Endogenous Sialylation of the Lipooligosaccharides of *Neisseria meningitidis*†

R. E. MANDRELL,^{1*} J. J. KIM,^{1,2} C. M. JOHN,³ B. W. GIBSON,³ J. V. SUGAI,^{1,2} M. A. APICELLA,⁴
J. M. GRIFFISS,^{1,2} AND R. YAMASAKI^{1,2}

Center for Immunochemistry, Veterans Administration Medical Center, 4150 Clement Street, San Francisco, California 94121¹; Department of Laboratory Medicine² and Department of Pharmaceutical Chemistry,³ University of California, San Francisco, California 94143; and Department of Medicine, State University of New York at Buffalo, Buffalo, New York 14215⁴

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Monoclonal antibodies (MAb) 3F11 and 06B4 recognize epitopes that are conserved on gonococcal lipooligosaccharides (LOS), present on some meningococcal LOS, and conserved on human erythrocytes. LOS of some group B and C prototype meningococcal LOS strains (LOS serotypes L1 to L8) treated with neuraminidase showed increased expression of the 3F11 and 06B4 MAb-defined epitopes. Neuraminidase-treated LOS separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained showed a shift in migration from a component with a mass of approximately 4.8 kDa to a component with a mass of between 4.5 and 4.6 kDa. The same strains grown in medium with excess CMP-*N*-acetylneuraminic acid had LOS that shifted in migration to a slightly higher component (mass, approximately 4.8 kDa). Chemical analysis of the neuraminidase-digested products from one LOS indicated it contained approximately 1.5% sialic acid. Covalent linkage between sialic acid and the LOS was confirmed by analysis of de-O-acylated and dephosphorylated LOS by liquid secondary ion mass spectrometry. These studies show that some meningococci contain sialic acid in their LOS, that the sialic acid is cleaved and lost in conventional acetic acid hydrolysis, and that the sialic acid alters the expression of MAb-defined epitopes.

The surface glycolipids (lipooligosaccharides [LOS]) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* are composed of multiple components that are antigenically and chemically distinct (21, 37). Many gonococcal and meningococcal strains contain LOS that bind monoclonal antibodies (MAbs) 3F11 and 06B4, usually to a 4.5-kDa LOS component. The shared epitopes defined by MAbs 3F11 and 06B4 (35) are conserved on gonococcal LOS (3, 4, 37) but variably expressed on LOS of meningococci (31, 35), *Neisseria lactamica* (30), and *Haemophilus ducreyi* (9). These MAbs also have been used to show that certain LOS components are immunochemically similar to molecules present in human erythrocytes and in other human cells (35). MAbs 3F11 and 06B4 bind to mammalian glycosphingolipids that have terminal Gal β 1 $\rightarrow$ 4GlcNAc structures (35).

Recent studies have shown that when gonococci are grown in the presence of CMP-*N*-acetylneuraminic acid (CMP-NANA), sialic acid is incorporated into the LOS component of 4.5 kDa that binds MAbs 3F11 and 06B4 (36, 44). The sialylated component no longer binds the MAbs (4, 36). Treatment of the LOS with neuraminidase restores these epitopes, both on organisms grown in vitro (36) and on organisms in vivo in urethral exudates (4). Although the 3F11 and 06B4 epitopes are of similar specificity and are both expressed on gonococcal LOS (37), the 06B4 epitope is expressed much better than is the 3F11 epitope on meningococcal LOS of group B and C strains (30, 35). Because of these results, we wondered whether the difference in the expression of the two MAb-defined epitopes on meningococci could be due to sialylation of their LOS. In the present

study, we confirm that sialic acid is present in the LOS of some meningococci and, as in gonococci, the presence of sialic acid modifies the epitopes defined by MAbs 3F11 and 06B4.

