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A glycolipid antigen specific to *Mycobacterium paratuberculosis*: Structure and antigenicity

(bovine paratuberculosis/Johne disease/species-specific glycolipid antigen/polar glycopeptidolipid/polar mycoside C)

RAYMOND T. CAMPHAUSEN, ROBERT L. JONES, AND PATRICK J. BRENNAN*

Department of Microbiology, Colorado State University, Fort Collins, CO 80523

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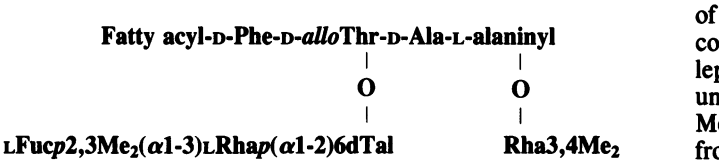
ABSTRACT *Mycobacterium paratuberculosis* (National Animal Disease Center strain 18 and American Type Culture Collection strain 19698), the causative agent of Johne Disease (bovine paratuberculosis), contains a major immunoreactive glycopeptidolipid (polar GPL-I) that has been isolated and characterized.



Thus, the glycolipid antigen belongs to the polar mycoside C glycopeptidolipid family present in other mycobacterial species. The distal 2,3-di-*O*-methyl-α-L-fucopyranosyl-(1→3)-α-L-rhamnopyranoside unit, the obvious antigen determinant, appears to be characteristic of *M. paratuberculosis*. The glycolipid can be recognized readily in isolates of *M. paratuberculosis* by TLC and its presence may be used as a characteristic marker of the infectious agent. The polar glycopeptidolipid was highly reactive in ELISA against serum from an animal hyperimmunized with *M. paratuberculosis* strain 18, indicating its basic immunogenicity.

Paratuberculosis (Johne Disease) is a chronic debilitating condition that remains virtually undetectable clinically until the onset of a copious, nontreatable diarrhea (1). In cattle, the disease is associated with infertility, prolonged intercalving intervals, and increased risk of culling (2). Paratuberculosis also causes large losses in revenue due in part to lower milk production and shorter life expectancy of affected cattle (2, 3). In the United States, 5–20% of cattle are infected and losses to the dairy industry exceed $1.5 billion per year (4, 5). Despite the insidious spread of the disease and its economic impact, there are no unequivocal means of identifying *Mycobacterium paratuberculosis*. Isolation of the organism from the feces or tissues of animals is considered to be the only valid criterion for diagnosis of disease (6). Specific serodiagnosis of presymptomatic paratuberculosis has been thwarted by lack of an antigen that could distinguish *M. paratuberculosis* from a host of environmental saphrophytic atypical mycobacteria.

The dominant immunogens of many mycobacteria are glycolipids. To date, three immunogenic glycolipid groups have been described (7). The first group, the glycopeptidolipids (GPLs), based on the so-called mycoside C, are composed of a monoglycosylated fatty acylated peptide which is further modified by small variable oligosaccharides, themselves responsible for the serospecificity of all immunotypes in the *M. avium/M. intracellulare/M. scrofulaceum* (MAIS) complex (8, 9). The second group, the lipooligosaccharides, are best represented in *M. kansasii* (10). They present unique immunochemical principles in that the putative reducing end of the oligosaccharide moiety consists of a nonreducing trehalose substituent. Yet, despite two nonreducing termini, only the distal end, containing the novel epitope *N*-acylkansosaminyl(1→3)Fuc*p*, is antigenic (11, 12). A representative of the third group, the phenolic phthiocerol-containing glycosides (13), has revolutionized the serological diagnosis of leprosy; the phenolic glycolipid from *M. leprae* contains the unique determinant 3,6-di-*O*-Me-β-D-Glc*p*(1→4)-2,3-di-*O*-Me-α-L-Rha*p*, which is highly reactive and specific for sera from lepromatous leprosy patients (14, 15). In the present report, we show that *M. paratuberculosis* is also marked by a major immunoreactive glycolipid which is of the GPL class and characterized by a unique distal disaccharide.

METHODS AND MATERIALS

***M. paratuberculosis*.** National Animal Disease Center strain 18 [originally American Type Culture Collection (ATCC) strain 12227] and ATCC strain 19698 were grown by R. S. Merkal (National Animal Disease Center, U.S. Department of Agriculture, Ames, IA) as surface pellicles in Roux bottles on a high-iron liquid medium (16). Cells were harvested after 3 weeks of growth at 38°C and lyophilized. Strain 18 was also grown at 37°C on 7H9 medium (17).

