Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.
Williams, R. C., Jr., & Detrich, H. W., III (1979) *Biochemistry 18*, 2499–2503.
Williams, R. C., Jr., & Lee, J. C. (1982) *Methods Enzymol. 85*, 376–385.
Williams, R. C., Sr. (1981) *J. Mol. Biol. 150*, 399–408.
Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., & Creswell, K. M. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 158–166.

Trehalose-Containing Lipooligosaccharides from Mycobacteria: Structures of the Oligosaccharide Segments and Recognition of a Unique *N*-Acylkanosamine-Containing Epitope†

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ABSTRACT: The structures of the oligosaccharide segments of nine trehalose-containing lipooligosaccharides (LOS) of *Mycobacterium kansasii* have been established by positive and negative fast-atom bombardment mass spectrometry, acetolysis, partial acid hydrolysis, methylation analyses, and nuclear magnetic resonance. Upon acetolysis, all produce the α,α-trehalose-containing tetraglucose (Glc_4) "core" -β-D-Glc*p*(1→3)-β-D-Glc*p*(1→4)-α-D-Glc*p*(1→1)-α-D-Glc*p*. The simplest (LOS I′) contains an additional α1→3-linked 3-*O*-methyl-L-rhamnopyranose (3-*O*-Me-L-Rha*p*) unit; those of intermediate complexity (LOS I–III) contain an additional D-xylopyranose (D-Xyl*p*) residue or xylobiose in β1→4 linkage; and those of ultimate complexity (LOS IV–VIII) contain further D-Xyl*p* residues and the distal *N*-acylkanosamine- (Kan*N*Acyl) and fucopyranosyl- (Fuc*p*) containing disaccharide Kan*N*Acyl(1→3)Fuc*p*. Thus, the structure of the oligosaccharide from LOS VII is Kan*N*Acyl(1→3)Fuc*p*(1→4)[-β-D-Xyl*p*(1→4)]$_6$-α-L-3-*O*-Me-Rha*p*(1→3)Glc_4. Polyclonal rabbit and murine monoclonal antibodies react only with the more complex Kan*N*Acyl-Fuc*p*-containing lipooligosaccharides, indicating that the Kan*N*Acyl distal end, not the trehalose end, contains the antibody binding site unique to *M. kansasii* and is responsible for the serological distinctiveness of *M. kansasii* among mycobacterial species.

The precise serological differentiation, most of the overt antigenicity, and features of the pathogenesis of many species within the *Mycobacterium* genus are due to a family of rather extraordinary trehalose-containing lipooligosaccharides (LOS)[1] (Brennan, 1984). In them, trehalose is found glycosidically linked as part of a tri- or a tetraglucosyl "core", which in turn is glycosidically modified in distinct fashion to confer species-specific antigenicity (Hunter et al., 1983; unpublished observations).[2] The sugar appendages unique to *Mycobacterium kansasii* are Kan*N*Acyl, Fuc, Xyl, and 3-*O*-Me-Rha (Hunter et al., 1983, 1984b). No other trehalose-containing lipooligosaccharide antigens have been examined in detail, although Saadat & Ballou (1983), working in a different context, found a dipyruvylated pentasaccharide, 4,6-(1-carboxyethylidene)-3-*O*-Me-β-D-Glc*p*(1→3)-4,6-(1-carboxyethylidene)-β-D-Glc*p*(1→4)-β-D-Glc*p*(1→6)-α-D-Glc*p*(1→1)-α-D-Glc*p*, and a related monopyruvylated tetrasaccharide, in *Mycobacterium phlei*, and these were lipid in origin. In this paper, a combination of chemical analyses and antibody reactivity using murine monoclonal antibodies was used to arrive at structures for the component oligosaccharides of the LOS family of *M. kansasii* and which allowed recognition of the segment of the oligosaccharide within the highly reactive polar lipooligosaccharides responsible for the specific antigenicity of *M. kansasii*.[3]

Experimental Procedures

Strain of M. kansasii. Previously we reported that the strain under examination was *M. kansasii* strain Forbes [No. 1201 in the Trudeau *Mycobacterial* Culture Collection (1980)]. In fact, the strain was Florisse, which has not been acceeded to the Trudeau mycobacterial collection. However, TMC strains 1201, 1214, 1217, and 1204 did contain the same array of LOS, both polar and apolar, seen in Florisse. *M. kansasii* was grown as described (Hunter et al., 1983). The entire sus-

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[1] Abbreviations: LOS, lipooligosaccharide; Ose, nonreducing oligosaccharide; FAB, fast-atom bombardment; MS, mass spectrometry; EI, electron ionization; GC, gas chromatography; TLC, thin-layer chromatography; Kan*N*Acyl, *N*-acylkanosamine [4,6-dideoxy-2-*O*,3-*C*-dimethyl-4-(2-methoxypropionamido)-α-L-*manno*-hexopyranose and -β-L-*manno*-hexopyranose]; Glc*p*, glucopyranose; Fuc*p*, fucopyranose; Rha*p*, rhamnopyranose; Xyl*p*, xylopyranose; Glc_4, tetraglucose "core"; Me, methyl or *O*-methyl; TMC, Trudeau mycobacterial collection.

[2] Work conducted with Virginia L. Barr.

[3] A preliminary account of this work has appeared (Hunter et al., 1984a).

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pension was autoclaved and dried at 50 °C in crystallizing dishes with 0.01% thimerosol added as preservative. Lipid was extracted from the glassy solid and washed (Folch et al., 1957). A typical 8-week-old 8-L culture yielded 110 g of dried solids from which 13 g of washed lipids was derived.

Purification of Individual LOS. Columns (3.7 × 30 cm) of Florisil were the primary means used for resolving the individual LOS. Application of 13 g of lipid to such a column and elution with 400 mL each of $CHCl_3$ and 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, and 25% CH_3OH in $CHCl_3$ removed 6.8 g of lipid of which about half was eluted with $CHCl_3$ and was nonspecific. The 5%–10% CH_3OH eluates contained the substance previously described as "cord factor" (dimycolyltrehalose) (Hunter et al., 1983). Further analysis of the product showed it to be monomycolyltrehalose.[4] The 12.5% and 15% CH_3OH eluted fractions contained mostly LOS I–IV; partial resolution could be achieved by collecting individual (20-mL) fractions. The 17.5% and 20% fractions contained mostly residual LOS III and IV. The 22.5% and 25% fractions contained mostly LOS V–VIII. Finer resolution was achieved by reapplying these fractions to long columns (1 × 100 cm) of Florisil with use of a similar elution regimen and smaller (2-mL) fractions. Final purification of individual LOS was achieved by centrifugally accelerated, preparative TLC using the Chromatotron device (Model 7924, Harrison Research, Palo Alto, CA) and radial rotors of silica gel PF-254 with $CaSO_4$ (type 60; E. Merck) and the solvent $CHCl_3$/CH_3OH/H_2O (60:35:8).

Preparation of Ose. Pure LOS (ca. 38 mg) was dissolved in $CHCl_3$/CH_3OH (2:1) and reacted with an equal volume of 0.2 N methanolic NaOH at 37 °C for 30 min. The reaction was stopped by neutralizing the mixture with glacial acetic acid, which was diluted with 4–5 volumes of $CHCl_3$/CH_3OH/H_2O (4:2:1). The upper aqueous phase of the biphasic mixture was applied to a column of mixed bed resin (MB-3) and eluted with water. The Ose was further purified by applying the preparation to columns (1 × 180 cm) of Bio-Gel P-4 (Ose I–III) or Bio-Gel P-6 (Ose IV–VIII). Carbohydrate was located in eluates with phenol–H_2SO_4 (Dubois et al., 1966). Individual fractions or mixed carbohydrate positive peaks were checked for purity by analytical silica gel TLC in 1-butanol/pyridine/water (6:4:3 or 7:3:3) and by paper chromatography in 1-butanol/pyridine/acetic acid/water (5:5:1:3). Alternatively, Ose preparations were acetylated and the products chromatographed on silica gel plates in benzene/acetone (4:1, 4:2, or 4:3). This ploy was also used to help separate peracetylated Ose I, II, and III, which were then deacetylated with $NaOCH_3$, deionized with Dowex 50 (H^+), and repurified on Bio-Gel P-4.