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MATERIALS AND METHODS

Materials. Two different preparations of neuraminidase were used for studies of the effect of neuraminidase on LOS antigens: *Clostridium perfringens* neuraminidase (type V; Sigma Chemical Co., St. Louis, Mo.; 1.3 U/mg of solid; proteinase, ≤ 0.001 U/mg of solid; *N*-acetylneuraminic acid-aldolase, 0.024 U/mg) or purified streptococcal neuraminidase (Genzyme Corp., Boston, Mass.; according to the manufacturer, this preparation contains other relevant glycosidases at levels of $<0.033\%$ by activity). The compound 4-butylphenylhydrazine (4-BPH) was a gift from E. M. Allen and J. W. Webb, Department of Chemistry, Illinois State University, Normal. Details of the synthesis will be published elsewhere. Trifluoroacetic acid was purchased from Pierce (Rockford, Ill.), and 48% aqueous HF solution was purchased from Mallinckrodt (Muskegon, Mich.). Anhydrous hydrazine was obtained from Sigma. Acetonitrile, water, and methanol were obtained from Burdick and Jackson (Muskegon, Mich.).

Bacterial strains. The prototype group B and C meningococcal LOS strains (38, 62) have been described previously. Gonococcal strains F62 and WR220 were kindly provided by Herman Schneider (Walter Reed Army Institute of Research, Washington, D.C.).

MAbs. The specificities and characteristics of anti-LOS MAbs 3F11 (3, 5, 35–37), 06B4 (4, 35–37), and 1-1-M (36, 37)

* Corresponding author.

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have been described previously. MAb 2-2-B, specific for meningococcal group B polysaccharide (39), was the kind gift of Wendell Zollinger.

LOS. The LOS used for solid-phase radioimmunoassay (SPRIA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purified as described previously (49, 56). We also purified larger amounts of a single LOS for chemical analysis. In brief, meningococcal group B strain 6275 was grown in 11 liters of liquid 4× Frantz medium (34) in a fermentor (Lab-line Bioengineering, Melrose Park, Ill.). Acetone-dried organisms were added to 50 mM phosphate-buffered saline (PBS)–5 mM EDTA (pH 7.4) to make a 10% solution. This mixture was blended to make a suspension, and lysozyme (grade I; Sigma) was added to a 0.2% final concentration. This mixture was stirred at 4°C for 2 h and then at 37°C for 20 min. $MgCl_2$ was added to a 20 mM final concentration, and the suspension was mixed. DNase and RNase (Sigma) were each added to a final concentration of 3 µg/ml, and the suspension was incubated and stirred at 37°C for 15 min. LOS was extracted from this solution by the hot phenol water procedure of Westphal and Jann (56). The final product had <2% nucleic acid and <2% protein, as assessed by the ratio of the optical densities of the sample at wavelengths of 280 and 260 nm. MAb 2-2-B (specific for the sialic acid-containing meningococcal group B polysaccharide) (39) was tested in a SPRIA inhibition assay with strain 6275 LOS and purified group B polysaccharide as inhibitors of the anti-group B polysaccharide MAb (61). The results indicated that antigenic group B polysaccharide contamination of the LOS was <0.3%.

OMCs. Outer membrane complexes (OMCs) were prepared from meningococci as described previously (63).

SDS-PAGE and immunoblotting. LOS samples were separated through polyacrylamide slab gels by the method of Laemmli (32). Immunoblotting (37) and silver staining (54) were done as described previously. The masses of LOS components were estimated by reference to the LOS of meningococcal strain 8032 (group Y), which has six LOS components with masses of 5.4, 5.1, 4.5, 4.0, 3.6, and 3.2 kDa (31), as determined by reference to the *Salmonella* rough mutant LOS (50).

SPRIA. SPRIA was performed as described previously (37, 60). Stock solutions of LOS or OMCs were diluted in Dulbecco PBS modified to contain 50 mM $MgCl_2$. A 25-µg/ml solution of LOS or OMC (based on protein concentration) was used to sensitize polyvinyl microtiter plate wells. In some assays, plates were sensitized with whole organisms as described previously (1).

Neuraminidase treatment of LOS. Neuraminidase treatment of antigens on polyvinyl wells was performed as described previously (36). In brief, after sensitization of polyvinyl microtiter plate wells with LOS (25 µl of a 25-µg/ml solution) for 1 h, the wells were washed with PBS (pH 6) and 25 µl of neuraminidase (0.01 to 10 mU/ml diluted in PBS [pH 6]) was added to the wells. PBS was added to duplicate wells as a negative control. After incubation for 2 h at 37°C, the enzyme or buffer was removed and the wells were processed with MAb and secondary antibody as described above. A control experiment with all reagents except for the MAb revealed no binding of the secondary antibody to enzyme-treated LOS. From these experiments, we determined that treatment of LOS with neuraminidase at a concentration of ≥ 25 mU/ml resulted in optimal binding of MAb 3F11. Enzyme treatment of SDS-PAGE-separated and nitrocellulose-immobilized LOS was performed as described above (25 mU of neuraminidase per ml).

