The arrows point to the type-specific polar dGPLs as distinct from the shared apolar GPLs.

sheets (Merck), and preparative TLC was performed with Silica gel G glass sheets (Fisher Scientific Co.).

RESULTS

Preparation of the type-specific dGPL antigen of serotype 2.

The scheme employed for the recovery and purification of the characteristic dGPL from *M. avium* serotype 2 relies on extraction, alkalinolysis, and absorption chromatography. Lyophilized cells, or cells dried together with spent media, were extracted with CHCl₃-CH₃OH (2:1) to recover the total lipid population and treated with 0.2 M NaOH to destroy nonspecific glycerides and deacetylate the variably acetylated GPLs. The characteristic polar dGPL was separated from common apolar dGPLs by column absorption chromatography. Chromatography on a second column usually resulted in a pure polar dGPL preparation. Total lipid accounted for approximately 10 to 15% of the mass of lyophilized cells; of this, roughly 5% comprised the characteristic polar dGPL.

Similarity of the specific dGPL antigen of *M. avium* serotype 2 and *M. paratuberculosis* strains. The patterns of sugars in the specific dGPL from serotype 2 was examined by GC of the alditol acetates (Fig. 1). GC-MS showed mass fragments of *m/z* 87, 89, 99, 115, 129, 131, 189, and 233, for the first peak, those of a 3,4-di-*O*-methyl-6-deoxyhexitol acetate; *m/z* 101, 117, 143, and 203 for the second peak, those of a 2,3-di-*O*-methyl-6-deoxyhexitol acetate; *m/z* 87, 101, 129, 143, 189, and 203 for the third peak, those of a 3-*O*-methyl-6-deoxyhexitol acetate; and *m/z* 115, 128, 157, 170, 187, 203, and 303 for the final two peaks, those of 6-deoxyhexitol acetates (22). A comparison of the retention times of these peaks with those of standards (7) allowed the identifications

FIG. 3. GC-MS of the alditol acetates derived from the perdeuteriomethylated r-Ose of the dGPL antigen of serotype 2. The total ion current chromatogram and formation of diagnostic ions are portrayed. Perdeuteriomethylation, hydrolysis, alditol acetate preparation, and GC-MS are described in Materials and Methods.

FIG. 4. Direct probe electron impact-MS of the perdeuteriomethylated r-Ose.

indicated in Fig. 1. Previously, we had demonstrated that this same set of sugars typified the major immunoreactive surface GPL (so-called polar GPL-I) of strains of *M. paratuberculosis* (8). This was confirmed in the present set of experiments (Fig. 1); clearly, the alditol acetates from both sources are identical. In addition, a preparation of dGPL from *M. paratuberculosis* 18, rich in polar dGPL-I, was compared by TLC to the unfractionated GPLs from several serotypes of *M. avium*, including serotype 2 (Fig. 2A). Perfect concordance was seen between the purified polar GPL-I of *M. paratuberculosis* and that from serotype 2 (Fig. 2B).

FIG. 5. NMR of the r-Ose. A, Proposed structure and assignment of proton and carbon signals. B, ^1H -NMR of r-Ose at 360 MHz with expansion of 5.4 to 4.8 region. C, ^{13}C -NMR at 360 MHz with wide band decoupling. The C-1 proton-coupled ^{13}C -NMR spectrum is included.

FIG. 6. Parallel TLC and TLC-ELISA of the polar dGPL of serotype 2 in $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (30:8:1). Plate A was subjected to ELISA as described in Materials and Methods. Plate B was charred with 10% (vol/vol) H_2SO_4 in $\text{C}_2\text{H}_5\text{OH}$ at 110°C for 5 min.

Isolation of the oligosaccharide hapten of serotype 2 as r-Ose. From past experience (3), it was presumed that the 3,4-di-*O*-methyl-rhamnose associated with the GPL was of the invariant core, and the remaining sugars comprised the appended oligosaccharide (see below). To isolate the oligosaccharide hapten, two lots of GPL (ca. 200 mg) were subjected to alkaline reductive β -elimination. After neutralization, evaporation, and a biphasic solvent separation, TLC of the organic soluble material in $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (45:5:0.5) showed that virtually all of the GPL was converted to a new, highly mobile lipid, the degraded GPL core (5). The aqueous phase was evaporated, suspended in a minimal amount of H_2O , and fractionated on a Bio-Gel P-2 column. Carbohydrate assays of column eluates showed a major component and four very minor oligosaccharide degradation products. A second fractionation of the major carbohydrate peak on Sephadex G-15 yielded a sharp, apparently homogeneous fraction. TLC of the r-Ose in 1-propanol- H_2O -concentrated NH_4OH (80:20:1) confirmed its purity. Recovery of purified r-Ose (85 mg) represented 22% by weight of the polar dGPL.

