Structural Analysis of Lipooligosaccharide Produced by *Neisseria gonorrhoeae*, Strain MS11mk (Variant A): A Precursor for a Gonococcal Lipooligosaccharide Associated with Virulence†

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ABSTRACT: We studied the structure of the lipooligosaccharide (LOS) that is produced by a variant A of strain MS11mk. This variant produces a single LOS that is recognized by monoclonal antibody (MAb) 2-1-L8. In a recent study of the pathogenesis of *Neisseria gonorrhoeae* in male volunteers, variant A gave rise to other phase variants that produce higher molecular weight LOSs, and these LOS were associated with virulence. Definition of the structure of the variant A LOS is important to understand the biosynthesis of LOS and its expression in vivo. The dephosphorylated oligosaccharide (OS) structure derived from the variant A LOS was analyzed by two-dimensional NMR and methylation analysis. The OS structure was found to be a truncated form of the LOS produced by strain F62 [Yamasaki et al. (1991) *Biochemistry 30*, 10566–10575]; the variant A OS is a hexamer, a β-lactosyl residue linked to a tetrasaccharide: Galβ1→4Glcβ1→4{GlcNAcα1→2Hepα1→3}Hepα1→KDO. We determined that the variant A LOS is a precursor for the synthesis of higher MW LOS. We also studied expression of the MAb 2-1-L8-defined epitope present on the variant A LOS. Our data indicate that the MAb-defined epitope is not a linear β-lactosyl residue but its specificity is directed toward the phosphorylated GlcNAc-Hep-Hep residue. Since this MAb binds to gonococci, at least part of the phosphorylated diheptose area is exposed on the gonococcal surface.

The lipooligosaccharides (LOSs)[1] of *Neisseria gonorrhoeae* are important pathogenic and antigenic outer membrane components. LOSs are cytotoxic and mediate most of the toxic damage to human fallopian tubes (Gregg et al., 1981). Normal human serum contains bactericidal antibodies directed against LOS (Apicella et al., 1986; Schoolnik et al., 1979; Ward et al., 1978). Additional antibodies against LOS develop in response to disseminated gonococcal infection (Rice & Kasper, 1977). Hence, LOSs are important in the host immune response against gonococci.

Gonococcal LOSs lack the O-repeating glycose units that are found in lipopolysaccharides (LPSs) of the enteric Gram-negative bacteria. Each gonococcal strain may produce several structurally different LOS components (Schneider, et al., 1984). These structural differences among LOSs are due to differences in the carbohydrate moiety, as demonstrated by immunochemical (Schneider et al., 1984; Yamasaki et al., 1991b; Mandrell et al., 1988) and structural (Gibson et al., 1989; John et al., 1991; Yamasaki et al., 1991a) analyses of LOS.

In addition to the ability of gonococci to produce multiple LOS components, a recent study on pathogenesis in human volunteers indicates that gonococcal LOS expression can shift with the stage of infection (Schneider et al., 1991). At the time of inoculation, a test strain produced a single LOS component. After urethral infection was established, the gonococci recovered from the urethral exudates were found to produce several LOS components that differed in their glycosyl structure. Thus not only can LOS structure vary between strains, but each strain can produce a variety of LOSs, depending on its environment.

Recently, we analyzed epitope expression of the LOS of strain F62 (Yamasaki et al., 1988, 1991b). F62 LOS consists of two major components that are recognized by murine IgM monoclonal antibodies (MAbs) 1-1-M and 3F11. The epitopes defined by the two MAbs are expressed in many gonococcal strains (Schneider et al., 1984; Mandrell et al., 1986). We determined the oligosaccharide (OS) structures of these F62 LOS components (Yamasaki et al., 1991a). The OS from the 1-1-M-defined LOS component was found to be a nonamer, GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hep{3←1αHep2←1αGlcNAc}α1→KDO. The pentasaccharide present at the nonreducing terminus of the longer oligosaccharide chain is identical to that of human asialo-G_3 ganglioside (Watanabe & Hakomori, 1979). The OS from the MAb 3F11-defined LOS component does not have a GalNAc that is present only in the MAb 1-1-M-defined LOS component. The exposed tetrasaccharide at the nonreducing terminus of the MAb 3F11-defined LOS component is identical to that of a human glycosphingolipid, paragloboside.

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[1] Abbreviations: 2D, two dimensional; COSY, chemical shift correlation spectroscopy; DQF, double quantum filtered; FID, free induction decay; Gal, Galactose; GalNAc, *N*-acetylgalactosamine; GC, gas chromatography; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; HF, hydrofluoric acid; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; HPAEC, high-performance anion-exchange chromatography; IgG, immunoglobulin G; IgM, immunoglobulin M; KDO, 2-keto-3-deoxymannooctulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharide; MAb, monoclonal antibody; MS, mass spectrometry; NMR, nuclear magnetic resonance; MW, molecular weight; NOE, nuclear Overhauser effect; OS, oligosaccharide; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TQF, triple quantum filtered.

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Thus, we determined that the antigenic similarity between the MAb 3F11-defined LOS component and paragloboside (Mandrell et al., 1988) is due to the presence of an identical OS structure.

We studied the structure of LOS produced by strain MS11mk variant A, the strain used in a study of the pathogenesis of *N. gonorrhoeae* (Schneider et al., 1991). In vivo, variant A exhibited phase shifts to two different variants, B and C, each of which produces higher MW LOS components than variant A. These higher MW LOS components bind MAbs 1-1-M and 3F11 and are associated with the gonococcal disease process. We determined that the variant A LOS is a precursor for the higher MW LOS components.