Extraction of Glycolipid Antigen. Cells (117 g) were twice extracted with $CHCl_3/CH_3OH$ (2:1, vol/vol; 40 ml/g) at 50°C for 18 hr. The dried lipid extracts were suspended in $CHCl_3/CH_3OH/H_2O$ (4:2:1, vol/vol) to give a biphasic mixture. The lower, $CHCl_3$ phase yielded 12.5 g of washed lipid. This lipid was applied to a 5 × 50-cm column of Florosil (60–100 mesh, Fisher) which then was developed sequentially with 2 liters each of $CHCl_3$ and 2%, 5%, 25%, and 60% (vol/vol) CH_3OH in $CHCl_3$. The amounts of lipid recovered from each step were 2.5, 2.8, 2.5, 1.3, and 2.4 g, respectively. Eluted fractions were monitored for glycolipid content by TLC (Silica Gel 60, Merck) in $CHCl_3/CH_3OH/H_2O$ (30:8:1), $CHCl_3/CH_3OH/H_2O$ (90:10:1), or $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (120:20:1:1), using a spray composed of 1% orcinol in 40% or of 10% (wt/vol) H_2SO_4 for detection.

Isolation of Alkali-Stable Glycolipids. Glycolipid-containing fractions were pooled, suspended in $CHCl_3/CH_3OH$ (2:1), and treated with an equal volume of 0.6 M NaOH in methanol at 50°C for 3 hr to destroy nonspecific acylglycerols. On occasion, fractions were treated with 0.1 M NaOH in benzene/methanol (1:2) at 37°C for 1 hr. Resulting mixtures were partitioned with $CHCl_3/CH_3OH/H_2O$ (4:2:1) and centrifuged. The $CHCl_3$-rich phase was washed with 0.2 volume of CH_3OH/H_2O (1:1), dried, dissolved in $CHCl_3$, applied to a silicic acid/Celite (2:1) column (2.5 × 30 cm), and eluted with $CHCl_3$ and 2% CH_3OH in $CHCl_3$ to remove fatty acids, followed by 25% CH_3OH in $CHCl_3$, to remove the alkali-

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Abbreviations: GPL, glycopeptidolipid; r-Ose, reduced oligosaccharide.

*To whom reprint requests should be addressed.

stable GPLs. Final purification was accomplished by chromatographing 10–15 mg samples of such glycolipid on preparative precoated glass TLC plates (Rediplates, Silica gel G, Fisher) developed in $CHCl_3/CH_3OH/H_2O$ (30:8:1). Opaque bands were located with a fine mist of H_2O, excised after drying, and eluted with $CHCl_3/CH_3OH$ (2:1).

Release of Oligosaccharide from Glycolipid. Alkaline borohydride reductive β-elimination (18) was performed on the total glycolipid preparation. To about 200 mg of lipid dissolved in 6 ml of C_2H_5OH/H_2O (5:1), 4 ml of 1 M NaOH, followed by 16 mmol of solid $NaBH_4$, were added. The mixture was sonicated for 15 min and allowed to react at 60°C for 24 hr. Excess hydride was destroyed with glacial acetic acid and boric acid was removed by codistillation with CH_3OH. The dried residue was partitioned between 2 volumes of $CHCl_3/CH_3OH$ (2:1) and 1 volume of CH_3OH/H_2O. The organic phase was reextracted with CH_3OH/H_2O (1:1). To desalt the reduced oligosaccharide (r-Ose) preparation, combined aqueous phases were dried, suspended in minimal H_2O, and applied to a Sephadex G-15 column (2.5 × 120 cm). Final purification was achieved on a 1 × 100-cm column of Sephadex G-10. Column eluates were monitored for total carbohydrate by the phenol/sulfuric acid method (19).

Other Analytical Procedures. r-Ose was perdeuteriomethylated by the Hakomori procedure (20) and the perdeuteriomethylated r-Ose was purified by preparative TLC in ether/acetone (5:1). To identify constituent amino compounds, GPL was hydrolyzed with 6 M HCl at 110°C for 48 hr and analyzed by HPLC on a 0.3 × 24-cm column of Dionex DC-4A; elution was with Dionex Hi-pH sodium buffer at a flow rate of 12 ml/hr. Temperature was maintained at 45°C for 28.5 min and then increased to 65°C.