Partial Hydrolysis of Per-CH_3-Ose. Partial acid hydrolysis was applied to the per-CH_3- or per-CD_3-Ose, not to the underivatized Ose. Each Ose was permethylated (Stellner et al., 1973), purified by preparative TLC in 1-butanol/acetic acid/ether/water (9:6:3:1), and hydrolyzed in 0.3 N HCl at 100 °C for 18 h. The hydrolysate was extracted with $CHCl_3$/CH_3OH (2:1), and the products were resolved by TLC in the same solvent.

Detection of Acyl Residues. Acetyl groups in individual LOS were observed as acetohydroxamate by paper chromatography in 2-propanol/NH_4OH (2:1) and the use of a $FeCl_3$ spray and as methyl acetate on a column of 10% Carbowax 20M as described (Hunter et al., 1983). 2,4-Dimethyltetradecanoate was observed initially as the methyl ester by TLC in hexane/ether (78:22) in which it had a R_f value identical with that of methyl palmitate and by GC on OV-1 at 190 °C followed by EI/MS by which the McLafferty base ion (m/z 88), m/z 101 and 129, and other fragments were recognized (Hunter et al., 1983).

Detection of KanNAcyl. Individual LOS were hydrolyzed with 2 M CF_3COOH at 100 °C for 3 h, and the hydrolysate was subjected to TLC in $CHCl_3$/CH_3OH (11:1). The presence of a bright yellow spot upon spraying with dichromate or H_2SO_4, with a R_f value of about 0.5, indicated the amino sugar. For confirmation of structure, the amino sugar was isolated from a Bio-Gel P-2 column (1 × 180 cm), O-acetylated, and examined by direct-probe EI/MS. The base peak m/z 186 and other intense peaks, such as m/z 143 and high mass peaks at m/z 242 and m/z 260, were recognized (Hunter et al., 1984b).

Detection of Glc_4 Core. Acetolysis of as little as 100 μg of the individual LOS was sufficient to allow detection of the tetraglucose. Dried sample was acetolysed in a mixture of acetic anhydride/acetic acid/H_2SO_4 (10:10:1, 500 μL) at 40 °C overnight, followed by the addition of water (1 mL) and $CHCl_3$ (2 mL) (Lee, 1966). The $CHCl_3$ phase was subjected to TLC in benzene/acetone (4:1) and sprayed with 0.1% orcinol in 40% H_2SO_4 (or 10% H_2SO_4). Acetylated Glc_4 (R_f 0.15) was readily recognized as the major product by comparison with the authentic material (Hunter et al., 1983). The acetylated Glc_4 from several Ose (III–VII) was purified by preparative TLC, deacetylated, and permethylated, and the per-CH_3-Glc_4 was examined by ^1H NMR. The two usually coincident anomeric proton signals of α,α-trehalose and the two β-anomeric signals were identified. In addition, the per-CH_3-Glc_4 was hydrolyzed and reduced, and the partially methylated alditol acetates were identified by GC/MS as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol, and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, as described (Hunter et al., 1983).

Identification of Sugar Linkages. Sugars and linkage patterns were identified through analysis of the partially methylated (or trideuteriomethylated) alditol acetates by GC on OV-225 or ECNSS-M and comparison with 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (Lindberg, 1972). With the exception of 1,5-di-*O*-Ac-2,3,4-tri-*O*-Me-Rha (relative retention time on OV-225 of 0.45 not 0.35), the exact relative retention times reported by Lindberg were applicable, when the recommended temperature (170 °C) was used. GC was routinely followed by GC/MS as described (Brennan et al., 1981a,b; Hunter et al., 1982, 1983, 1984b). Recourse to these and another reference (Janssen et al., 1976) allowed us to make the following assignments on the basis of the given m/z values: 2,3,4-tri-*O*-CH_3-Xyl(2,3,4-tri-*O*-CD_3-Xyl), 58 (61), 71 (74), 87 (90), 101 (107), 117 (120), and 161 (167); 2,3-di-*O*-CH_3-Rha(2-*O*-CD_3-3-*O*-CH_3-Rha), 101 (101), 101 (104), 117 (120), 143 (143), 161 (164), and 203 (203); 2,3,4,6-tetra-*O*-CH_3-Glc(2,3,4,6-tetra-*O*-CD_3-Glc), 45 (48), 71 (74), 87 (90), 101 (107), 117 (120), 129 (132), 145 (154), 161 (167), and 205 (214); 2,3-di-*O*-CH_3-Xyl(2,3-di-*O*-CD_3-Xyl), 87 (90), 101 (107), 117 (120), 129 (132), 161 (167), and 189 (192); 2,4,6-tri-*O*-CH_3-Glc(2,4,6-tri-*O*-CD_3-Glc), 45 (48), 87 (90), 99 (102), 101 (107), 117 (120), 129 (132), 161 (167), 201 (204), and 233 (239); 2,3,6-tri-*O*-CH_3-Glc(2,3,6-tri-*O*-CD_3-Glc), 87 (90), 99 (102), 101 (107), 113 (119), 117 (120), 129 (132), 131 (137), 161 (167), and 233 (239); 2,4-di-*O*-CH_3-Fuc, 89, 101, 117, 131, 143, 159, 173, and 233.

Analytical Procedures. Details on column, thin-layer, gas, and paper chromatography have been described (Brennan et

[4] Unpublished observations with Dianna M. Wynn.

al., 1981a,b; Hunter et al., 1983). Further details are provided in the text. Procedures for GC/MS and ^1H and ^{13}C NMR have also been described (Hunter et al., 1982, 1983).

Fast-Atom Bombardment Mass Spectrometry. The mass spectrometer/computer system used was a Kratos MS50/DS55 operating at 6-kV accelerating potential. A primary bombarding atom beam of Xe was produced from a saddle field ion source (FAB11NF, Ion Tech Ltd., Teddington, U.K.) operating with a tube current of ~1.5 mA at an energy of ~7 keV. The mass scale was calibrated by the data system using cesium iodide cluster ions. The oligosaccharides were subjected to both positive (glycerol matrix) and negative (triethanolamine matrix) FAB-MS analysis with computer recording of the spectra. Whenever possible, two to five individual scans were averaged to improve the signal to noise ratio.

Animals and Immunization Protocol. M. kansasii Forbes was grown in 100 mL of 7H11 medium for 5 weeks, shaking at 37 °C. Fourteen milliliters of 5% phenol in water (w/v) was added, and the flask stored at 37 °C for 48 h. Cells were washed with sterile normal saline and resuspended to an optical density of 0.3 at 525 nm in a Coleman Junior spectrophotometer. The suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Sigma Chemical Co.) and used as inoculum. Five female BALB/c mice (Charles Rivers Laboratories), 8 weeks old, were inoculated intraperitonally with 0.1 mL of the suspension, boosted 20 days later with a similar inoculum, and finally bled on the 30th day. The animal having the highest titer of antibody against *M. kansasii* total lipid in ELISA was used for hybridoma production.