For neuraminidase treatment of LOS run in SDS-PAGE, 50 µg of purified LOS was suspended in 25 µl of PBS (pH 6.0) and the suspension was mixed with an equal volume of the same buffer containing 50 mU of enzyme (type V; Sigma) or the same buffer containing 50 mU of enzyme inactivated by heating at 100°C for 5 min. The mixtures were incubated at 37°C for 2 h, and the reaction was stopped by the addition of 50 µl of SDS-PAGE sample buffer (32).

Growth of organisms in medium with CMP-NANA. *N. meningitidis* strains were grown overnight on GC agar base (Difco Laboratories, Detroit, Mich.) containing 1% Kellogg supplement (57). The organisms were grown in the presence of CMP-NANA as described previously (36). In brief, the organisms were collected, washed once with PBS, and diluted to a concentration of approximately 0.2×10^6 CFU/ml. The organisms were mixed with an equal volume of a 500-µg/ml solution of CMP-NANA in PBS (pH 7.4), and the mixture was spread on GC agar plates. After overnight growth, the organisms were removed from the surface of the medium and suspended in Dulbecco PBS (optical density at 620 nm, 0.2) for whole-cell SPRIA (1) or in SDS-PAGE sample buffer for treatment with proteinase K (25).

Gold particle IEM of neuraminidase-treated bacteria. Immunoelectron microscopy (IEM) was done as follows. Strain 89I was grown on nutrient agar, collected in PBS (pH 6), washed once with PBS, and suspended in PBS to an optical density at 620 nm of 1.0. The organisms were divided into two aliquots: one was treated with 0.01 U of neuraminidase per ml and one was treated with neuraminidase that had been inactivated by heating at 100°C for 5 min. The bacteria were incubated at 37°C for 2 h, washed three times with PBS (pH 7.4), and fixed with 3% paraformaldehyde. A Formvar-coated nickel grid was placed on a 75-µl drop of organisms; excess liquid was removed, and the grid was blocked with normal goat serum. The bacteria on the grid were treated with MAbs and gold particle secondary antibodies essentially as described previously (5). The results represent the phenotype of >98% of the organisms examined.

Analysis of released neuraminic acid from strain 6275 LOS by anion-exchange chromatography with pulsed amperometric detection. Strain 6275 LOS was suspended in 25 mM sodium acetate at pH 6.8 (2 mg/ml) and sonicated for 2 min. Neuraminidase (10 mU) or heat-inactivated neuraminidase (100°C for 30 min) was added to 1-ml samples of LOS in glass test tubes. After the samples were incubated at 37°C for 2 h, an additional 10 mU of intact or inactivated enzyme was added and the mixtures were incubated again at 37°C for 2 h. A 50-µl aliquot was removed for analysis by SDS-PAGE and SPRIA, and the remainder was lyophilized. The lyophilized LOS-neuraminidase-buffer mixture was resuspended in 2 ml of water. The mixture was sonicated for 1 min and filtered with a 0.22-µm-pore-size membrane. To remove neuraminidase, we loaded the filtrate onto a Sep-Pak C18 cartridge (Waters, Milford, Calif.) and washed the cartridge with water. The eluate and the washes were pooled and concentrated, and the combined solution was analyzed for sialic acid with a CarboPac PA1 column (4 by 250 mm) and a carbohydrate analysis system (Dionex Corp., Sunnyvale, Calif.). The system consisted of a chromatographic module, pulsed amperometric detector, eluent degas module, and postcolumn delivery system. The analysis was carried out in the isocratic mode (eluant, 375 mM NaOH; flow rate, 1 ml/min; detector setting, 300 to 1,000 nA). Under these conditions, sialic acid had a retention time of 6.9 min; sodium acetate remaining in the sample did not affect the retention time of sialic acid. Sialic acid in the LOS sample

was quantified with xylose and authentic sialic acid as internal standards.