Structure of the r-Ose from serotype 2. The sugar composition of the r-Ose (0.5 mg) was determined by GC and GC-MS of the ^2H -alditol acetates. Three components were observed in roughly equimolar concentration (results not shown; Fig. 1). The most volatile acetate comigrated with 2,3-di-*O*-methyl-fucitol acetate, and the mass fragment ions (m/z 87, 101, 102, 118, 143, 162, and 203) were consistent with those of a 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-6-deoxy-[1- ^2H]hexitol (22). The acetate of a second sugar cochromatographed with rhamnitol acetate prepared from the authentic sugar and the mass fragment ions (m/z 115, 129, 157, 171, 188, 231, 290, and 303) were in accord with those of a penta-*O*-acetyl-6-deoxy-[1- ^2H]hexitol. The least volatile acetate comigrated with 6-deoxytalitol acetate, derived from the authentic sugar, and the mass fragmentation pattern (ions at m/z 115, 128, 157, 170, 187, 231, 289, and 303) showed concordance with that of a penta-*O*-acetyl-6-deoxyhexitol with no 1- ^2H , since this alditol was generated during the reductive β -elimination which employed NaBH_4 rather than $\text{NaB}[^2\text{H}]_4$. These results established that the reduced oligosaccharide was a diglycosyl alditol and that linkage of the native trisaccharide to the GPL core must be mediated through the 6-deoxytalose residue.

FIG. 7. ELISA inhibition and the pure polar dGPLs of *M. paratuberculosis* 18 and *M. avium* serotype 2. Plates were coated with the pure polar dGPL from strain 18 (1 μg/ml; 50 ng/well). The anti-strain 18 serum, diluted 1:4,000, preexposed to various concentrations of the pure polar dGPL from serotype 2, was added to the plates before developing the reaction.

To establish the sugar sequence and linkage positions, the r-Ose was perdeuteriomethylated, purified by preparative TLC, hydrolyzed, and derivatized. GC-MS of the partially perdeuteriomethylated alditol acetates (Fig. 3) revealed two major components that were present in roughly equimolar quantities; one comigrated with authentic 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-fucitol, and the other comigrated with authentic 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol acetate. The mass fragment ions (m/z 92, 102, 104, 118, 134, 162, and 178 and m/z 92, 107, 121, 128, 134, 205, and 240, respectively) established that one was 1,5-di-*O*-acetyl-2,3-di-*O*-methyl-4-*O*-[^2H]₃methyl-fucitol and the other was 1,3,5-tri-*O*-acetyl-2,4-di-*O*-[^2H]₃methyl-rhamnitol (20, 22). A third peak, apparent on GC in less than stoichiometric amounts, is believed to be the highly volatile partially perdeuteriomethylated 6-deoxytalitol acetate; its fragmentation pattern (ions at m/z 62, 74, 90, 107, 132, 154, 167, and 214) is consistent with that of a 2-*O*-acetyl-1,3,4,5-tetra-*O*-[^2H]₃methyl-6-deoxyhexitol. These results established the linkage arrangement 2,3-di-*O*-methyl-fucose-(1→3)-rhamnose-(1→2)-6-deoxytalitol.

Evidence gained from direct insertion electron impact MS of the perdeuteriomethylated r-Ose and application of the principles of Kochetkov and Chizhov (24), Kovacic et al. (25, 26), and Sharp and Albersheim (34) helped establish the sequence and linkage pattern. Ions at m/z 192 (aA₁, Fig. 4) and m/z 217 (ald J₂) arise from the nonreducing 2,3-di-*O*-methyl-6-deoxyhexosyl and the 6-deoxyhexitol termini (Fig. 4, inset), respectively. The ions at m/z 372 (baA₁) and m/z 543 (alditol cleavage) confirm the presence of the internal 6-deoxyhexosyl residue. The ion at m/z 263 (ald J₀) indicates that the internal 6-deoxyhexosyl residue is 3 linked, since this ion, 46 mass units greater than the old J₂ ion, has been shown to occur only in the case of 3-linked residues (25). Although the b ald J₂ ion at m/z 397 was not observed, an ion at m/z 457 (b ald J₁) demonstrates that the terminal 6-deoxyhexosyl residue (residue a, Fig. 4) contains an OCH₃, not an OC[^2H]₃, group at C-3; the methyl group in the C-3 is present in the J₁ ion. The origin of the prominent ion at m/z 353 cannot be predicted at this time.