Purified GPL and degradation products were hydrolyzed with 2 M CF_3COOH for 3 hr at 100°C to obtain constituent sugars. Alditol acetates were prepared and then were chromatographed on a 1.8-m column of 3% SP-2340 on 100–120 mesh Supelcoport (Supelco, Bellefonte, PA) at 160°C at an N_2 flow rate of 45 ml/min, as described (10). GC/MS of alditol acetates was performed with a VG Instruments MM-16F low-resolution mass spectrometer and data system with a capillary GC inlet, direct probe, and both electron impact and chemical ionization capabilities.

^{1}H-NMR spectra were recorded on a Nicolet NT-360 and Nicolet 1180 computer operating in the Fourier-transform mode. Chemical shifts are reported with respect to internal tetramethylsilane.

Immunoassay. Polar GPL-I was suspended in absolute ethanol with the aid of a probe sonicator. Aliquots (50 μl) of the suspension were applied to U-bottom wells of polystyrene microtiter plates (Dynatech, Alexandria, VA) and allowed to dry at 37°C overnight. Wells were washed with 10 mM NaCl/100 mM phosphate, pH 7.4/0.1% Tween 80 (P_i/NaCl/Tween) and incubated at room temperature in a humid chamber with P_i/NaCl/Tween/5% normal rabbit serum (100 μl per well) for 1 hr. Wells then were incubated for 1 hr with bovine serum diluted 1:100 in P_i/NaCl/Tween/5% normal rabbit serum (50 μl per well), washed, and incubated for 1 hr with rabbit anti-bovine IgG-horseradish peroxidase conjugate (Cappel Worthington, Malvern, PA) diluted 1:1000 in P_i/NaCl/Tween/5% normal rabbit serum (50 μl per well). Wells were washed and 50 μl of substrate (20 mg *o*-phenylenediamine dihydrochloride and 20 μl of 30% H_2O_2 in 50 ml of citrate/phosphate buffer, pH 5.0) was added to each well. Incubation was for 1 hr in the dark. The reaction was stopped with an equal volume of 1.25 M H_2SO_4; absorbances were read at 490 nm with a Dynatech MR 600 Microplate Reader.

Normal bovine sera were obtained from cattle that were fecal culture-negative for *M. paratuberculosis*. Serum was also supplied by R. S. Merkal from a bull (animal 7248) that had been hyperimmunized with *M. paratuberculosis*. Sera were also obtained from nine clinically affected animals with culture-positive feces.

RESULTS

Recognition of Distinct GPLs in *M. paratuberculosis*. A preliminary examination of *M. paratuberculosis* strains 18 and ATCC 19698 for immunoreactive glycolipids of the lipooligosaccharide and phenolic glycolipid classes, by described procedures (10–13), was not fruitful. By virtue of an amidylated fatty acid residue, immunoreactive glycolipids of the GPL class, unlike, for instance, the lipooligosaccharides, survive mild alkalinolysis (8). Indeed, some form of alkalinolysis was necessary to expose the species-specific GPLs of *M. paratuberculosis*; otherwise, they were obliterated by a mass of nonspecific lipid. The conditions selected for optimal recovery of GPLs and destruction of the nonspecific mono- and dimycolyltrehalose and other neutral lipids, were 0.3 M NaOH in $CHCl_3/CH_3OH$ (1:2) or 0.1 M NaOH in benzene/CH_3OH (1:2).

When the products of such alkalinolysis were fractionated on silicic acid/Celite (2:1) and subjected to TLC, a characteristic profile of glycolipids was seen (Fig. 1). Based on a typical yellow-gold color response to orcinol/H_2SO_4 (8) and on the work described below, these were designated apolar GPL-I–IV and polar GPL-I. Recovery of total purified GPLs was ≈600 mg from 117 g of dry cells (0.5% of the cell mass). The infrared spectrum of the GPLs as a group (Fig. 2*A*) showed absorption bands at 3300, 1640, and 1540 cm^{-1}, attributable to peptide and fatty acyl amide linkages. The spectrum was qualitatively similar to that of the mycoside C GPLs (8). Amino acid analysis showed the presence of alanine, threonine, phenylalanine, and alaninol (Fig. 2*B*) in approximately equimolar proportions, allowing for the differing responses of the various amino compounds (8). Thus, in these highly determinative respects, the family of glycolipids from *M. paratuberculosis* conform to the specifications of the mycoside C GPLs.