De-O-acylation of LOS. LOS from *N. meningitidis* 6275 was de-O-acylated by a previously described procedure (24). LOS (0.5 to 2.0 mg) was placed in 25-ml glass centrifuge tubes with screw caps, and 150 to 300 μ l of anhydrous hydrazine was added. The tubes were shaken in a water bath at 37°C for 20 min with intermittent sonication. Acetone chilled at -20°C (0.75 to 1.5 ml) was added slowly, the solution was centrifuged at 12,000 $\times$ g for 20 min, and the supernatant was discarded. The addition of cold acetone and centrifugation were repeated. The precipitated de-O-acylated LOS was dissolved in 0.5 ml of distilled water, and the solution was lyophilized.

Preparation of OS. A modification of a previously described procedure was used to purify and separate oligosaccharides (OS) from LOS (18). Approximately 12 mg of LOS from meningococcal strain 6275 was dissolved in 1% acetic acid (2 mg/ml), and the mixture was stirred at 80°C for 2 h. The samples were centrifuged (5,000 $\times$ g, 20 min, 4°C), and the supernatant was removed and lyophilized. The resulting OS fraction was dissolved in distilled water and centrifuge filtered through 0.45- μ m-pore-size Microfilterfuge tubes (Rainin, Emeryville, Calif.). The acid-hydrolyzed OS were further purified by gel filtration chromatography with two BioSil TSK-125 columns (7.5 by 600 mm; Bio-Rad, Richmond, Calif.) in series. The OS were eluted (isocratic mode; 1% acetic acid; flow rate, 1 ml/min) and monitored by refractive index measurements. Fractions containing OS were pooled and evaporated to dryness with a Speed Vac evaporator (Savant).

Dephosphorylation of de-O-acylated LOS and OS. Free OS or de-O-acylated LOS (0.1 to 1.0 mg) was placed in a polypropylene tube and diluted to a final 1- μ g/ μ l concentration in 48% aqueous HF. The samples were incubated for 16 to 20 h at 4°C, and the excess HF was removed by drying under vacuum over NaOH in a polypropylene dessicator with an N₂ stream and an in-line NaOH trap.

Derivatization with 4-BPH. The derivatization reaction mixture consisted of 125 μ g of dephosphorylated meningococcal strain 6275 OS, 35 μ g of 4-BPH, 10 μ l of distilled water, 40 μ l of methanol, and 1 μ l of acetic acid. The solution was vortexed, heated at 80°C for 30 min, and dried under N₂. The OS samples derivatized with 4-BPH were purified on a reverse-phase C18 column (Vydac; 25 by 4.6 cm) with UV detection at 335 nm. A linear gradient of 0 to 55% acetonitrile-water (1 ml/min) containing 0.05% trifluoroacetic acid was used for elution of the OS.

LSIMS. For liquid secondary ion mass spectrometry (LSIMS) analysis, the OS samples derivatized with 4-BPH were dissolved in water, and the de-O-acylated LOS samples were dissolved in methanol-water (1:1). Analysis was performed with a Kratos MS50S mass spectrometer with a cesium ion source (13, 18). Approximately 1 μ l of glycerol-thioglycerol (1:1) was applied to the stainless steel probe tip, and portions of the dissolved samples were added. The primary ion (Cs⁺) ion beam energy was 10 keV, and the accelerating voltage of the secondary ions was 8 kV. The spectra were acquired in the negative-ion mode.

RESULTS

Binding of MAbs 3F11 and 06B4 to neuraminidase-treated LOS of serotype L1 to L8 prototype strains. Table 1 shows the binding of MAbs 3F11 and 06B4 to meningococcal L1 to L8 prototype strains before and after the strains were treated

TABLE 1. Binding of MAbs 3F11 and 06B4 to neuraminidase-treated and untreated LOS^a

Strain	Group ^b	L type	Binding of the following MAb to antigen treated as indicated:			
			3F11		06B4	
			PBS	Neuraminidase	PBS	Neuraminidase
126E	C	L1	<1	<1	<1	<1
35E	C	L2	<1	53	1	58
6275	B	L3	<1	84	1	100
89I	C	L4	<1	100	47	98
M981	B	L5	<1	6	1	8
M992	B	L6	<1	<1	1	1
6155	B	L7	15	69	82	94
M978	B	L8	<1	5	2	22

^a Purified LOS was used as the antigen; 25 mU of streptococcal neuraminidase (Genzyme) or PBS was added to each microtiter plate well. Values are reported as the percentage of maximum binding: 20,275 cpm of ¹²⁵I-labeled goat antimouse immunoglobulin M was bound by MAb 3F11 to strain 89I LOS, and 52,687 cpm was bound by MAb 06B4 to strain 6275 LOS (see Materials and Methods).