ELISA. The assay is based on a modification of procedures described by Smolarsky (1980) and Voller (1979). Total lipid from *M. kansasii* was sonicated in ethanol (2 μg/mL) and 50 μL dried overnight at room temperature in polystyrene microtiter plates (Dynatech Laboratories.). The wells were blocked for 45 min with PBS containing 1% Tween 80. Anti-*M. kansasii* rabbit serum (Schaefer, 1965) diluted in PBS–Tween or undiluted tissue culture fluid (see below) was added and the plate incubated for 1 h at room temperature in a humid chamber. The plate was washed 4 times with PBS and peroxidase-conjugated goat anti-mouse IgG–IgM–IgA (Cappel Laboratories), or the individual immunoglobulins, diluted 1:1000 in PBS-Tween, were added. The plate was again incubated for 30 min and washed as before. Fifty microliters of substrate was added and the plate incubated in the dark for 30 min. The reaction was stopped with an equal volume of 2.5 N H_2SO_4 and the absorbance read on an automatic ELISA reader (Dynatech Laboratories) at 490 nm.

Hybridoma Production. Four days prior to fusion, the selected mouse was injected intravenously with 0.1 mL of the whole cell suspension. Spleen cells were perfused, washed twice in Hank's balanced salt solution (K. C. Biological), and counted with trypan blue. SP2/O-Ag14 mouse myeloma cells (Center for Disease Control, Fort Collins, CO), selected with 10 μg/mL 8-azaguanine 2 months prior to fusion and maintained at log phase of growth, were counted simultaneously. Cell counts for spleen and myelomas were 5.64×10^7 and 1.5×10^7, respectively. The fusion protocol was that described by Hudson & Hay (1980). When cell growth was three-fourths confluent in each well, the tissue culture fluid was collected and tested for antibody by ELISA against total *M. kansasii* lipid antigen. Positive wells were cloned by limiting dilution. A suspension of macrophage feeder layers and cells (1 cell/50 μL of suspension) was plated over 96 wells, grown to three-fourths confluency, and again tested for antibody. Limiting clone dilution was performed 3 times to ensure specificity. One cell from each original and positive well was grown to 2-ml cultures, to 25-ml cultures, and to 75-ml cultures. Cells were collected and injected into pristane-treated mice for ascites fluid.

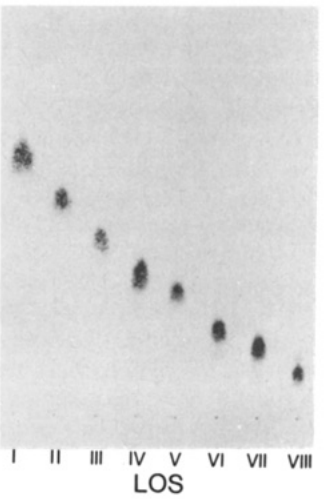

FIGURE 1: Thin-layer chromatography of LOS I–VIII in $CHCl_3$/CH_3OH/H_2O (10:5:1). Plates were sprayed with 10% H_2SO_4 and heated at 110 °C.

Table I: Qualitative Analysis on LOS I–VIII

LOS	Acyl[a]	Glc_4[b]	3-*O*-Me-Rha[c]	Xyl[c]	Fuc[c]	Kan*N*Acyl[d]
I	+	+	1	±	0	0
II	+	+	1	1	0	0
III	+	+	1	2	0	0
IV	+	+	1	4	1	+
V	+	+	1	4	1	+
VI	+	+	1	6	1	+
VII	+	+	1	6	1	+
VIII	+	+	1	6	1	+

[a] Both 2,4-dimethyltetradecanoate and acetate were present. [b] See Experimental Procedures for details on the isolation of Glc_4 from LOS. [c] Semiquantitatively analyzed by GC of alditol acetates as described in Figure 2. [d] Based on its recognition among acid hydrolysis products by TLC in $CHCl_3/CH_3OH$ (11:1) (see Experimental Procedures for further details).

RESULTS

Properties of the Individual LOS. Figure 1 shows a thin-layer chromatogram of the individual LOS after final purification by centrifugally accelerated TLC. Sugar analysis of each by GC/MS of the alditol acetates showed that Glc, Xyl, and 3-*O*-Me-Rha were the constant sugars and that LOS IV–VIII, in contrast to LOS I–III, contained additional Fuc (Figure 2). There also appeared to be a progressive increase in the amount of Xyl present in LOS I through LOS VII. Analyses were also conducted on each for Kan*N*Acyl by TLC of hydrolysates in $CHCl_3/CH_3OH$ (11:1), 2,4-dimethyldecanoate by GC/MS, acetate by paper chromatography of the hydroxamate, and Glc_4 core by acetolysis of the corresponding Ose followed by TLC of the acetylated Glc_4 in benzene/acetone (4:1). The results of this largely qualitative study are summarized in Table I.

The results indicate that each LOS is based on an identical trehalose-containing tetraglucose core and share a common 3-*O*-Me-Rha unit, and all contain an unknown number of 2,4-dimethyltetradecanoyl and acetyl substituents. LOS I contains less than the expected quantity of Xyl, presumably because some preparations contain a LOS (LOS I′, see below) devoid of any Xyl residues. The obvious difference among members of the LOS family is in the amounts of Xyl present

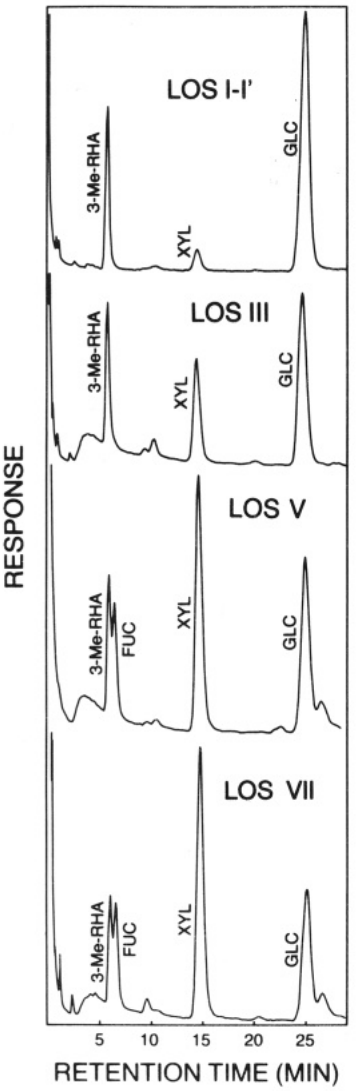

FIGURE 2: Sugar composition of some individual LOS. GC of the alditol acetates was conducted on SP-2340, initially at 190 °C for 8 min, followed by temperature programming at 1 °C/min for 30 min.

and in the presence or absence of Fuc and Kan*N*Acyl. Fuc and Kan*N*Acyl always appear together and are present only within the most polar of the family, namely, LOS IV–VIII.

Sugar Sequence in Ose I–VIII. The negative ion FAB mass spectrum of Ose I revealed an intense $(M - H)^-$ ion at m/z 957 (Figure 3A). The peak at m/z 825 (957 − 132) most likely arises from subsequent loss of Xyl; the m/z 665 (825 − 160) fragment arises from loss of 3-*O*-Me-rha, m/z 503 (665 − 162) from loss of Glc, and m/z 341 (503 − 162) from loss of another Glc. The ion at m/z 446 is from the triethanolamine matrix (149 + 149 + 148). The other ions are unassigned at this time. The positive ion spectrum revealed an intense natriated molecular ion at m/z 981 (958 + 23) with a fragment at m/z 849, probably resulting from loss of the terminal Xyl residue (Figure 3B). Thus, FAB MS suggests the sequence Xyl-3-*O*-Me-Rha-Glc_4 for Ose I.