Polar GPL-I. Previous work had shown that the most polar of the GPLs in *M. avium/M. intracellulare* immunotypes were multiglycosylated at the threonine hydroxyl group and highly antigenic and species-specific (7, 9). Contrarily, the apolar GPLs were singly glycosylated, nonantigenic, and common to all immunotypes (8). Accordingly, the polar GPL-I from both strains were purified by preparative TLC. Comparative TLC indicated that both were identical (Fig. 3*A*). The polar GPL from strain 18 was hydrolyzed and the

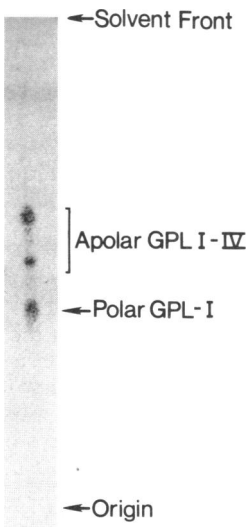

FIG. 1. TLC of total GPLs from *M. paratuberculosis* strain 18. Total washed lipid from *M. paratuberculosis* grown on 7H9 medium was treated with 0.1 M NaOH in benzene/methanol and fractionated on silicic acid/Celite (2:1). A 50-μg sample of the 25% $CH_3OH/CHCl_3$ eluate was applied to a thin-layer plate and developed in $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (120:20:1:1). The plate was sprayed with 10% H_2SO_4 and heated at 110°C for 5 min.

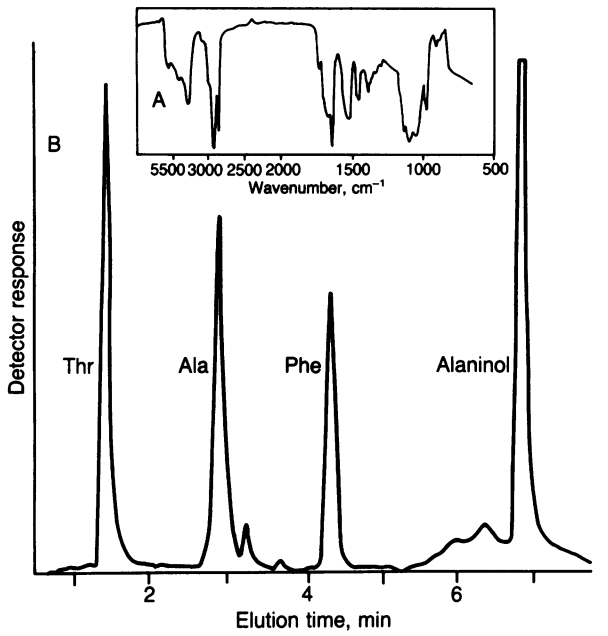

FIG. 2. Infrared spectroscopy and amino acid analysis of polar GPL-I from *M. paratuberculosis* strain 18. (*A*) Infrared spectrum of the intact polar GPL-I (4 mg/ml in $CHCl_3$). (*B*) Amino compounds in polar GPL-I. Polar GPL-I (0.5 mg) was hydrolyzed and analyzed as described in *Methods and Materials*.