MATERIALS AND METHODS

N. gonorrhoeae MS11mk (piliated) has been previously described (Swanson et al., 1988) and has been redesignated MS11mk variant A by Schneider et al. (1991). For brevity's sake, and to minimize confusion over strain names, it will be referred to here as variant A. This variant produces a single lipooligosaccharide (LOS) that is recognized by MAb IgG 2-1-L8 (Schneider et al., 1991). MAb 2-1-L8 (mouse IgG) was a kind gift from Dr. Wendell D. Zollinger (Walter Reed Research Institute, Washington, DC). We prepared the variant A LOS from acetone-powdered variant A organisms using the hot phenol–water extraction method (Westphal & Jann, 1972). We treated the LOS with exo- and endogalactosidases and analyzed the enzyme-treated LOSs for TLC immunostaining as described previously (Yamasaki et al., 1991b). Lactosylceramide was purchased from Biocarb Chemicals (Lund, Sweden). The variant A LOS and enzyme-treated LOSs were analyzed by polyacrylamide gel electrophoresis (PAGE) using a Bio-Rad Mini-Protean 2 Cell (Bio-Rad, Richmond, CA) (Yamasaki et al., 1988, 1991b). One gel was silver stained (Tsai & Frasch, 1982) and the other was electroblotted to nitrocellulose paper. Electroblotted LOSs were immunostained as follows: (1) treatment first with MAb 2-1-L8 and then with a secondary antibody (goat anti-mouse IgG, alkaline phosphatase conjugate, 1:1000 dilution, Sigma Chemical Co., St. Louis, MO) and (2) staining with 3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate and Fast Red (both from Sigma Chemical Co.) in Tris-HCl buffer (pH 7.6).

The variant A LOS was modified as described previously (Yamasaki et a., 1988). The variant A LOS (5 mg) was dephosphorylated in HF (48%, 250 μL) for 24–48 h at 4 °C. Partial deacylation of the LOS was done by treating the LOS (10 mg) in 50 mM NaOH (0.5 mL) for 0.5–1 h at 80 °C. The carboxyl groups of the LOS (10 mg) were reduced by Anderson's method (Anderson & Stone, 1988). The modified LOSs were dialyzed in water and then lyophilized. TLC dot-blot analysis of the variant A LOS and its derivatives was done using glass-fiber sheets impregnated with silica gel (Gelman Science, Ann Arbor, MI) (Yamasaki et al., 1988, 1991b). For the detection of the binding of MAb 2-1-L8 to the LOSs, the TLC sheet was treated with a goat anti-mouse IgG (peroxidase conjugate, Kirkegaard & Perry Labaratories, Gaithersburg, MD) and then with a solution of 4-chloronaphthol and hydrogen peroxide in 50 mM Tris-HCl (pH 7.4).

The variant A oligosaccharide (OS) (dephosphorylated unless otherwise stated) was prepared and analyzed as previously described (Yamasaki et al., 1991a,b): (1) hydrolysis of the lipooligosaccharide (LOS) in 1% AcOH (2 h at 100 °C), (2) fractionation by Bio-Gel P-4 chromatography (Bio-Rad Laboratories, Richmond, CA) (<400 mesh, 2.6 × 90 cm, 100 mM ammonium acetate) and desalting by Bio-Gel P-2 chromatography (<400 mesh, 2.6 × 90 cm, water), and (3) dephosphorylation of the intact OS with aqueous hydrofluoric acid (48%, 24 h at 4 °C). The OS components of *N. gonorrhoeae* F62 prepared in a previous study (Yamasaki et al., 1991a) were also used for the analysis of heptoses.

The variant A OS was also analyzed by high-performance anion-exchange chromatography (HPAEC) [a 20-min gradient of 100 mM NaOH–100 mM NaOAc → 100 mM NaOH–125 mM NaOAc; detection, amperometric (100 nA)]. In addition to the major OS (7.1 min), two minor OS components (8.5 and 10.4 min) were detected. The HPAEC system consisted of a quaternary gradient pump, a pulsed amperometric detector with gold electrode, a liquid chromatographic module (LCM-2), a basic post column delivery system, and a Shimatz C-R3A integrator–recorder. A CarboPac PA 1 column (4.6 × 250 mm) and a CarboPac PA guard column (3 × 25 mm) (Dionex, Sunnyvale, CA) were used.

Carbohydrate components of variant A oligosaccharide (OS) were analyzed by hydrolyzing the OS with 2–4 M TFA (2 h at 100 °C) and followed by HPAEC (Hardy et al., 1988) as previously described (Yamasaki et al., 1991a). KDO was analyzed by the thiobarbituric acid assay (Yamasaki et al., 1988, 1991a). The TFA hydrolysate was also acetylated and reduced with $NaBD_4$ (Cambridge Isotopes, Cambridge, MA) (Blakeney et al., 1983), and the resulting alditol acetates were analyzed by GC/MS as will be described below. Monosaccharides (Glc, Gal, GlcNAc, GalNAc, and Hep) were converted to their alditol acetate derivatives in a similar manner.

Methylation (Ciucanu & Kerek, 1984) of the dephosphorylated variant A OS was not successful due to its poor solubility in dimethyl sulfoxide. The variant A OS was first acetylated with pyridine and acetic anhydride in the presence of 4-dimethylaminopyridine; the mixture was stirred at room temperature for 24 h, and the acetylated OS was extracted with chloroform. Then the acetylated OS was methylated, and this methylated OS was hydrolyzed, reduced with $NaBD_4$, and acetylated (Levery & Hakomori, 1987; Yamasaki et al., 1991a). A carboxy-reduced OS was prepared by acid hydrolysis (1% AcOH, 2 h at 100 °C) of a carboxy-reduced LOS (the preparation of this LOS was described above). This OS was methylated and processed as described above. Alditol acetates derivatives of the methylated carbohydrates were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) under the same conditions as previously described (Yamasaki et al., 1991a). Alditol acetate derivatives of monosaccharides were analyzed using a different temperature program [the program for the splitless mode: $T1$ = 35 °C for 4 min, 18 °C/min; $T2$ = 160 °C/min; $T3$ = 280 °C for 10 min; column, DB1 (0.25 mm × 30 m, J & W, Folsom, CA); carrier gas, helium (16 psi)]. Under these conditions, the typical retention times of alditol acetate derivatives of Glc, Gal, GlcNAc, and L- and D-*glycero*-D-*manno*-heptoses were 24.28, 24.39, 27.29, 29.18, and 28.42 min, respectively.