^b Capsular serogroup of strain.

with neuraminidase. The LOS of L2, L3, and L4 showed a marked increase in the binding of MAb 3F11 after treatment with neuraminidase (<1% of maximum binding before neuraminidase as compared with 53 to 100% of maximum binding after neuraminidase). The L5, L7, and L8 LOS showed small or moderate increases in the binding of MAb 3F11 after enzyme treatment. MAb 06B4 also showed minimal binding to the L2 and L3 LOS but bound well to the L4 LOS; a marked increase in binding occurred after treatment of these LOS with neuraminidase. In numerous studies with native LOS (non-enzyme treated), we have noted that the L2, L3, and L4 LOS often are negative for binding of MAb 3F11 but vary in their binding of MAb 06B4 (data not shown).

Nitrocellulose-immobilized LOS modified by neuraminidase. Meningococcal LOS that showed a significant increase in the expression of the MAb 3F11-defined epitope after neuraminidase treatment (L2, L3, and L4; Table 1) were separated by SDS-PAGE, transferred to nitrocellulose, treated with neuraminidase, and tested with MAb 3F11. For some of the strains, an OMC antigen was also tested to represent a more native form of the antigen (Fig. 1). Both purified LOS and OMC preparations of strains 35E, 6275, and 89I showed greater binding of MAb 3F11 after neuraminidase treatment (panel C) than did control LOS treated only with PBS (panel B). Untreated LOS of strains 35E, 6275, and 89I bound MAb 3F11 slightly (panel B, lanes 1 to 6), in contrast to an absence of binding in SPRIA. For the strain 6275 LOS sample shown in lane 4, only 0.05 μ g of LOS was applied, as compared with 1 μ g for the other LOS samples. This relatively small amount of LOS reflected the absence of MAb binding to the untreated LOS (panel B, lane 4); however, MAbs bound even to approximately 0.05 μ g of the neuraminidase-treated LOS (panel C, lane 4). Untreated LOS of strains M981 and M978 bound MAb 3F11 (Table 1 and Fig. 1B, lanes 7 to 9), but the binding was increased after neuraminidase treatment of the LOS (Table 1 and Fig. 1C, lanes 7 to 9). Of the untreated LOS that did not bind MAb 3F11 in SPRIA (Table 1, strains 35E, 6275, and 89I), all bound MAb 3F11 to a component of approximately 4.5 kDa after being treated with neuraminidase. MAb 3F11 bound to two components of M981 LOS of approximately 4.0 and 5.0 kDa and to a component of M978 LOS slightly larger than

FIG. 1. Binding of MAb 3F11 to meningococcal LOS. Meningococcal prototype LOS were separated by SDS-PAGE, transferred to nitrocellulose, treated with purified neuraminidase or PBS, and treated with MAb 3F11 and 125 I-labeled secondary antibody. (A) silver stain. (B) Immunoblot, PBS treated. (C) Immunoblot, neuraminidase treated. Lanes: 1, 35E OMC; 2, 35E LOS; 3, 6275 OMC; 4, 6275 LOS; 5, 89I OMC; 6, 89I LOS; 7, M981 OMC; 8, M981 LOS; 9, 978 LOS. Approximately 1 μ g of LOS was used for each of the samples, except for a 0.05- μ g sample for 6275 LOS (lane 4). The arrowheads in panel A point to the LOS component that binds MAb 3F11. The arrowheads in panels B and C (lanes 8) point to aggregates (dimers or trimers) of LOS that bind MAb 3F11.

the 4.0-kDa component. Some of the immobilized LOS also bound MAb 3F11 to a component (or mixture of components) detected as a diffuse smear in immunoblots (Fig. 1C, lanes 1 to 3 and 6 to 8). The smearing in the enzyme-treated samples could have been due to increased epitope expression on LOS dimer or trimer aggregates (Fig. 1A and B, lane 8) and/or the presence of other forms of sialylated LOS that do not resolve into defined bands and do not stain well with silver.