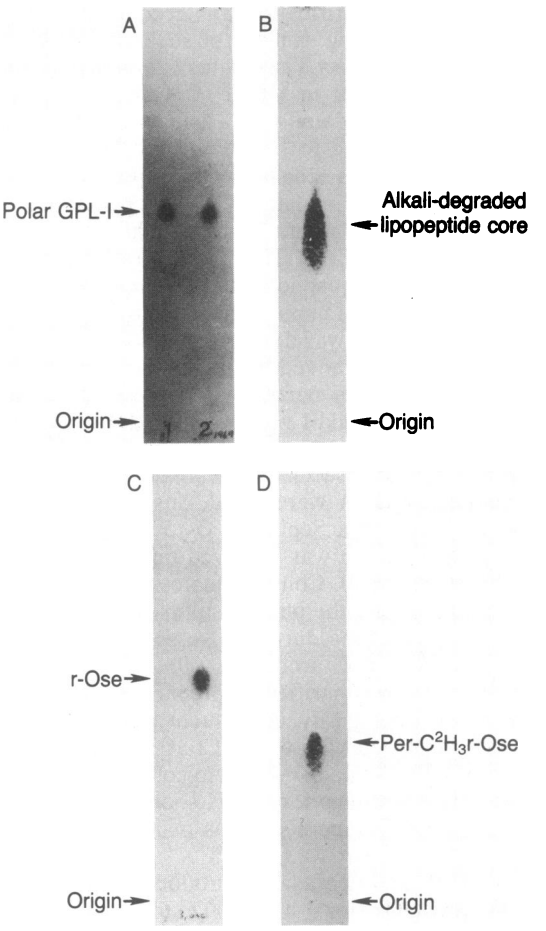

FIG. 3. TLC of GPL-I and degradation products. (*A*) Polar GPL-I from *M. paratuberculosis* strain 18 (lane 1) and ATCC strain 19698 (lane 2), developed in $CHCl_3/CH_3OH/H_2O$ (30:8:1), (*B*) Residual core after reductive β-elimination of polar GPL-I, in $CHCl_3/CH_3OH/H_2O$ (45:5:0.5), (*C*) r-Ose from reductive β-elimination of total GPL, in 1-butanol/pyridine/H_2O (10:3:3). (*D*) Perdeuteriomethylated r-Ose, in ether/acetone (5:1) Plates were sprayed with 10% H_2SO_4 and heated at 100°C.

constituent sugars were examined, as the alditol acetates, by GC and electron impact GC/MS. A highly characteristic profile of five sugar derivatives was recognized by GC (Fig. 4*A*). The 3,4-di-*O*-MeRha was identified on the grounds that its alditol acetate comigrated with that of authentic 3,4-di-*O*-MeRha (8) on SP-2340 and the mass fragmentation pattern (*m/e* 87, 89, 99, 115, 129, 131, 189, and 233) was that of a 1,2,5-tri-*O*-Ac-3,4-di-*O*-Me-6-deoxyhexitol (22). The acetates of the sugars identified as rhamnitol and 6-deoxytalitol comigrated with those prepared from the authentic sugars (8) and the fragment ion spectra (*m/e* 115, 128, 157, 170, 187, 231, and 303) were those of penta-*O*-Ac-6-deoxyhexitols (22). The 3-*O*-Me-6-deoxytalitol was recognized as such based on coelution of the acetate with that of the authentic sugar derived from the cardiac glycoside acovenoside A (21). The mass fragment ions (*m/e* 87, 101, 129, 143, 189, and 203) were also those of a 1,2,4,5-tetra-*O*-Ac-3-*O*-Me-6-deoxyhexitol (22). The 2,3-di-*O*-MeFuc was identified by comigration of the acetate with that of 2,3-di-*O*-MeFuc, derived from the cardiac glycoside strebloside (21), and the mass spectrum (Fig. 4 *Inset*) showed perfect concordance with that of a 1,4,5-tri-*O*-Ac-2,3-di-*O*-Me-6-deoxyhexitol (22). These data, taken in conjunction with structural information on the type-specific GPL antigen of *M. avium/M. intracellulare*, suggested that the 3,4-di-*O*-MeRha was glycosidically linked to the alaninol of the lipopeptide core, whereas 2,3-di-*O*-MeFuc, rhamnose, 6-dTal and, perhaps, the 3-*O*-Me-6dTal, were part of an oligosaccharide substituent linked to *allo*-threonine.

r-Ose from Polar GPL-I. Alkali-catalyzed reductive β-elimination was conducted on total GPL rather than polar GPL-I, to allow greater recovery of r-Ose. The products were partitioned between H_2O and $CHCl_3$, resulting in the appearance of the *allo*-threonine-linked oligosaccharide as the r-Ose in the aqueous phase and the alkali-degraded lipopeptide-containing core in the $CHCl_3$ layer. The latter was purified by preparative TLC (Fig. 3*B*), and sugar analysis showed that it contained the 3,4-di-*O*-MeRha unit (Fig. 4*B*).