The variant A oligosaccharide (OS) (1–1.5 mg) was repeatedly lyophilized in D_2O and dissolved in 99.999% D_2O to give a final volume of 0.4 mL. All NMR experiments were run on a GE 500-MHz spectrometer. ^1H chemical shifts were referenced to the chemical shift of HOD that was carefully calibrated relative to 3,3,3-trimethyl[2,2,3,3-$^2H_4$]propionate as a function of temperature. The one-dimensional (1D) experiments were run at 25 and 50 °C, and the number of

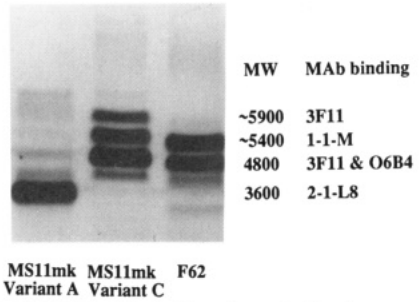

FIGURE 1: SDS–PAGE (14% gel) analysis of gonococcal LOSs: lane 1, MS11mk variant A; lane 2, MS11mk variant C; lane 3, F62. The LOS components were visualized by silver staining. The MWs of the LOS components are according to the estimation by PAGE (Mandrell et al., 1990). The apparent PAGE mobility difference of the two major components of variant C and F62 is probably due to the overloading of the lane F62 LOS.

acquisitions was 64. Two-dimensional (2D) NMR data were acquired at 25 °C with a spectral width of 3205 Hz in both dimensions, and the number of acquisitions was 16 unless otherwise noted. These data were transferred to an off-line SUN workstation (SPARC) and processed using the NMR processing programs developed in the Pharmaceutical Chemistry Department, University of California at San Francisco.

The double-quantum-filtered COSY (DQF-COSY) (Rance et al., 1983; Neuhaus et al., 1985) spectrum was acquired in the phase-sensitive mode using time-proportional phase incrementation (Redfield & Kunz, 1975). The 800 × 4K data points were processed with a shifted sine-squared bell function in both dimensions. The final dimensions was 1K × 4K real points after zero-filling in t_1. Homonuclear Hartmann–Hahn (HOHAHA) (Bax, et al., 1985; Yamasaki et al., 1991a) spectra were acquired with 256 × 2K data points (τ_m = 60, 130, and 200 ms), and each data matrix was processed with a Gaussian function and zero-filled in both dimensions to give a final number of data points of 1K × 4K. The triple-quantum-filtered COSY (TQF-COSY) (Piantini et al., 1982) spectrum was acquired with 512 × 4K data points, and the number of acquisitions was 64. The data matrix was processed using a shifted sine-squared function in both dimensions and zero-filled in t_1 to give a final number of real data points of 1K × 4K. The pure absorption 2D NOE spectrum (Jeener et al., 1979) (τ_m = 150 ms) was obtained (States et al., 1982; Yamasaki et al., 1991a) as 512 × 4K data points and processed with a shifted sine-squared function. The preparation delay was 5 s. The 1K × 4K real data points were obtained after zero-filling.

RESULTS AND DISCUSSION

Variant A of strain MS11mk produces almost entirely one single lipooligosaccharide (LOS) that is recognized by MAb 2-1-L8 (Figure 1). This variant phase shifts in vivo to different variants that produces higher MW LOS components (Schneider et al., 1991), and the LOS components produced by variant C are shown as an example in the figure. Variant C does not produce the LOS of MW 3600 but produces higher MW components that are recognized by MAbs 1-1-M and 3F11.

The variant A oligosaccharide (OS) was prepared by hydrolysis of the LOS (1% AcOH, 2 h at 100 °C) and followed by dephosphorylation (48% HF for 24 h at 4 °C) and Bio-Gel P-4 chromatography (<400 mesh, 2.6 × 90 cm, 100 mM ammonium acetate, K_d = 0.40) as described previously (Yamasaki et al., 1988, 1991a). The variant A OS preparation (dephosphorylated) was found to contain two minor components by high-performance anion-exchange chromatography (HPAEC) but their amounts were 7% of the major OS (estimated by integration). Carbohydrate analysis by GC (alditol acetate derivatization) (Blakeney et al., 1983) and HPAEC (Yamasaki et al., 1991a) indicated that the variant A OS is a hexamer of which the carbohydrate composition is Gal:Glc:GlcNAc:Hep:KDO 1:1:1:2:1.

Table I: ^{1}H Chemical Shifts[a] and Coupling Constants[b] of Carbohydrate Components[c] of Dephosphorylated Variant A OS

	Galβ1 → V	4Glcβ1 → IV	4Hep III	{3←1αHep2 I	←1αGlcNAc} II
			^{1}H Chemical Shifts (ppm)		
H-1	4.481	4.556	5.078	5.562	5.085
H-2	3.549	3.464	4.132	4.128	3.902
H-3	3.704	3.655	~4.14	3.977	3.805
H-4	3.939	3.480	4.098	3.647	3.533
H-5	3.939	3.613	3.725	4.061	3.882
H-6	3.548	3.728	4.261	3.639	3.442
H-6′	3.704	4.058			3.542
H-7			~4.13	3.724	
H-7′			~4.13	4.061	
			Coupling Constants (Hz)		
$J_{1,2}$	8	8	3	3	3
$J_{2,3}$	10	10	2	3	10
$J_{3,4}$	4	8	4	6	9
$J_{4,5}$	3	10	8	7	9
$J_{5,6}$		6		6	4
$J_{5,6'}$		3			6
$J_{6,6'}$		12			12
$J_{6,7}$	7				
$J_{6,7'}$	5				
$J_{7,7'}$	10				

[a] Chemical shifts are with reference to the HOD (4.78 ppm) signal at 25 °C. [b] The coupling constant values of the endocyclic protons were measured from the DQF-COSY spectrum. The values of the exocyclic protons were estimated by comparative cross-peak analysis of the DQF-COSY and TQF-COSY spectra. Error approximately ±1 Hz. [c] Roman numerals refer to each carbohydrate residue. Hep, heptose; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Glc, glucose.

Figure 2A shows the one-dimensional spectrum of the dephosphorylated variant A OS at 25 °C. The spectral pattern of the variant A OS is almost identical to the partial spectra of the F62 OS which will be decreased later. The presence of five anomeric protons was confirmed: I (5.562 ppm), II (5.085 ppm), III (5.078 ppm), IV (4.556 ppm), and V (4.481 ppm). The rest of the endocyclic and exocyclic protons of each carbohydrate component were located in the range 4.30–3.40 ppm. The methyl resonance of NHAc of GlcNAc was located at 2.05 ppm, and the deoxy resonances (A–D of Figure 2A) due to a KDO residue were in the range 1.00–2.50 ppm.

The identity of each carbohydrate component I–V and the assignments of their endocyclic and exocyclic protons were determined by DQF-COSY and HOHAHA analysis as reported previously (Yamasaki et al., 1991a). The analysis of the exocyclic protons was also aided by a TQF-COSY experiment (Piantini et al., 1982) that filters all cross peaks except those due to three or higher spin systems. The oligosaccharide sequence was analyzed by 2D NOE experiments (Jeener et al., 1979; States et al., 1982) and methylation analysis as previously described (Yamasaki et al., 1991a).