Shift in the LOS component(s) after neuraminidase treatment. In SDS-PAGE, a silver-stained band of neuraminidase-treated and SDS-PAGE-separated LOS of strains 35E and 89I migrated at a slightly lower mass (Fig. 2, lanes 2 and 4) than did that of non-neuraminidase-treated LOS (Fig. 2, lanes 1 and 3). These results indicated that neuraminidase cleaved the high-mass component (Fig. 2, top arrow) of these LOS and that the resulting product migrated to an identical, or very similar, mass as did the lower-mass component (Fig. 2, bottom arrow).

Shift in the migration of LOS from organisms grown with CMP-NANA. Strains 35E, 6275, and 89I were grown in the absence and presence of CMP-NANA, and the organisms were tested for expression of the MAb 3F11-defined epitope (Fig. 3). Silver stains of SDS-PAGE-separated proteinase K lysates of strains 35E, 6275, and 89I showed two closely migrating bands (panel A, CMP-NANA -). The same strains grown with CMP-NANA (panel A, CMP-NANA +) had LOS that migrated predominately as a component with a mass similar to that of the high-mass component of strains grown without CMP-NANA. MAb 3F11 bound to the lower-mass LOS component of the untreated organisms but less well to the same component of the CMP-NANA-treated organisms (panel B). MAb 06B4 bound to LOS of both the

FIG. 2. Shift in SDS-PAGE migration of neuraminidase-treated meningococcal LOS. Purified LOS of meningococcal strains 35E (lanes 1 and 2) and 89I (lanes 3 and 4) were treated with buffer (PBS) (lanes 1 and 3) or neuraminidase (lanes 2 and 4), separated by SDS-PAGE, and silver stained. The arrowheads point to the 4.5-kDa (MAb 3F11 and 06B4 binding-positive) band and the 4.8-kDa band sensitive to neuraminidase.

treated and the untreated organisms but, similarly, bound better to LOS of untreated organisms (panel C). The relatively low level of binding to the CMP-NANA-treated LOS could have been due to the presence of residual unsialylated lower-mass LOS. These results indicated that the sialic acid

FIG. 3. Binding of MABs 3F11 and 06B4 to meningococcal LOS of strains grown in the presence or absence of CMP-NANA. Strains 35E, 6275, and 89I were grown on medium either with (+) or without (-) CMP-NANA, and proteinase K lysates of the organisms were separated by SDS-PAGE, transferred to nitrocellulose, and tested with MAb 3F11 (B) or MAb 06B4 (C). A duplicate silver-stained gel is shown in panel A. The arrowheads show the two closely migrating bands present in the LOS of strains grown in the absence of CMP-NANA. The lower band has a mass of approximately 4.5 kDa and binds MABs 3F11 and 06B4.

FIG. 4. Incorporation of radiolabeled CMP-NANA into LOS. Meningococcal strains 126E (Mab 3F11 binding negative) and 6275 (Mab 3F11 binding positive) were grown on solid medium with or without CMP-[14 C]NANA, collected, treated with proteinase K, and separated by SDS-PAGE. The gels were silver stained (A), dried, and incubated with film for autoradiography (B). LOS shown in the first and second lanes of each panel are from strains grown in the absence or presence of CMP-[14 C]NANA, respectively. The arrowheads point to the approximate location of the strain 6275 LOS component that has incorporated radioactivity.

is on the high-mass component of these LOS and that group B and C strains grown in the presence of exogenous CMP-NANA have more complete sialylation of their LOS.

Incorporation of CMP-[14 C]NANA into 4.5-kDa-component-positive LOS. To determine whether the incorporation of sialic acid was responsible for the shift in mass in the LOS, we grew strains 6275 (positive for the component 4.5-kDa and for Mab 3F11 binding) and 126E (negative for both) on plates in the presence of CMP-[14 C]NANA. Figure 4 shows the silver stain (panel A) and autoradiograph (panel B) of LOS from these organisms. Strain 6275 incorporated radioactive NANA in its LOS (panel B), but strain 126E did not. In addition, there was no obvious difference in the LOS profiles of organisms grown with and without CMP-NANA (panel A).