Upon gel filtration, the r-Ose from the aqueous phase yielded a highly pure product (Fig. 3*C*). Sugar analysis (Fig. 4*C*) showed the presence of the 2,3-di-*O*-MeFuc, rhamnose, and 6-deoxytalose associated with the original GPL, and the relative amounts of these sugars indicated that the r-Ose was a trisaccharide alditol. The origin of the small quantities of 3-*O*-Me-6dTal in the polar GPL-I preparation is not known; perhaps it results from the presence of small amounts of a trisaccharide based on 3-*O*-Me-6dTal rather than 6-deoxytalose.

The r-Ose from strain 18 was perdeuteriomethylated and then purified by preparative TLC in ether/acetone (5:1), to yield a single entity (Fig. 3*D*). It was hydrolyzed and the alditol acetates were analyzed. These were identified as 2-*O*-Ac-1,3,4,5-tetra-*O*-C^2H_3-6-deoxytalitol (*m/e* 62, 74, 90, 107, 132, 154, 167, and 214), 1,5-di-*O*-Ac-2,3-di-*O*-CH_3-4-*O*-C^2H_3-fucitol (*m/e* 92, 101, 104, 117, 118, 134, 161, and 178), and 1,3,5-tri-*O*-Ac-2,4-di-*O*-C^2H_3-rhamnitol (*m/e* 92, 107, 120, 127, 134, 162, 176, 193, 204, and 209). As shown in Fig. 5, 360-MHz ^{1}H-NMR of the perdeuteriomethylated r-Ose showed the presence of three CH—CH_3 signals ($\delta \approx 1.2$) and two O—CH_3 signals ($\delta \approx 3.5$) and two anomeric protons at 5.042 ($J_{1,2}$ = 1.52 Hz) and 5.137 ($J_{1,2}$ = 1.60 Hz) indicative of two α-anomeric linkages (9, 13). Thus, the structure 2,3-di-*O*-Me-α-L-Fuc*p*(1→3)-α-L-Rha*p*(1→2)6-deoxytalitol is proposed for the r-Ose from strain 18.

The Apolar GPLs. The apolar-GPL group (Fig. 1) was subfractionated into four individual GPLs by preparative TLC