In previous work with other GPL antigens, we had never established the absolute (enantiomeric) configurations of any of the glycosyl residues. Accordingly, the r-Ose was demethylated with Li, and the efficacy of the reaction was determined by GC of the alditol acetates. Although incomplete, the demethylation reaction substantially decreased the concentration of 2,3-di-*O*-methyl-fucitol acetate relative to the rhamnitol and 6-deoxytalitol acetates. In addition, a new acetate cochromatographing with the acetate derived from L-fucose was observed. Consequently, (+)-2-butyl glycosides were prepared from the demethylated r-Ose (250 μg) and then trimethylsilylated. GC analyses of the derivatized (+)-2-butyl glycosides obtained from the demethylated r-Ose revealed components cochromatographing with trimethylsilylated (+)-2-butyl-L-rhamnoside and (+)-2-butyl-L-fucoside prepared from enantiomerically pure sugars. These results indicate that the 2,3-di-*O*-methyl-fucosyl and the rhamnosyl residues in the parent r-Ose are in the L-enantiomeric configuration.

^1H -NMR and ^{13}C -NMR of r-Ose (62 mg in D₂O) (Fig. 5) confirmed the basic trisaccharide alditol structure and were used to establish the anomeric configuration of the 2,3-di-*O*-methyl-fucopyranosyl and rhamnopyranosyl residues. ^1H -NMR (Fig. 5B) revealed three CH-CH₃ signals ($\delta \approx 1.15$, $J_{5,6} \approx 6.5$), two O-CH₃ signals ($\delta \approx 3.3$), and two anomeric signals, one at δ 5.29 ($J_{1,2} = 3.8$) and the other at δ 4.82 ($J_{1,2} = 1.42$). The coupling constant and chemical shift of the proton at δ 5.29 suggested the signal be assigned to the 2,3-di-*O*-methyl-fucopyranosyl residue and indicated an α configuration (8). The anomeric signal at δ 4.82 has a small coupling constant and was therefore assigned to the rhamnopyranosyl residue (8). The anomeric configuration of the rhamnopyranosyl residue could not be established by ^1H -NMR, because a chemical shift of δ 4.82 is consistent with either an α or β configuration. Therefore, the C1-H1 coupling of the rhamnopyranosyl residue was established by proton-coupled ^{13}C -NMR (Fig. 5C) and found to be 168.2 Hz. The rhamnopyranosyl residue was thus determined to be in the α -anomeric configuration, because a C1-H1 coupling of ~ 160 Hz is expected for β -rhamnopyranosyl residues, and a C1-H1 coupling of 170 Hz is expected for α -rhamnopyranosyl residues (23).

Based on these findings, the structure 2,3-di-*O*-methyl- α -L-fucopyranosyl-(1→3)- α -L-rhamnopyranosyl-(1→2)-6-deoxytalose is proposed for the type-specific oligosaccharide segment of the GPL antigen of *M. avium* serotype 2. We had previously proposed an identical structure, based on much less information, for the oligosaccharide hapten of strains of *M. paratuberculosis* (8).

Immunoreactivity of the GPL from serotype 2. The immunoreactivity of the pure dGPL was established by a variation on the TLC-ELISA procedure of Magnani et al. (28) (Fig. 6). GPL was spotted in parallel on TLC strips and developed in solvent. One-half was reacted with 10% H₂SO₄, and the other was reacted with rabbit anti-serotype 2 followed by anti-rabbit IgG-M-A enzyme-conjugated antibody and substrate. A purple precipitate (Fig. 6) formed at the spot corresponding to the chemically identified GPL, demonstrating the immunoreactivity of the glycolipid. Thus, the dGPL from serotype 2 is highly reactive for antibodies raised with whole killed bacteria in which the antigen is probably in its acetylated, undegraded form. This is not an invariant rule among *M. avium* complex serotypes; in the case of serotype 9 only the native, acetylated GPL reacts with the corresponding antibodies (H. Gaylord and B. Ranchoff, unpublished observations).

In an attempt to support the evidence for structural homology between the specific polar GPLs of serotype 2 and *M. paratuberculosis*, antigenic homology was also sought by inhibition ELISA. ELISA plates were coated with 1 µg (50 ng/well) of the pure polar GPL-I from *M. paratuberculosis* per ml. Rabbit antibodies already titrated against the homologous glycolipid and diluted 1:4,000 were preincubated with different concentrations of the pure glycolipid from serotype 2 and reacted against the solid-phase antigen (Fig. 7). The rabbit antibodies, the majority of which are known to be anti-glycolipid (40), bound to the heterologous glycolipid and, as demonstrated, could not react with the homologous glycolipid.