Figures 2B and 3 show the H1–H2 and 4.40–3.40 ppm portions of the DQF-COSY spectrum of the variant A OS, respectively. In Figure 3, the lines traces the connectivities for each carbohydrate residue with the exception of residue III. Figures 4 and 5 show the HOHAHA and TQF-COSY spectra, respectively. The chemical shifts and the coupling constants data of each carbohydrate residue are presented in Table I. The differences of the chemical shifts of the variant

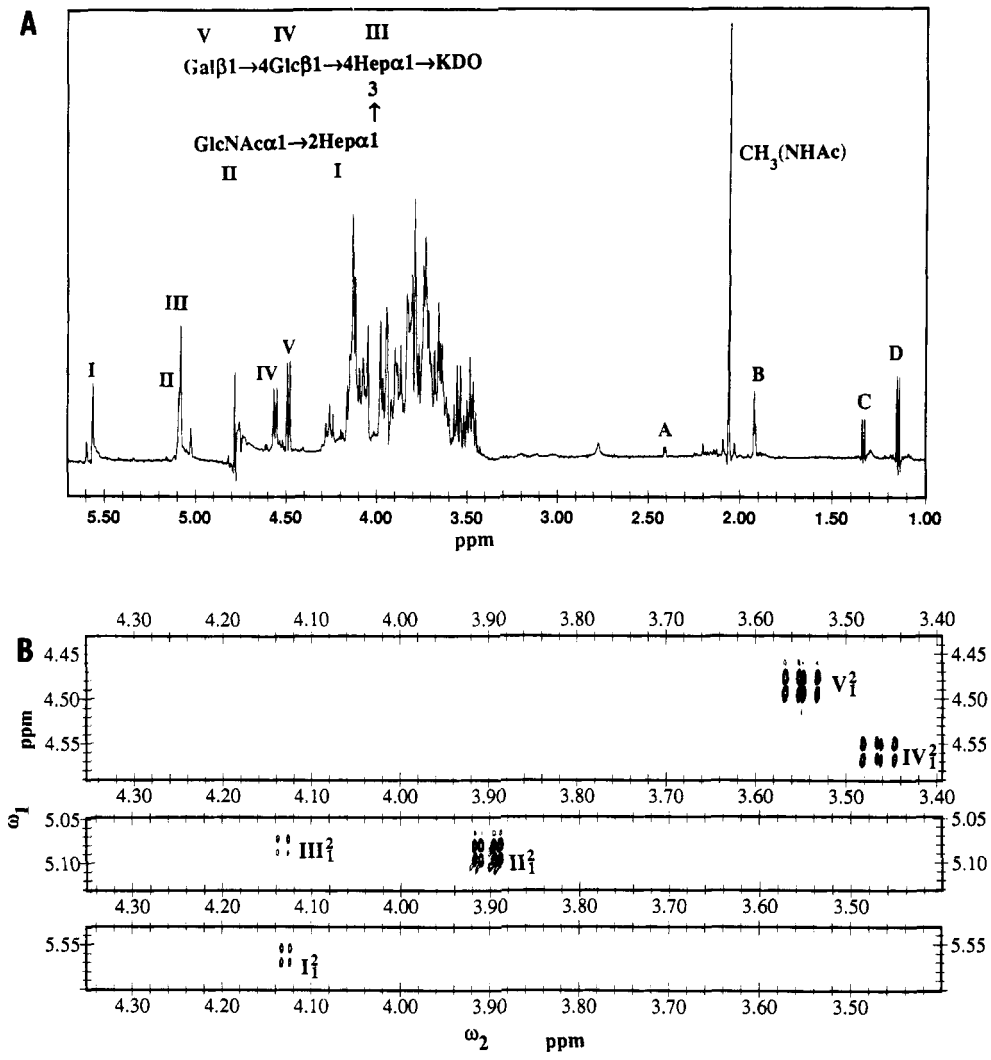

FIGURE 2: (A) Structure of the variant A OS and one-dimensional spectrum of the OS at 25 °C. The Roman numerals refer to the five different carbohydrate residues as shown above. The uppercase letters refer to the upfield peaks of the deoxy protons of KDO (see Table II). The absence of anomeric protons around the HOD signal (4.78 ppm, partially saturated in the spectrum) was confirmed by examining the spectrum at 50 °C (data not shown). (B) Parts of the DQF-COSY spectrum that show the H1–H2 cross peaks. The Roman numerals refer to the carbohydrate residue (see the structure) and the Arabic numerals refer to the proton in the respective carbohydrate residue. The subscript refers to the proton whose chemical shift is given on the ω_1 axis and the superscript refers to the protons whose chemical shift is given on the ω_2 axis. The digital resolution of the DQF-COSY spectrum was 0.002 and 0.010 ppm/point in ω_2 and ω_1, respectively.

Table II: ^{1}H Chemical Shifts[a] and Coupling Constant[b] Data for KDO Protons

	A	B	C	D
	Chemical Shifts[c] (ppm)			
a	2.406	1.914	1.329	1.144
b	3.945	4.122	4.108	3.881
c	4.054	4.113		3.435
d				3.457
e				3.535
f				3.557
	Coupling Constants (Hz)			
$J_{a,b}$	5	9	14	13
$J_{b,c}$	2	2	4	4

[a] Chemical shifts are with reference to the HOD (4.78 ppm) signal at 25 °C. [b] The coupling constant values were obtained from the DQF-COSY spectrum (error approximately ±1 Hz). [c] Line a: the chemical shift of each of the KDO deoxy protons A–D that are shown in Figure 2A. Line b: the chemical shift of a proton which is directly coupled to each of the deoxy protons, taken from the DQF-COSY spectrum. Lines c–f: each proton connectivity was determined by analyzing the HOHAHA spectra (τ_m = 60, 130, and 220 ms).

A carbohydrate residues and the corresponding F62 carbohydrate resides (Yamasaki et al., 1991a) were within 0.01 ppm except for the terminal Gal (V).