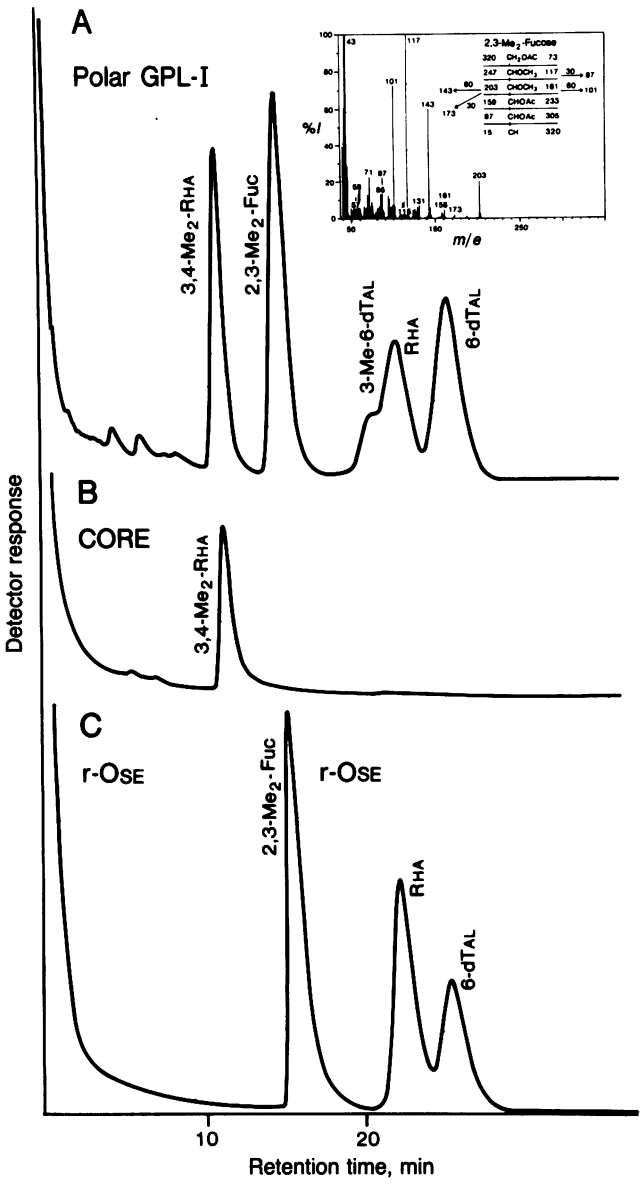

FIG. 4. GC/MS of alditol acetates from polar GPL-I and degradation products. (*A*) Pure polar GPL-I from strain 18. (*B*) Residual core after β-elimination of polar GPL-I. (*C*) r-Ose from β-elimination of polar GPL-I. Details of hydrolysis, alditol acetate preparation, and GC are described in *Methods and Materials*. (*Inset*) Electron-impact mass spectrum of the putative 1,3,4-tri-*O*-Ac-2,3,-di-*O*-Me-fucitol at 75 eV. *I*, total ion current.

in $CHCl_3/CH_3OH/H_2O$ (45:5:0.5), and the two principal entities were characterized by methods already described (8). Both were shown to contain a 3,4-di-*O*-MeRha unit linked to alaninol and a single unit of either 3-*O*-Me-6dTal or 6-deoxytalose attached at the *allo*-threonine position (results not shown).

Immunoreactivity of Polar GPL-I. Polar GPL-I was highly reactive against hyperimmune rabbit antiserum raised against *M. paratuberculosis* (results not shown). Fig. 6 *A* and *B* show the activity observed against serum from a bull hyperimmunized with *M. paratuberculosis* strain 18 in ELISA in plates coated with polar GPL-I. The saturating coating concentration of antigen was 5–6 μg/ml (ethanol), suggesting a highly reactive antigen. No activity was observed against serum specimens from animals that were fecal culture-negative for *M. paratuberculosis*. Sera from nine clinically affected animals also were tested for antibodies against polar GPL-I. Of these, only one was highly reactive (Fig. 6 *C* and *D*).

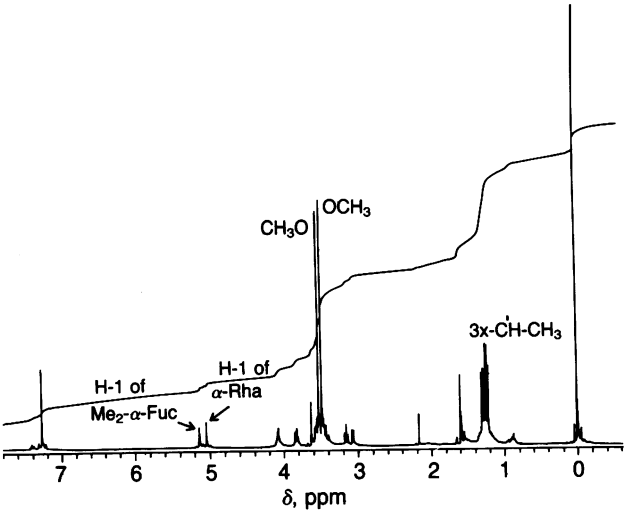

FIG. 5. ^{1}H-NMR (360 MHz) of 3 mg of TLC-purified perdeuteriomethylated r-Ose in C^2HCl_3.

DISCUSSION

The diagnosis of paratuberculosis has long been hampered by lack of sensitive and specific assays of infection and lack of tools to distinguish *M. paratuberculosis* from other mycobacteria. Mycobactin dependence is a phenotypic characteristic that can be lost, and, in any case, some strains of *M. avium* and *M. intracellulare* show mycobactin dependence (23). In addition, in a study of 22 strains of *M. paratuberculosis*, consistent biochemical traits could not be identified (5), and seroagglutination, the primary means of identifying many mycobacteria (24), was largely ineffective because of consistently rough morphology. Without reliable serodiagnostic assays and consistent phenotypic traits by which to identify the organism, accurate estimates of the prevalence of paratuberculosis and its economic impact are not available.

Mycobacteria favor lipids of myriad structure in reactions with their external environment. Thus, glycolipids may act as agents of toxicity (25), inhibitors of phagosome–lysosome fusion (26), and overt surface antigens (7). Accordingly, identification of mycobacteria, based on immunoreactive glycolipids, with chemical (27) and immunological (14) tools has reached a reliable status. Jenkins (28) was unable to detect a characteristic pattern of glycolipids in *M. paratuberculosis* isolates by using TLC, perhaps due to the considerable body of nonspecific acyltrehaloses which tends to mask the species-specific GPLs. The simple ploy of gentle alkalinolysis, preferably with benzene/methanol as solvent, revealed a characteristic array of GPLs, and this feature can now be used to differentiate *M. paratuberculosis* from other species within the genus, particularly members of the *M. avium/M. intracellulare* complex ubiquitous within cattle herds and other domestic and wild animals (29). Thus, a major requirement for success of the planned epidemiological surveys of the incidence of paratuberculosis within the United States (5)—identification of the etiologic agent—may be satisfied.