The negative ion FAB mass spectrum of Ose II showed an intense $(M - H)^-$ ion at 1089. The other intense peaks at m/z 957 (1089 − 132), m/z 825 (957 − 132), and m/z 665 (825 − 120) presumably arose from sequential loss of Xyl and xylobiose (Xyl_2), followed by 3-*O*-Me-Rha. The positive ion spectrum revealed an intense natriated molecular ion at m/z 1113 (1090 + 23) and m/z 981 (1113 − 132), the latter due to loss of the terminal Xyl residue. Thus, both negative and

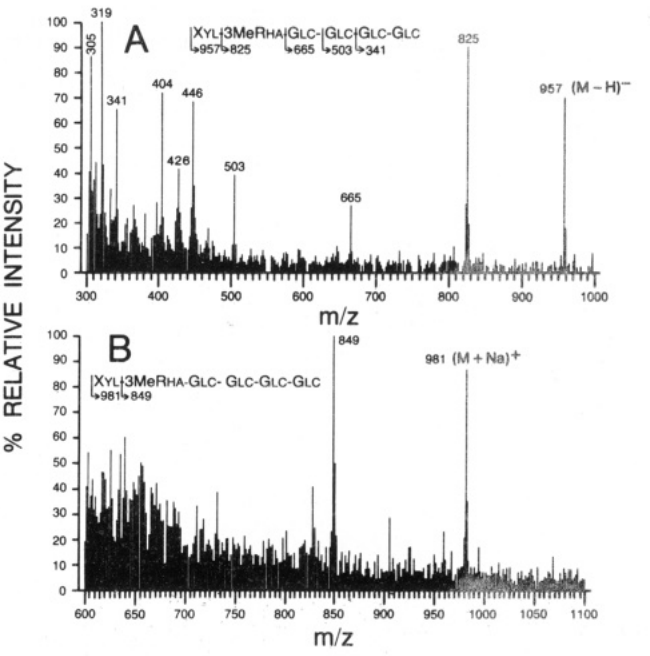

FIGURE 3: FAB mass spectra of Ose I: (A) negative ion FAB MS (trethanolamine matrix); (B) positive ion FAB MS (glycerol matrix).

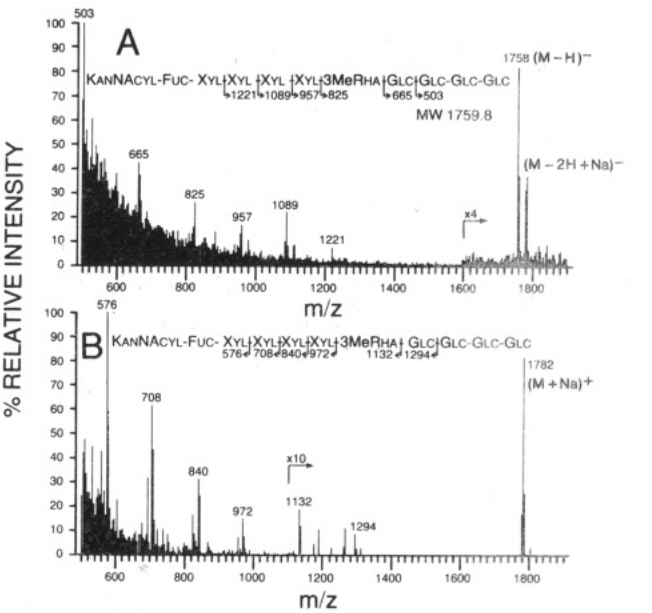

FIGURE 4: FAB mass spectra of Ose V: (A) negative ion FAB MS (triethanolamine matrix); (B) positive ion FAB MS (glycerol matrix).

positive spectra suggested the sequence Xyl-Xyl-3-*O*-Me-Rha-Glc_4. The FAB mass spectra of Ose II and III were identical, and thus, both appear to contain the same sequence of sugars.

Both Ose V and Ose VI also showed identical positive and negative FAB mass spectra suggesting that they also contain the same sugar sequence. The results (Figure 4) clearly suggest the sequence Kan*N*Acyl-Fuc-Xyl_4-3-*O*-Me-Rha-Glc_4. Interestingly, the fragments in the positive ion spectrum of Ose V arise from the distal Kan*N*Acyl end of the Ose, in contrast to the fragments from the negative ion spectrum; these arise from the trehalose end. This difference is presumably due to the influence of the amino sugar; compare the positive spectrum of Ose V with that of Ose III, which is devoid of the Kan*N*Acyl.

The negative ion FAB mass spectrum of Ose VII revealed an intense $(M - H)^-$ ion at m/z 2022. A further intense peak

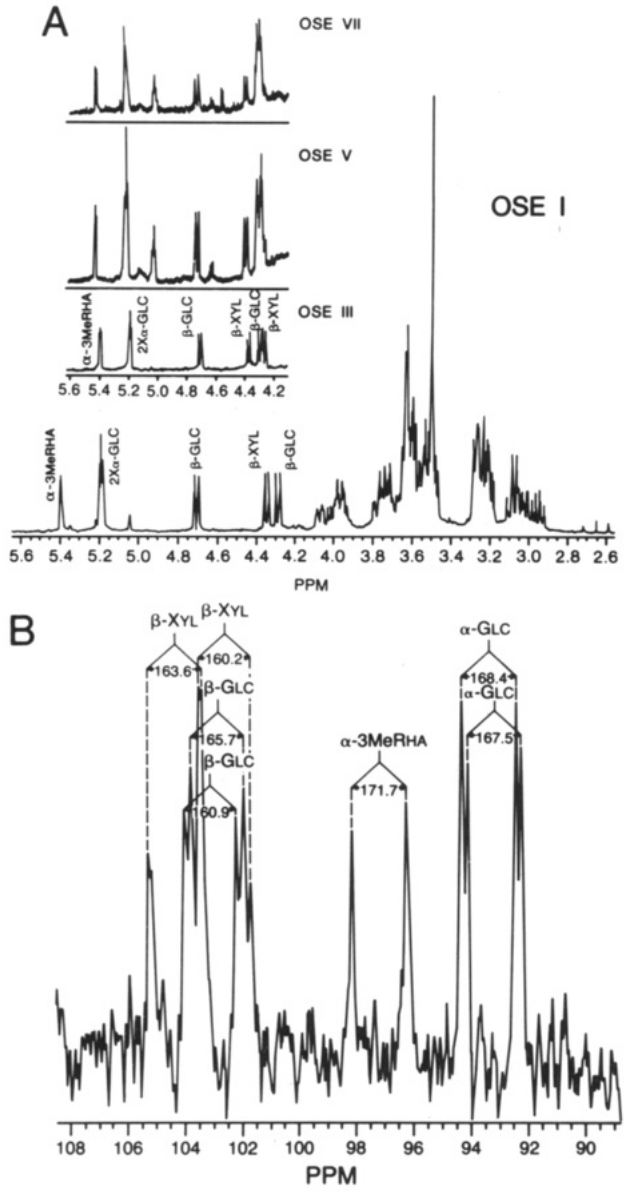

FIGURE 5: NMR of per-CH_3-Ose: (A) proton NMR of per-CH_3-Ose I, III, V, and VII at 360 MHz; (B) ^{13}C NMR (undecoupled) of 50 mg of per-CH_3-Ose III. All spectra were obtained on solutions in $CDCl_3$.