The coherence transfers of H1 to the exocyclic protons were almost complete with the hexose residues II and IV (Figure 5) having the gluco configuration (τ_m = 130 ms; Yamasaki et al., 1991a), and residues II and IV were determined to be GlcNAc and Glc, respectively, as will be described later. Residues I and III were found to be Hep, and residue V was found to be Gal. The coherence transfer of I-1 to I-5 was clear from Figure 5. The exocyclic protons of residue I, H6, H7, and H7′, were assigned to be 3.639, 4.061, and 3.724 ppm, respectively, from careful inspection of their TQF-COSY cross peaks, which partially overlapped with those due to IV-5–IV-6′ and IV-6–IV-6′ (see Figures 3 and 4).

The bond connectivity of Gal residue V could be traced to H4 (Figure 3), but the assignments of H5, H6, and H6′ were not possible by the DQF-COSY and HOHAHA analysis because of their overlap with H-2, H-3, and H-4. The overlap was indicated by the intensities of the HOHAHA cross peaks (Figure 5) due to H-1–H-3 and H-1–H-4. By the TQF-COSY analysis (Figure 4), H-6 and H6′ were assigned to be 3.548 and 3.704 ppm, respectively, showing characteristic coupling pattern of the geminal protons. Thus, H-5 was determined to be overlapped with H-4.

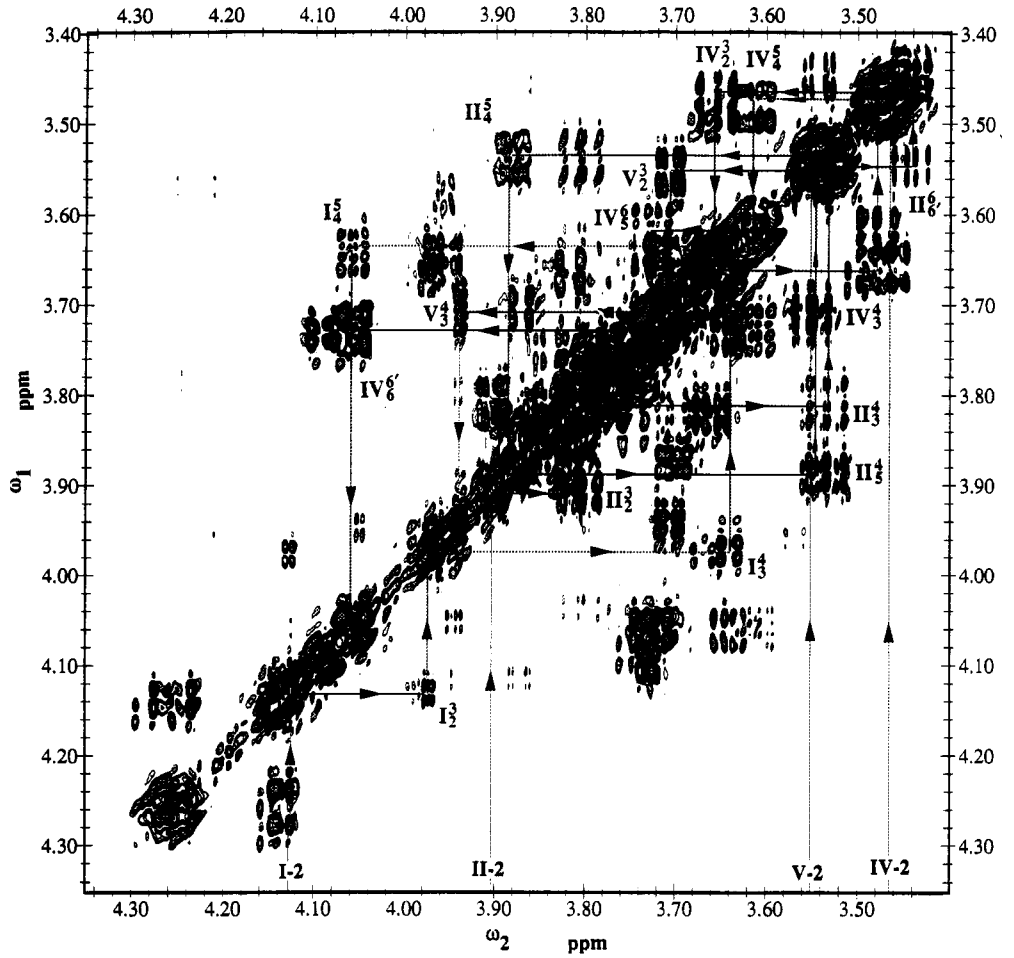

FIGURE 3: Part of the DQF-COSY spectrum (4.35–3.40 ppm) of the MS11mk OS at 25 °C. Negative levels are shaded, and the lines trace the connectivity for the carbohydrate components of the OS. Each line begins at the H1–H2 cross peak (see Figure 2B) and goes up to the diagonal and across to the next cross peak. It continues in this manner until the connectivity is traced for each carbohydrate residue. The cross peaks are labeled in the manner explained in Figure 2B.

The assignment of the residue III protons, except for H-1 and H-2, was difficult because H-2–H-5 resonated in a narrow range similar to that found in F62 OS (Yamasaki et al., 1991a). This was indicated by the 1D spectrum (Figure 2A) as well as the intensity and width of the relay cross peak due to III-1 and III-2 (Figure 4). H-3 and H-4 were assigned to be at 4.136 and 4.098 ppm, respectively, similar to F62 OS (Yamasaki et al., 1991a). The assignment of H-4 was also confirmed from its interresidual NOE with H-1 of Glc IV, as will be described below. Two cross peaks, one major and one minor, due to two sets of protons (4.133–4.261 and 4.150–4.265 ppm, Figures 3 and 4) were found to be parts of two independent sets of three spin systems. The former cross peak was assigned to be III-6 (at 4.261 ppm) and III-7 (at 4.133 ppm). This cross peak is not be due to the geminal protons, H7 and H7′, since large active couplings were not present in the cross peak (below the diagonal) of the DQF-COSY spectrum (Figure 3). H-5 and H-7′ probably overlapped with H-3. The other TQF-COSY cross peak mentioned above and other unidentified three-spin systems are probably due to the exocyclic protons of the KDO residue or due to minor OS components as already described. It is not yet clear whether the minor OS components detected in the variant A OS were derived from minor LOS components present in the variant A LOS preparation or due to the equilibrium of the reducing KDO, which will be discussed later.