The polar GPL-I of *M. paratuberculosis* conforms to the previously defined specifications of a species-specific antigen (7): the combination of sugars at the distal nonreducing end of the oligosaccharide, 2,3-di-*O*-Me-α-L-Fuc*p*(1→3)-α-L-Rha*p*, has not been encountered before; the particular glycolipid does not correspond in TLC mobility to those previously encountered; and the glycolipid is highly reactive against hyperimmune rabbit and cattle serum raised against whole *M. paratuberculosis*. However, the polar GPL was highly reactive against sera from only one animal of nine

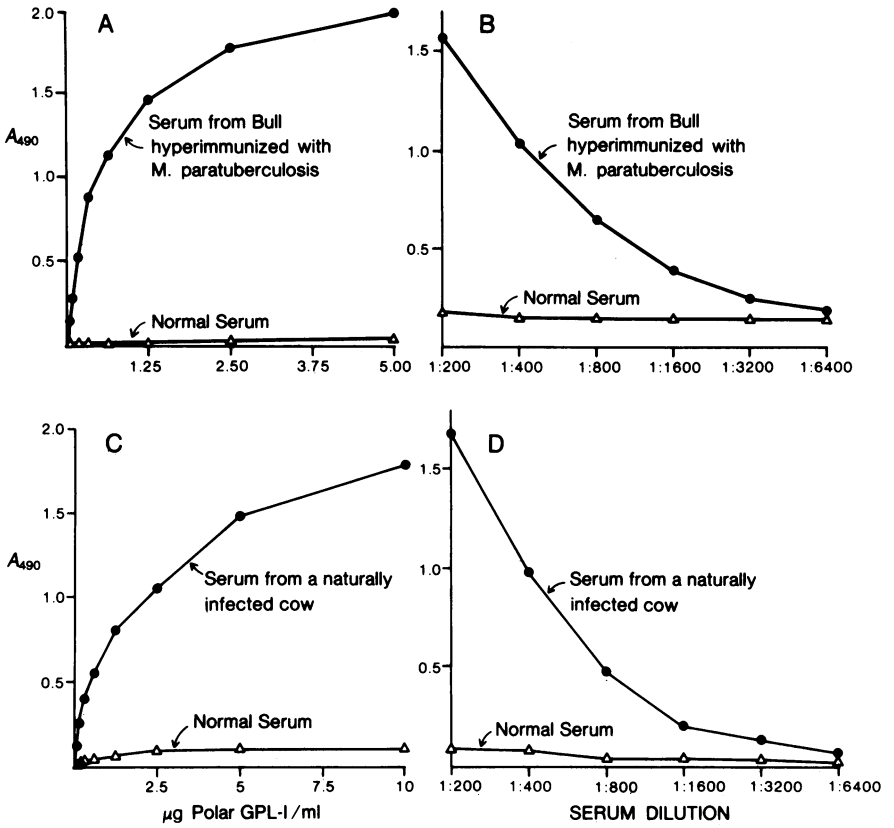

FIG. 6. Anti-polar GPL-I IgG antibody activity in serum from cattle with paratuberculosis. Serial 2-fold dilutions of polar GPL-I were tested against serum (diluted 1:100) from an animal hyperimmunized with *M. paratuberculosis* (*A*) or naturally infected with *M. paratuberculosis* (*C*). Serial 2-fold dilutions of reactive serum from an animal hyperimmunized with *M. paratuberculosis* (*B*) or naturally infected (*D*) were assayed against polar GPL-I (5 μg/ml). ELISA conditions are described in *Methods and Materials*.

with overt clinical paratuberculosis. Perhaps not all infected animals mount a vigorous humoral response to GPL-I. Alternatively, perhaps paratuberculosis, like many forms of human nontuberculous mycobacterioses, is caused by not one infectious agent but a host of biochemically and pathogenically identical but antigenically different subspecies of *M. paratuberculosis*.

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