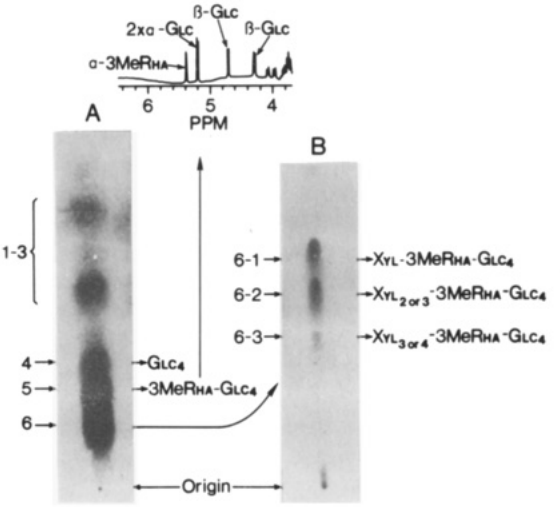

FIGURE 6: Partial acid hydrolysis of per-CH_3-Ose V. (A) The TLC-purified material was hydrolyzed in 0.3 N HCl at 100 °C for 1.5 h, and the products were chromatographed on a thin-layer plate in ether/acetone (4:1) and sprayed with H_2SO_4, followed by heating. (B) Fragment 6 was deuteriomethylated and rechromatographed in ether/acetone (5:1). The derivation of the given structures is described in the text. (Inset) ^1H NMR of the methylated 3-Me-Rha-Glc_4.

at m/z 1089 (2022 − 933) is presumably due to loss of the entire Kan*N*Acyl-Fuc-Xyl_4 unit, and further intense peaks at m/z 957 and 825 are probably due to loss of additional Xyl units. Thus, the sequence Kan*N*Acyl-Fuc-Xyl_6-3-*O*-Me-Rha-Glc_4 is suggested. Incidentally, the ion at m/z 2023 [possibly the $(M - H)^-$ ^{13}C isotope ion] is more intense than the ^{12}C isotope ion at m/z 2022 possibly because of the large number of carbons in the molecule at this mass. The negative ion spectrum of Ose VIII was identical with that of Ose VII, indicating a similar sequence of sugars. Positive ion FAB on Ose VII and VIII was not successful.

Anomeric Configurations. Each of Ose I–VII was per-methylated, and the products were shown to be single entitites by TLC in 1-butanol/acetic acid/ether/water. ^1H NMR on each (Figure 5A) showed the presence of the two superimposed anomeric proton signals of α,α-trehalose (δ 5.193, $J_{1,2}$ = 3.40 Hz) and two others (δ 4.672, $J_{1,2}$ = 7.84 Hz; δ 4.287, $J_{1,2}$ = 7.96 Hz) attributable to two β-D-glucosidic links (Hunter et al., 1983). The assignment of the δ 4.367 signal ($J_{1,2}$ = 7.68 Hz) to β-D-xylose was based on the similarity of the chemical shift value to that reported (Lemieux & Stevens, 1966) and the large coupling constant. The chemical shift of H_1 of 3-*O*-Me-Rha (δ 5.40, $J_{1,2}$ = 1.70 Hz) is closer to that of synthetic phenyl α-L-rhamnoside (δ 5.52) than to that of synthetic phenyl 2,3,4-tri-*O*-acetyl-β-L-rhamnoside (δ 4.74) (Hunter et al., 1982) (acetyl groups have little effect on the chemical shift of the H_1). Also, the chemical shift of the C_1 of 3-*O*-Me-Rha in per-CH_3-Ose III (δ 97.4) (Figure 5B) is close to that of synthetic phenyl α-L-rhamnoside (δ 97.9) (Hunter et al., 1982). Most importantly, the large C_1–H_1 coupling constant ($J_{C_1-H_1}$ = 171.7 Hz) indicates an α configuration (Kasai et al., 1979). The ^{13}C NMR spectra of Ose IV and the higher Ose were too complex to analyze, and hence, the anomeric configurations of Fuc and Kan*N*Acyl are not known at this time.

Linkage Analysis on Ose V. The strategy used to establish linkage positions is best exemplified with Ose V. Per-CH_3-Ose V was exposed to the mild acid hydrolysis conditions described under Experimental Procedures, the acid was removed by evaporation, and the products were extracted with $CHCl_3$ and subjected to TLC in ether/acetone (Figure 6A, left lane).

Fragments 1–3 were apparently mostly monosaccharides. Fragments 4–6 were each isolated by centrifugal TLC. The TLC mobility of fragment 4 corresponded to that of the methylated Glc_4 described previously (Hunter et al., 1983), and upon hydrolysis and GC/MS of the alditol acetates, it was shown to produce 2,3,4,6-tetra-*O*-Me-Glc, 2,3,6-tri-*O*-Me-Glc, and 2,4,6-tri-*O*-Me-Glc in the approximate proportions of 1:1:2. Thus, methylation analysis confirmed the earlier results from acetolysis that Ose V is based on β-D-Glc*p*(1→3)-β-D-Glc*p*(1→4)-α-D-Glc*p*(1→1)-α-D-Glc*p* and suggested that further glycosylation is at the 3-OH of the distal Glc. More direct evidence on this latter point was sought by deuteriomethylation of the already partially methylated fragment 4 and reanalysis of the alditol acetates. The new distal tri-*O*-methylmono-*O*-[$^2H_3$]methylglucitol acetate could be distinguished from the old tetra-*O*-methylglucitol acetate by GC/MS (m/z 104, 120, 164, and 208 compared to m/z 101, 117, 161, and 205). However, one could not categorically distinguish between 3-*O*-CD_3 or 4-*O*-CD_3 residues (m/z 161 could arise from $C_{1,2,3}$ or $C_{4,5,6}$ fragments). Hence, the issue of the linkage of 3-*O*-Me-Rha to the Glc_4 subunit rests on the

evidence that more 2,4,6-tri-*O*-Me-Glc arises from fragment 4 than any other methylated glucose.

Upon hydrolysis, fragment 5 yielded an additional 2,3-di-*O*-Me-Rha. Fragment 5 was deuteriomethylated, and the alditol acetates were again examined by GC/MS. The 2,3-di-*O*-Me-Rha was converted to 2,3-di-*O*-CH_3-4-*O*-CD_3-Rha, further confirming that in the intact Ose V the 4-OH of the 3-*O*-Me-Rha was the point of further glycosylation. Again, the relative amounts of 2,4,6-tri-*O*-Me-Glc and 2,3,6-tri-*O*-Me-Glc were approximately 2:1, further suggesting that 3-*O*-Me-Rha was 1→3 linked to Glc. ^1H NMR of fragment 5 confirmed its pentasaccharide structure (Figure 6).

Fragment 6 was also further deuteriomethylated, and the products were resolved by twice developing the TLC plate in ether/acetone (Figure 6B, right lane). Fragments 6-1, 6-2, and 6-3 were isolated by preparative TLC and the alditol acetates analyzed. Fragments 6-2 and 6-3 were qualitatively similar, yielding the acetates of 2,3-di-*O*-CH_3-4-*O*-CD_3-Xyl, 2,3-di-*O*-CH_3-Rha, 2,3,4,6-tetra-*O*-CH_3-Glc, 2,4,6-tri-*O*-CH_3-Glc, and 2,3,6-tri-*O*-CH_3-Glc. The relative amounts of 2,3-di-*O*-CH_3-4-*O*-CD_3-Xyl and 2,3-di-*O*-CH_3-Xyl indicated that fragment 6-2 contained two Xyl residues and fragment 6-3 contained three such residues. Fragment 6-1 yielded 2,3-di-*O*-CH_3-4-*O*-CD_3-Xyl, in addition to the di-*O*-Me-Rha and the *O*-Me-Glc derivatives, indicating that it contained but one Xyl residue. Thus, all of the Xyl residues are 1→4 linked.