Determination of the oligosaccharide (OS) sequence was based on the results of 2D NOE data (Figure 6) and methylation analysis (Table III). Because the variant A OS is smaller in MW than F62 OS, the NOE intensities detected with the former were smaller than those with the latter. Methylation of the dephosphorylated variant A OS resulted in very low yields of the methylated OS due to its poor solubility in dimethyl sulfoxide. However, this problem was solved by acetylation of the OS and subsequent methylation of the acetylated OS.

Table III: Methylation Analysis of Dephosphorylated Variant A OS[a]

	t_R
2,3,4,6-tetra-*O*-methylgalactitol	1.00
2,3,6-tri-*O*-methylglucitol	1.06
2-acetamido-2-deoxy-3,4,6-tri-*O*-methylglucitol	1.26
3,4,6,7-tetra-*O*-methylheptitol	1.22
2,6,7-tri-*O*-methylheptitol	1.27

[a] Relative retention times (t_R) are referenced to 2,3,4,6-tetra-*O*-methylgalactitol. The alditol acetate derivatives of the partially methylated carbohydrates in the table gave fragmentation patterns identical to those reported previously (Yamasaki et al., 1991a).

The Gal(V)β1→4Glc(IV) structure was confirmed by the presence of both terminal Gal and 4-linked Glc, as well as the interresidual V-1–IV-4 NOE (Figure 6). Since the 4-linked GlcNAc was not detected in the methylation analysis, IV and II were determined to be Glc and GlcNAc, respectively. The above lactosyl residue was determined to be β1→4 linked to the Hep (III) of the same tetrasaccharide, {GlcNAc-(II)α1→2Hep(I)α1→3}Hep(III)α→KDO, as found in the

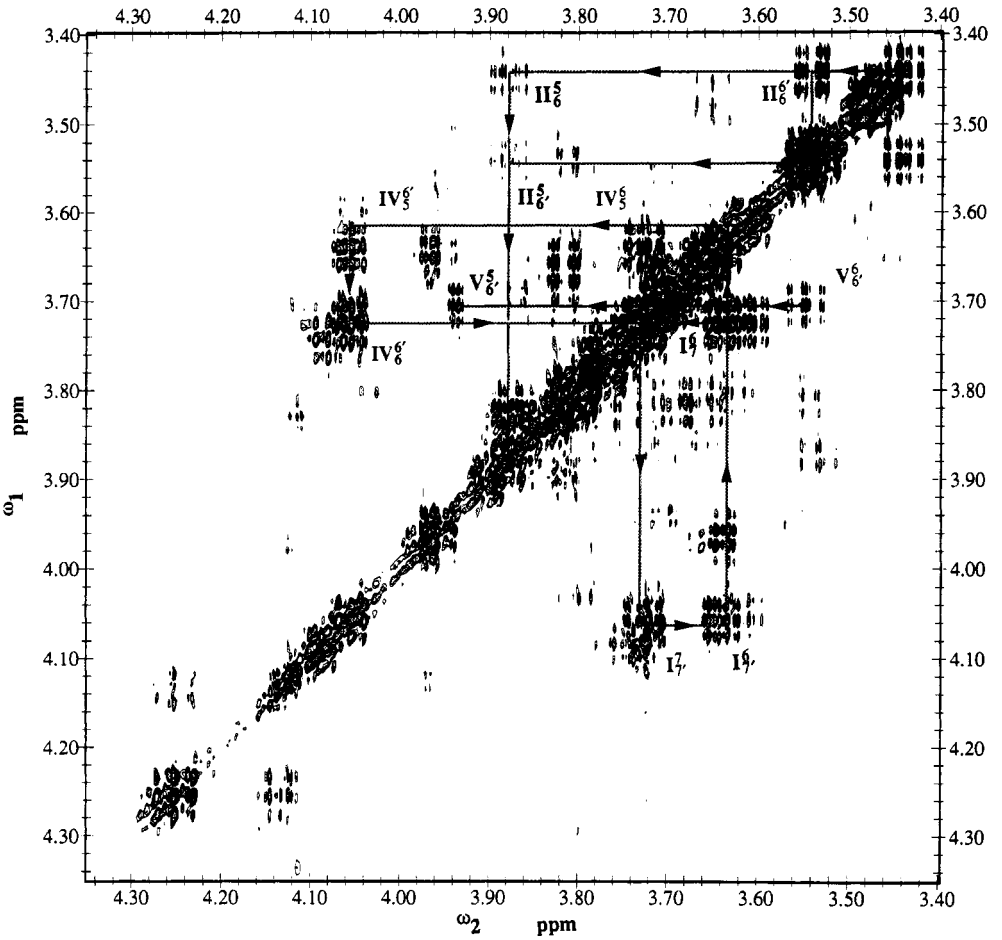

FIGURE 4: Part of the TQF-COSY spectrum (4.35–3.40 ppm) of the MS11mk OS at 25 °C. The lines connect the proton cross peaks for each three-spin network. The cross peaks are labeled as explained in Figure 2B. The digital resolution of the spectrum was 0.002 and 0.010 ppm/point in ω_2 and ω_1, respectively.

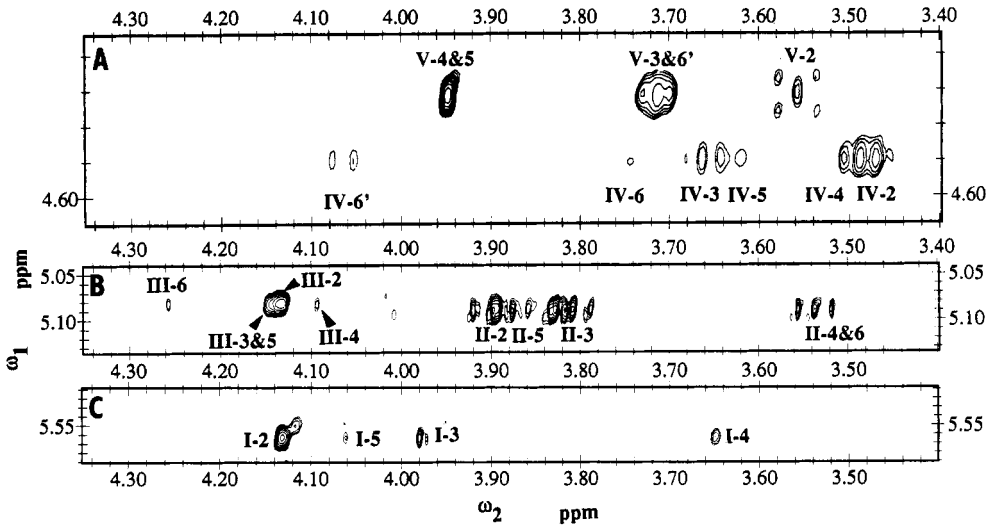

FIGURE 5: Parts of the HOHAHA spectrum of the variant A OS at 25 °C. (A) τ_m = 130 ms; (B, C) τ_m = 220 ms. The cross peaks are labeled as explained in Figure 2B. The digital resolution of the spectrum was 0.002 and 0.010 ppm/point in ω_2 and ω_1, respectively.