DISCUSSION

This current work demonstrates that the glycopeptidolipid described in fine detail here conforms to the specification of the type-specific antigen of *M. avium* serotype 2. The particular glycolipid does not correspond in TLC mobility to those from other *M. avium* complex serotypes, the glycolipid is singularly reactive to antiserum against the homologous serotype and unreactive against hyperimmune rabbit sera raised against most other serotypes (40), and the distal nonreducing end of the oligosaccharide, 2,3-di-*O*-methyl- α -L-fucopyranosyl-(1→3)- α -L-rhamnopyranoside, has not been encountered in other serotypes. Accordingly, we can propose the complete structure

for the specific GPL antigen of *M. avium* serotype 2 and the 2,3-di-*O*-methyl-fucopyranosyl-(α 1→3)-L-rhamnopyranosyl-(α 1→) as its own characteristic antigen determinant. Previously we proposed, with lesser detail than presented here and accordingly with certain assumptions, this same structure as the characteristic surface antigen of strains of *M. paratuberculosis* (8). We have also shown (R. T. Camphausen, R. L. Jones, and P. J. Brennan, unpublished data) that other strains of *M. paratuberculosis* possess a glycolipid identical to that of *M. avium* serotype 8, which is characterized by a 4,6-(1'-carboxyethylidene)-3-*O*-methyl- β -D-glucopyranosyl terminal unit (4), whereas other strains of *M. paratuberculosis* appear to be rough variants of *M. avium*. For the past several years, we have been using the precise chemical and immunological features of the various type- and species-specific antigens of mycobacteria to identify the etiological agent responsible for several forms of human mycobacterioses; examples par excellence are *Mycobacterium leprae*, characterized by a 3,6-di-*O*-methyl-D-glucopyranosyl-(β 1→4)-2,3-di-*O*-methyl-L-rhamnopyranosyl-(α 1→2)-3-*O*-methyl-L-rhamnopyranosyl unit on a highly antigenic phenolic glycolipid (19), and *M. avium* serotype 4, the cause of the vast majority of disseminated atypical mycobacterioses in patients with acquired immunodeficiency syndrome (16) and characterized by a 4-*O*-methyl-L-rhamnopyranosyl-(α 1→4)-2,3-di-*O*-methyl-fucopyranosyl residue on its GPL antigen (M. McNeil, A. Tsang, and P. J. Brennan, unpublished observations). Accordingly, key developments from this present work are, first, that this approach is also applicable to animal mycobacterioses, namely, paratuberculosis (Johne's disease), a devastating

illness of cattle herds (9), and, second, that a close relationship, if not complete homology, exists between *M. avium* serotype 2 and the causative agent of at least some forms of paratuberculosis. The implications of these findings, particularly in the context of serodiagnosis of disease, are being explored.

Undoubtedly, *M. avium* serotypes arise from our surroundings, airways, soil, and waterways (13). How the structures presented here and elsewhere (4) bear on the ecological niche of *M. avium* serotypes 4 and 8 and their propensity for humans and *M. avium* serotype 2 and related *M. paratuberculosis* strains and their proclivity for animals is not known. It has been suggested that the ubiquity of *M. avium* serotype 2 in the environment and as a cause of chronic infections in humans and animals is mitigated by surface hydrophobicity and a consequent propensity for aerosolization (10, 31), all, perhaps, contingent on its peculiar surface composition. The persistence of *M. avium* within the intracellular milieu has also been attributed to its copious filamentous capsule (11), composed mostly of polar GPLs (1), and Furuchi and Tokunaga (14) and Goren et al. (17) had implicated at least the apolar GPLs (those singly glycosylated at the Thr position) in D4-phage absorption to *Mycobacterium smegmatis*. Perhaps a more pressing concern with *M. avium* serotypes is their notorious resistance to antituberculosis drugs (39). Rastogi and colleagues (32) have long reasoned that drug resistance in nontuberculous mycobacteria may be due to failure of antibiotics to reach

the cytoplasmic membrane, and undefined carbohydrates have been implicated. The GPLs, dominated by amide-linked fatty acids, amino acids in the D configuration, and extensively methylated 6-deoxyhexoses, do present a peculiarly inert facade, but also an ideal target for selective chemotherapy.

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