The Distal KanNAcyl(1→3)Fuc. Although present in Ose V, neither Fuc nor Kan*N*Acyl was recognized among the products of partial acid hydrolysis of per-CH_3-Ose V, possible due to comigration with the many hydrolysis products on TLC or to the relative instability of the terminal glycosidic links. Likewise, methylation analysis on whole per-CH_3-Ose V following the standard 2 M CF_3COOH hydrolysis did not reveal any recognizable methylated fucitol product. However, GC analysis of the alditol acetates prepared from milder hydrolysis of the per-CH_3-Ose V on ECNSS-M showed small quantities of 2,4-di-*O*-Me-Fuc (*m/z* 89, 101, 117, 131, 143, 159, 201, 203, and 233). Since this was the only recognizable Fuc product arising from a number of hydrolysis conditions, it is concluded that Fuc is not the distal nonreducing terminus but is part of the linear chain of Ose IV–VIII. This conclusion is in accord with the results of FAB MS.

Previous work had shown that Kan*N*Acyl has the structure 4,6-dideoxy-2-*O*,3-*C*-dimethyl-4-(2-methoxypropionamido)-L-*manno*-hexopyranose (Hunter et al., 1984b). Accordingly, if Kan*N*acyl is at the distal terminus, the product arising from permethylated Ose V will be the 3-*O*-Me derivative, i.e., 4,6-dideoxy-2-*O*,3-*O*,3-*C*-trimethyl-4-(2-methoxypropionamido)hexose, whereas, if it is between two sugar residues, the 3-OH will be engaged in glycosidic linkage and the native sugar itself should be found among the products of acid hydrolysis. With these considerations in mind, per-CH_3-Ose V was hydrolyzed with 2 M CF_3COOH, the reducing sugars were converted to the methyl glycosides, and the Kan*N*Acyl methyl glycosides were selectively extracted with $CHCl_3$ and compared with the permethylated Kan*N*Acyl, which had been isolated as described previously and methylated by the Hakomori procedure (Hunter et al., 1984). Both products had identical retention times on SE-30 (4.3 min at 175 °C), and the mass spectra were similar (*m/z* 44, 59, 71, 88, 102, 115, 157, 118, and 288) and in accord with those reported for other derivatives of Kan*N*Acyl (Hunter et al., 1984b). Thus, Kan*N*Acyl occupies the distal terminus and is apparently 1→3 linked to the penultimate Fuc residue. Accordingly, the structure Kan*N*Acyl(1→3)Fuc(1→4)[-β-D-Xyl(1→4)]$_4$-β-L-3-*O*-Me-Rha(1→3)Glc$_4$ is proposed for Ose V.

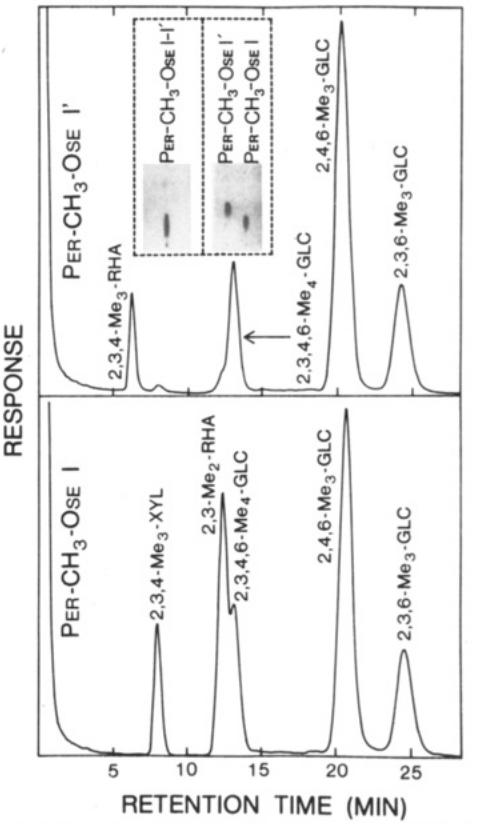

FIGURE 7: Isolation of per-CH_3-Ose I and I′ and GC of alditol acetates. (Inset) TLC in ether/acetone (5:1) of a natural mixture of per-CH_3-Ose I and I′ and of separated per-CH_3-Ose I and I′ after development of the plate 3 times in ether/acetone (5:1). GC was conducted on OV-225 at 170 °C for 8 min followed by 1 °C/min to 180 °C.

Linkage Analysis on Ose I′, I, II, and III. Some preparations of per-CH_3-Ose I contained small quantities of a contaminant (Ose I′); the two were resolved with some difficulty by repeated development in ether/acetone (Figure 7, inset). Alditol acetate analysis of the two (Figure 7) showed that per-CH_3-Ose I produced 2,3,4-tri-*O*-Me-Xyl, 2,3-di-*O*-Me-Rha, 2,3,4,6-tetra-*O*-Me-Glc, 2,4,6-tri-*O*-Me-Glc, and 2,3,6-tri-*O*-Me-Glc, whereas per-CH_3-Ose I′ yielded 2,3,4-tri-*O*-Me-Rha and the same Glc products but no Xyl derivative. Thus, Ose I′ probably has the structure α-L-3-*O*-Me-Rha-(1→3)Glc$_4$, whereas Ose I is β-D-Xyl(1→4)-α-L-3-*O*-Me-Rha(1→3)Glc$_4$. The per-Ac-Glc$_4$ was isolated from each of these, thereby providing further corroboration for the proposed structure. Results of like analysis on the Xyl-containing Ose II and Ose III showed the presence of 2,3,4-tri-*O*-Me-Xyl, 2,3-di-*O*-Me-Xyl, and the same *O*-Me-Rha and Glc products seen in Ose I (Table II). This evidence combined with that from FAB MS indicates the structure β-D-Xyl*p*(1→4)-β-D-Xyl*p*(1→4)-α-L-3-*O*-Me-Rha*p*(1→3)Glc$_4$ for both. Thus, the parental LOS II and III must differ in the number of acyl or acetyl groups.

The application of methylation analyses to Ose VI–VIII showed in each case the same products as were previously observed in Ose V, i.e., the alditol acetates of 2,4-di-*O*-Me-Xyl, 2,4-di-*O*-Me-Fuc, 2,3-di-*O*-Me-Rha, 2,4,6-tri-*O*-Me-Glc, 2,3,6-tri-*O*-Me-Glc, and 2,3,4,6-tetra-*O*-Me-Glc by GC on OV-225 or ECNSS-M and the methyl glycoside of 3-*O*-Me-Kan*NAcyl* by GC on SE-30 (Table II). However, there was more of the 2,3-di-*O*-Me-Xyl in Ose VII than in Ose V. Thus, the combined results of FAB MS and methylation analyses suggest the following. Ose VI is of the same structure as Ose

Table II: Summary of Results of Methylation Analysis and FAB MS on Per-CH_3-Ose I–VIII