F62 OS components (Yamasaki et al., 1991a). This structure was confirmed by the presence of 2-linked Hep, 3,4-linked Hep, and t-GlcNAc (Table III). The variant A OS structure was also supported by the following NOE data (Figure 6): (1) the IV-1–III-4 interresidual NOE and (2) the II-1–I-2 and I-1–III-3 interresidual NOEs. These NOE cross peaks partially overlapped with the intraresidual ones, III_1^2 and I_1^2. The present study identified an interresidual NOE cross peak, IV-1–III-6, which was suggested in a previous study. This assignment was supported by the TQF-COSY analysis.

As observed previously with F62 OS (Yamasaki et al., 1991a), four sets of resonances (A–D in Figure 2) due to the KDO deoxy protons were located at 1.144, 1.329, 1.914, and 2.406 ppm, and no cross peaks were observed among the deoxy protons. As shown in Table II, A and B show small couplings with directly coupled protons whereas C and D show large

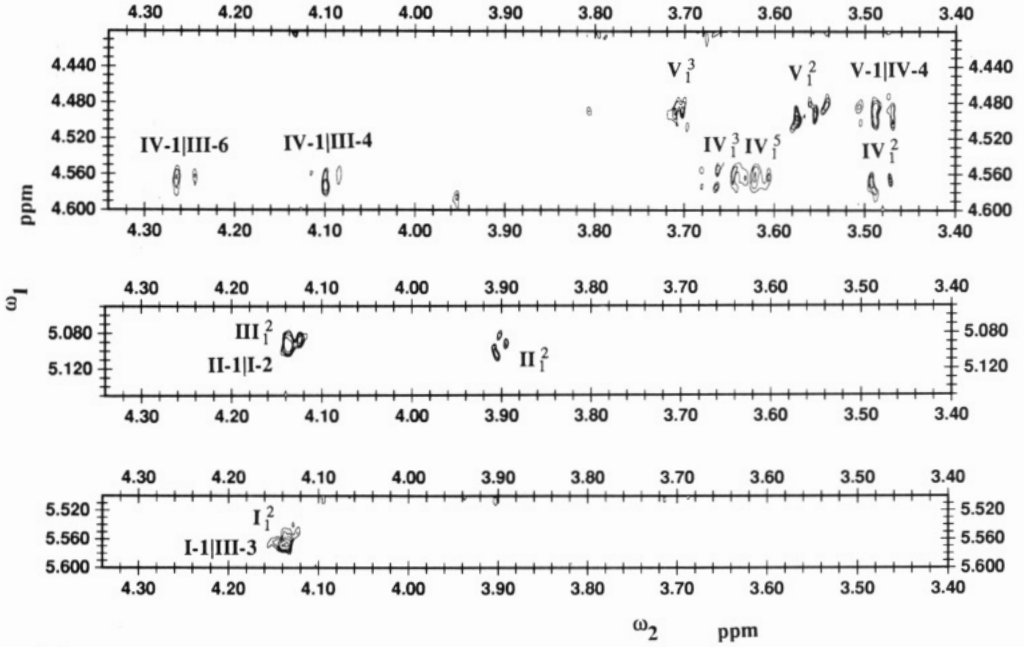

FIGURE 6: Parts of the pure absorption 2D NOE spectrum (τ_m = 150 ms) of variant A at 25 °C. They show the intra- and interresidual NOE cross peaks of the anomeric protons of I(Hep), II(GlcNAc), III(Hep), IV(Glc), and V(Gal). The cross peaks are labeled as explained in Figure 2B. The digital resolution of the spectrum was 0.002 and 0.010 ppm/point in ω_2 and ω_1, respectively.

couplings. Although $J_{a,b}$ of both C and D are large, their chemical shifts support neither form of furanose, pyranose, or 2,8-anhydro derivative, as described previously (Yamasaki et al., 1991a). Our current data suggest that the KDO may be in an equilibrium of A–D. In order to determine the linkage between the heptose (I) and KDO, the carboxyl groups of the LOS (~100 μg) were reduced (Yamasaki et al., 1988), and the OS obtained from the resulting LOS by hydrolysis was analyzed by methylation. However, we were not able to detect a 5-linked KDO that has been found in a gonococcal LOS produced by a mutant strain (John et al., 1991). We are currently investigating the linkage involved by using a preparative scale of OS and LOS.

The identity of the two heptoses was suggested to be L-*glycero*-D-*manno*-heptose; the retention times in HPAEC and GC (as alditol peracetate) analysis of the heptose detected were identical to those of the L-*glycero*-D-*manno*-heptose, and the heptose structure was also confirmed by the GC/MS analysis. The identity of the heptoses present in the F62 OS samples was also suggested to be same as those of the variant A OS by the two different chromatographic methods. However, as was the case with the F62 OS (Yamasaki et al., 1991a), the $J_{3,4}$ of the heptose I was smaller than the expected value for the $^4C_1$ conformer in which the H3 and H4 are diaxially oriented. The $J_{3,4}$ value obtained indicate that the configuration of the endocyclic protons may be talo if the heptose exists in the $^4C_1$ conformation. If the configuration is talo, the identical chromatographic retention times happened to be coincident. If not, the ring structures of the 2- or 2,3-substituted mannoheptoses may be in an equilibrium between the chair ($^4C_1$) and boat ($^{3,0}B$) conformation as found in the glucuronate residue in chondrosine (Lamba et al., 1991). The identity and conformations of the heptoses present in the variant A will be determined by preparation of L- and D-*glycero*-*talo*-heptoses and subsequent HPAEC analysis.