Ose	composition of permethylated Ose	proposed structure of oligosaccharides
I′	2,3,4-tri-*O*-Me-Rha, 2,4,6-tri-*O*-Me-Glc, 2,3,6-tri-*O*-Me-Glc, 2,3,4,6-tetra-*O*-Me-Glc	3-*O*-Me-Rha(1→3)Glc_4[a]
I	2,3,4-tri-*O*-Me-Xyl, 2,3-di-*O*-Me-Rha, 2,4,6-tri-*O*-Me-Glc, 2,3,6-tri-*O*-Me-Glc, 2,3,4,6-tetra-*O*-Me-Glc	Xyl(1→4)3-*O*-Me-Rha(1→3)Glc_4
II	2,3,4-tri-*O*-Me-Xyl, 2,3-di-*O*-Me-Xyl, 2,3-di-*O*-Me-Rha, 2,4,6-tri-*O*-Me-Glc, 2,3,6-tri-*O*-Me-Glc, 2,3,4,6-tetra-*O*-Me-Glc	Xyl_2(1→4)3-*O*-Me-Rha(1→3)Glc_4
III	same as II	Xyl_2(1→4)3-*O*-Me-Rha(1→3)Glc_4
IV	3-*O*-Me-Kan*N*Acyl, 2,4-di-*O*-Me-Fuc, 2,3-di-*O*-Me-Xyl, 2,3-di-*O*-Me-Rha, 2,4,6-tri-*O*-Me-Glc, 2,3,6-tri-*O*-Me-Glc, 2,3,4,6-tetra-*O*-Me-Glc	Kan*N*Acyl(1→3)Fuc(1→4)Xyl_4(1→4)3-*O*-Me-Rha(1→3)Glc_4
V	same as IV	Kan*N*Acyl(1→3)Fuc(1→4)Xyl_4(1→4)3-*O*-Me-Rha(1→3)Glc_4
VI	same as V	Kan*N*Acyl(1→3)Fuc(1→4)Xyl_4(1→4)3-*O*-Me-Rha(1→3)Glc_4
VII	same as VI but more 2,3-di-*O*-Me-Xyl	Kan*N*Acyl(1→3)Fuc(1→4)Xyl_6(1→4)3-*O*-Me-Rha(1→3)Glc_4
VIII	same as VII	Kan*N*Acyl(1→3)Fuc(1→4)Xyl_6(1→4)3-*O*-Me-Rha(1→3)Glc_4

[a] Glc_4: β-D-Glc*p*(1→3)-β-D-Glc*p*(1→4)-α-D-Glc*p*(1→1)-α-D-Glc*p*.

V, and presumably, LOS VI and V differ in the number of acyl groups; Ose VII is of the structure Kan*N*Acyl(1→3)-Fuc*p*(1→4)[-β-D-Xyl*p*(1→4)]$_6$-α-L-3-*O*-Me-Rha*p*(1→3)Glc_4, which is also shared with Ose VIII.

KanNAcyl(1→3)Fuc: Specific Antigen Determinant of M. kansasii. We had reported that only the more polar LOS showed distinct immunoprecipitation bands against anti-*M. kansasii* rabbit antiserum in gel diffusion and that the less polar LOS did not react at all (the latter showed weak reactions in the more sensitive ELISA) (Hunter et al., 1983). Moreover, the reactions were species specific. Thus, the possibility was raised that the unique Kan*N*Acyl(1→3)Fuc unit confers specific immunoreactivity on the family of LOS.

From the final hybridoma exercise described under Experimental Procedures, which involved four plates and 384 wells, 80 wells were shown to contain colony growth. Each was screened by ELISA, and eight were found to be reactive against total *M. kansasii* lipid. These were cloned; four were subsequently lost while the remaining four were stable. These were designated A5/B1, IIB2/E12/F4, D12/D8/E6, and E2/B7/B3. Each was of the IgG class, 2b subclass, and appeared similar in specificity when screened against the individual LOS. The results from the use of one monoclonal antibody, A5/B1, are depicted in Figure 8. At 1:64 dilution A5/B1 reacted with LOS IV–VIII but showed little activity against LOS I–III, monomycolyltrehalose, or two lipooligosaccharides from *M. szulgai*, which are based on a trehalose-containing triglucosyl core and contain Rha and 2-*O*-Me-Fuc at the distal end of the oligosaccharide.[2] Anti-*M. kansasii* rabbit serum behaved in a similar fashion. Thus, the antigenicity of the LOS family primarily resides in the Kan*N*Acyl distal terminus and not in the α,α-trehalose terminus.

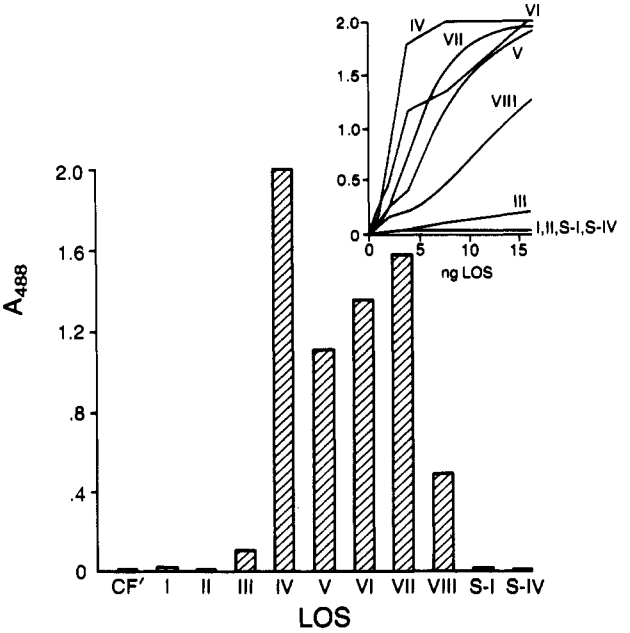

FIGURE 8: ELISA reactivity of monoclonal antibody to various LOS. Each well was coated with 8 ng of glycolipid, and an excess (ca. 50 μL) of monoclonal A5/B1 (diluted 1:64) was added. (Inset) Titration pattern of A5/B1 against various glycolipids. S-I and S-IV are LOS antigens from *M. szulgai* (see text for further details). CF′ is a monomycolated cord factor (monomycolated trehalose). See Experimental Procedures for details on isolation of CF′.

Discussion

The fast-atom bombardment mass spectrometry technique has been applied to reducing oligosaccharides, small polysaccharides, and glycolipids (Forsberg et al., 1982; Dell & Ballou, 1983; Dell et al., 1983). In the positive ion recording mode, underivatized reducing oligosaccharides usually show signals for the protonated, cationized (Na^+, K^+) species, together with fragment ions from these species (Bosso et al., 1984). Interestingly, the trehalose-containing oligosaccharides, devoid as they are of a reducing terminus, conform to the principles observed with reducing oligosaccharides, as does their acquisition of the molecular anion in negative-mode recording. In addition, the trehalose-containing oligosaccharides behave in both modes as the methylglucose lipopolysaccharides (Forsberg et al., 1982; Dell & Ballou, 1983), the only other nonreducing oligosaccharides examined in detail, albeit nonreducing by virtue of a glycerate aglycon, but still highly relevant to the lipooligosaccharides by virtue of their mycobacterial origin and some semblances in composition.

Both nonreducing termini of the lipooligosaccharide antigens present features extraordinary in the context of carbohydrate structures. We had separately reported on the structure of *N*-acylkanosamine (Hunter et al., 1984) prior to the realization of its distal location and linkage to the penultimate fucose within the polar antigenic lipooligosaccharides. The principle of glycosidically linked α,α-trehalose and, consequently, of two nonreducing sugar terminii had been established by Kandler and colleagues. Selaginose [α-D-glucopyranosyl(1→2)-α-D-glucopyranosyl(1→1)glucopyranoside] was originally identified in *Selaginella kraussiana* (Fisher & Kandler, 1975). Its occurrence is restricted to two small clusters of *Selaginella* species and, hence, is of chemotaxonomic relevance (Kandler & Hopf, 1980). In pteridophytes, selaginose, like the parental α,α-trehalose, serves as the main water-soluble reserve carbohydrate, usurping the normal role of sucrose (Kandler & Hopf, 1980). Thus, it does not appear to arise from an acylated, lipid relative. Nevertheless, the selaginose type of unit, namely, a glucosyl-α,α-trehalose trisaccharide, may be more widespread among mycobacterial species than the tetraglucosyl

core inherent to the *M. kansasii* lipooligosaccharides. Saadat & Ballou (1983) reported on the presence of a β-D-Glc*p*(1→6)-α-D-Glc*p*(1→1)-α-D-Glc*p* as part of two pyruvylated glycolipids in *M. smegmatis*, and we have observed the same unit as part of a series of species-specific hexa- and heptasaccharide-containing antigenic lipooligosaccharide in *M. szulgai*.[2]

The use of murine monoclonal antibodies clearly implicates the unique *N*-acylkanosaminyl(1→3)fucoside as the specific antigen determinant of *M. kansasii* and suggests that this unit is probably responsible for the serological distinctiveness of this species within the *Mycobacterium* genus (Schaefer, 1980). However, one cannot yet rule out a contribution to antigenicity from the more distal units of the xylo hexasaccharide chain; attempts to selectively remove the distal disaccharide or uncover fully xylosylated but terminal disaccharide-defective lipooligosaccharides have not been fruitful. Nevertheless, the principle that the terminal mono- or disaccharide of an assortment of glycolipids is the primary antibody binding site and responsible for much of the exquisite specificity of mycobacterial species and subspecies is well documented (Brennan, 1984). For instance, the only difference between immunotypes 9 and 25 of the *M. intracellulare* complex lies in two methoxy groups on the terminal fucobiose (Brennan et al., 1980a,b), and on the antigen from immunotype 8 differs from antigens in all other immunotypes merely in the presence of a terminal 4,6-(carboxyethylidene)-3-*O*-methyl-β-D-glucopyranoside on an otherwise common antigen (Brennan et al., 1980a). The species-specific glycolipid antigen of the leprosy bacillus, phenolic glycolipid I, is also noteworthy in this respect. It is distinguished by a unique triglycosyl unit, 3,6-di-*O*-Me-β-D-Glc*p*(1→4)-2,3-di-*O*-Me-α-L-Rha*p*(1→2)-3-*O*-Me-α-L-Rha*p* (Hunter et al., 1982). Glycolipids devoid of the terminal di-*O*-Me-Glc*p* will not bind anti-glycolipid IgM from human lepromatous sera (Fujiwara et al., 1984) or anti-glycolipid murine monoclonal IgM (Young et al., 1984) or IgG (Mehra et al., 1984). In addition, glycolipids in which the terminal 3-*O*-methyl substituent is absent show only about one-third the activity of the phenolic glycolipid I (Fujiwara et al., 1984). This evidence for the primary location of specificity within the terminal sugar has led in turn to synthesis of artificial antigens based on the di-*O*-methylglucopyranoside, which have proved highly specific for the serodiagnosis of leprosy (Cho et al., 1984). The present evidence that the antigenicity of *M. kansasii* is located in the terminal Kan*N*Acyl segment provides a basis for an analogous synthetic strategy. *M. kansasii*, the "yellow" bacillus, is an important pathogen in the context of a variety of immune-deficiency states (Gombert et al., 1981). Use of a highly specific and reactive semisynthetic antigen for serodiagnosis of disease could result in timely chemotherapy and reduced mortality.

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REFERENCES

Bosso, C., Defaye, J., Heyraud, A., & Ulrich, J. (1984) *Carbohydr. Res. 125*, 309–317.

Brennan, P. J. (1984) in *Microbiology 1984* (Leive, L., & Schlessinger, D., Eds.) pp 366–375, American Society for Microbiology, Washington, DC.

Brennan, P. J., Meyer, H., Aspinall, G. O., & Nam Shin, J. E. (1981a) *Eur. J. Biochem. 115*, 7–15.

Brennan, P. J., Aspinall, G. O., & Nam Shin, J. E. (1981b) *J. Biol. Chem. 256*, 6817–6822.

Cho, S.-N., Fujiwara, T., Hunter, S. W., Rea, T. H., Gelber, R. H., & Brennan, P. J. (1984) *J. Infect. Dis. 150*, 311–322.

Dell, A., & Ballou, C. E. (1983) *Carbohydr. Res. 120*, 95–111.

Dell, A., Morris, H. R., Egge, H., Von Nicolas, H., & Strecker, G. (1983) *Carbohydr. Res. 115*, 41–52.

Dubois, M., Gilles, K. A., Hamilton, J. K., Robins, P. A., & Smith, F. (1966) *Anal. Chem. 28*, 350–356.

Fischer, M., & Kandler, O. (1975) *Phytochemistry 14*, 2629–2633.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Forsberg, L. S., Dell, A., Walton, D. J., & Ballou, C. E. (1982) *J. Biol. Chem. 257*, 3553–3563.

Fujiwara, T., Hunter, S. W., Cho, S.-N., Aspinall, G. O., & Brennan, P. J. (1984) *Infect. Immun. 43*, 245–252.

Gombert, M. E., Goldstein, E., Corrado, M. L., Stein, A. J., & Batt, K. H. (1981) *Ann. Intern. Med. 94*, 486–487.

Hudson, L., & Hay, F. C. (1980) in *Practical Immunology*, pp 303–327, Blackwell Scientific Publications, Oxford.

Hunter, S. W., & Brennan, P. J. (1983) *J. Biol. Chem. 258*, 7556–7562.

Hunter, S. W., Fujiwara, T., & Brennan, P. J. (1982) *J. Biol. Chem. 257*, 15072–15078.

Hunter, S. W., Murphy, R. C., Clay, K., Goren, M. B., & Brennan, P. J. (1983) *J. Biol. Chem. 258*, 10481–10487.

Hunter, S. W., Brennan, P. J., & Jardine, I. (1984a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2036.

Hunter, S. W., Fujiwara, T., Murphy, R. C., & Brennan, P. J. (1984b) *J. Biol. Chem. 259*, 9729–9734.

Janssen, P. E., Kenne, L., Liedgren, H., Lindberg, B., & Lonngren, J. (1976) *Chem. Commun., Univ. Stockholm No. 8*, 1–75.

Kandler, O., & Hopf, H. (1980) in *The Biochemistry of Plants: A Comprehensive Treatise* (Stumpf, P. K., & Conn, E. E., Eds.) Vol. 3, pp 221–170, Academic Press, New York.

Kasai, R., Okihara, M., Asakawa, J., Mizutani, K., & Tanaka, O. (1979) *Tetrahedron 35*, 1427–1432.

Lee, Y. C. (1966) *J. Biol. Chem. 241*, 1899–1908.

Lemieux, R. U., & Stevens, J. D. (1966) *Can. J. Chem. 44*, 249–262.

Lindberg, B. (1972) *Methods Enzymol. 28*, 178–195.

Mehra, V., Brennan, P. J., Rada, E., Convit, J., & Bloom, B. R. (1984) *Nature (London) 308*, 194–196.

Mycobacterial Culture Collection (1980) NIH Publication 80-289, pp 113–118, U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, MD.

Saadat, S., & Ballou, C. E. (1983) *J. Biol. Chem. 258*, 1813–1818.

Schaefer, W. B. (1965) *Am. Rev. Respir. Dis. 92* (Suppl.), 85–93.

Schaefer, W. B. (1980) *Methods Microbiol. 13*, 323–344.

Smolarsky, M. (1980) *J. Immunol. Methods 38*, 85–93.

Stellner, K., Saito, H., & Hakomori, S.-I. (1973) *Arch. Biochem. Biophys. 155*, 464–472.

Voller, A., Bidwell, D. E., & Bartlett, A. (1979) in *The Enzyme-Linked Immunosorbent Assay (ELISA)*, pp 23–125, Dynatech Laboratories, Inc., Alexandria, VA.

Young, D. B., Khanolkar, S. R., Barg, L. L., & Buchanan, T. M. (1984) *Infect. Immun. 43*, 183–188.