Neither the variant A LOS nor its OS was digested with either exo- or endo-β-galactosidase. This inactivity toward the enzymes is probably due to steric hindrance by the presence of a branched diheptose structure as observed with the F62 OS components (Yamasaki et al., 1991a). Since sequential

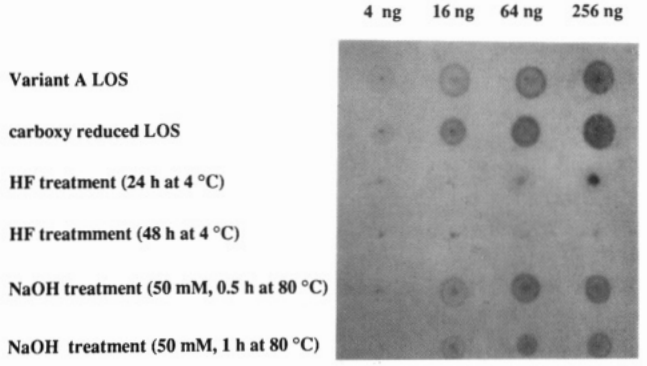

FIGURE 7: TLC immunostaining of variant A LOS and its chemically modified derivatives. Each LOS was spotted on a TLC sheet. The sheet was blocked with 0.5% casein solution and then treated sequentially with MAb 2-1-L8, goat anti-mouse IgG (conjugate with peroxidase), and a mixture of 4-chloro-1-naphthol and 0.001% hydrogen peroxide in 50 mM Tris-HCl (pH 7.4).

glycosidase cleavages of the variant A LOS were not possible, we investigated expression of the MAb 2-1-L8-defined epitope by chemical modification of the variant A LOS and then by examining the binding of MAb 2-1-L8 to the modified LOSs. Reduction of the KDO carboxyl group(s) of the LOS did not affect the antigenicity (Figure 7) as observed with MAb 3F11 and F62 LOS (Yamasaki et al., 1988). Further, the MAb bound the LOS even after treatment with 50 mM NaOH for 1 h at 80 °C for partial deacylation. However, MAb 2-1-L8 did not bind to the variant A LOS when it was treated with HF for dephosphorylation (Yamasaki et al., 1988). As expected, MAb 2-1-L8 did not bind to lactosylceramide.

This preliminary study indicate the following: (1) The MAb 2-1-L8-defined epitope is not a linear β-lactosyl residue. (2) KDO is not involved in the epitope. (3) The specificity of MAb 2-1-L8 is probably directed toward the GlcNAc-Hep-Hep area where phosphate groups are presumably present as expected from structural data for gonococcal LOS (Gibson et al., 1989; John et a., 1991). (4) Partial deacylation of F62 LOS under the conditions used in the present study resulted in the loss of expression the MAb 3F11-defined epitope (Yamasaki et al., 1988). The stability of MAb 2-1-L8-defined

Table IV: Structures of Gonococcal LOS and Human Glycosphingolipids

	binding MAbs	OS structures[a]
	Gonococcal LOS	
variant A (3.6 kDa)	2-1-L8	Galβ1→4Glcβ1→R
F62 (4.5 kDa)	3F11	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→R
F62 (4.8 kDa)	1-1-M	GalNAcβ1-3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→R
	Human Glycosphingolipids	
lactosylceramide		Galβ1→4Glcβ1→*O*-ceramide
asialo-GM2		GalNAcβ1→3Galβ1→4Glcβ1→*O*-ceramide
paragloboside	3F11	Galβ1→4GlcNAβ1→3Galβ1→4Glcβ1→*O*-ceramide
asialo-GM1		Galβ1→3GalNAcβ1→3Galβ1→4Glcβ1→*O*-ceramide

[a] GalNAc, *N*-acetylgalactosamine; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Hep, heptose; KDO, 3-deoxy-2-ketooctulosonic acid; R (diheptose-KDO core), 4Hep{3←1αHep2←1αGlcNAc}α1→KDO.

epitope may be 2-fold; removal of some fatty acids in the lipid A may not affect the stereochemistry of the epitope since it is close the reducing end of the oligosaccharide moiety, and the α-linked trisaccharide, GlcNAc-Hep-Hep, could be very stable. (5) Since MAb 2-1-L8 bind to gonococci (Stein et al., 1988), this MAb-defined epitope, at least a part of the phosphorylated GlcNAc-Hep-Hep residue, is expressed on the gonococcal surface. The exposure of this area on gonococcal surface was not known before. We will further define the MAb 2-1-L8-defined epitope by characterizing the structure of the LOS derivatives, and the results will be published elsewhere.

We have here determined the oligosaccharide (OS) structure of lipooligosaccharide (LOS) that MAb 2-1-L8 binds and have determined that the variant A OS is the building block that precedes the synthesis of larger LOS structure, first, one that binds MAb 3F11, and second, a larger MW one that binds to MAb 1-1-M (Table IV and Figure 1). Our structural data indicate that the OS structure of a gonococcal LOS is the result of sequential addition of glycosyl building blocks to one of the heptoses. Interestingly, MAb 3F11 binds a LOS component whose MW is larger than 4500 as shown in Figure 1. Structural analysis of this higher MW LOS component will provide further information whether the gonococci can produce a repeating lactosamine structure as found in human glycosphingolipids (Fukuda et al., 1985) or a lactosamine structure is simply linked to the GalNAc of the MW 4800 LOS.

Although the variant A strain has a LOS whose nonreducing disaccharide is β-lactose, this structural similarity to lactosylceramide does not necessary result in the antigenic mimicry. However, variant A of strain MS11mk has a membrane protein, distinct from the pili and PII, that binds to purified lactosylhydroxyceramide, asialo-GM_1, and asialo-GM_2 (Table IV) (Stromberg et al., 1988). Lactosylceramide and gangliotriaosylceramide were present in glycolipid preparations of an epithelial cell line derived from a human cervical carcinoma. Deal and Krivan (1990) also indicated that both variant A and nonpiliated variant B2 bind to paragloboside. These results indicate that the gonococcal LOS may play some role in the adhesion of the gonococci on human cell surfaces.

We have studied the LOS produced by variant A of strain MS11mk and determined that this LOS is a truncated form of the higher MW LOS components that are recognized by MAb 1-1-M and 3F11. Our present study indicates that the variant A LOS is a precursor for the higher MW LOS components that are associated with *N. gonorrhoeae* diseases. We also determined that the MAb 2-1-L8-defined epitope present on the variant A LOS is not a linear β-lactosyl residue. Its specificity is directed toward the phosphorylated GlcNAc-Hep-Hep residue.

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