IEM. We tested neuraminidase-treated meningococci by IEM to determine whether the exposed 3F11 epitope was surface expressed. Figure 5 shows the binding of Mab 3F11

FIG. 5. IEM of strain 89I treated with intact or heat-inactivated neuraminidase. Strain 89I was incubated with either purified intact (B) or heat-inactivated purified neuraminidase (A), washed, fixed, and treated with Mab 3F11 and gold-conjugated secondary antibody (see Materials and Methods).

to strain 89I in whole-cell mounts of organisms treated with either neuraminidase (panel B) or heat-inactivated neuraminidase (panel A). Organisms treated with neuraminidase circumferentially bound Mab (panel B), in contrast to the minimal binding with organisms treated with inactivated neuraminidase (panel A). These results indicated that Mab 3F11 bound to a surface-expressed epitope of the desialylated LOS.

Detection of sialic acid in products of neuraminidase-treated strain 6275 LOS by strong anion-exchange chromatography with pulsed amperometric detection. To confirm that the neuraminidase-sensitive activity in LOS was due to sialic acid, we treated purified strain 6275 LOS with either intact or heat-inactivated neuraminidase (100°C) and analyzed it by anion-exchange chromatography. (In preliminary anion-exchange experiments with neuraminidase-treated LOS, we noted that neuraminidase from one company [Sigma] cleaved both sialic acid and glucose from strain 6275 LOS [data not shown]; therefore, for this study, we used purified neuraminidase [Genzyme].) No neutral or amino sugars were detected in the sample prepared from neuraminidase-treated LOS, and sialic acid (retention time, 6.9 min) was the only carbohydrate component detected. The sample and an authentic sialic acid standard coeluted. A mixture of sialic acid and 2-keto-3-deoxyoctulosonic acid (KDO) standards was clearly resolved, with a retention time for the KDO standard of approximately 15.6 min. The neuraminidase-treated LOS sample had a single peak at a retention time equivalent to that of sialic acid. An identical sample of LOS treated with heat-inactivated neuraminidase (100°C for 30 min) had no detectable peaks (data not shown). The sialic acid in the LOS was calculated to be 15.3 μ g/mg of LOS (1.5% sialic acid).

LSIMS analysis of partially deacylated strain 6275 LOS. Strain 6275 LOS that was sequentially de-O-acylated and dephosphorylated was analyzed by LSIMS to confirm the presence of a covalent linkage between the sialic acid and the LOS. The peak at m/z 2508 is consistent with the $(M - H)^-$ ion for de-O-acylated lipid A attached to an OS of the composition $\text{Hex}_3\text{HexNAc}_2\text{Hep}_2\text{KDO}_2$ (Fig. 6A). However, the peak at m/z 2799 $(M - H)^-$ has an additional 291 Da and corresponds to the addition of sialic acid (*N*-acetylneuraminic acid). The molecular ion at m/z 871 corresponds to a monophosphorylated lipid A moiety that was not completely cleaved by dephosphorylation, whereas the $(M - H)^-$ ion at m/z 2799 contains no phosphate moieties. The mass of the peak at m/z 1231 (Y_3) is consistent with a reducing terminal fragment containing two KDO moieties plus the nonphosphorylated lipid A moiety (791 Da). The peak at m/z 1011 (Y_3 minus KDO) corresponds to a single KDO moiety plus the nonphosphorylated lipid A moiety. The difference between the molecular ions at m/z 871 and m/z 1273 could be explained by the addition of KDO and one lauric acid. Several smaller peaks at low masses (m/z 977, 1169, 1389) could not be readily explained in terms of the structures shown and may have been the result of heterogeneity in the LOS or artifacts of the chemical modifications (18). At this stage in our structural analysis of strain 6275 LOS, we do not know whether the two KDOs are branched or linked in series. LSIMS analysis of a mutant gonococcal LOS of strain FA5100 suggests that one of the two KDOs is terminal and nonreducing (47) and, therefore, is consistent with the branched structure shown in Fig. 6A.

LSIMS analysis of the acid-hydrolyzed OS moiety after conversion to its 4-BPH derivative is shown in Fig. 6B. This OS no longer contains sialic acid, because it is quantitatively

hydrolyzed by the conditions used to cleave lipid A. A dominant $(M - H)^-$ peak observed at m/z 1659 corresponds to $\text{Hex}_3\text{HexNAc}_2\text{Hep}_2\text{KDO-BPH}$. The $(M - H)^-$ ion at m/z 1701, which is 42 Da larger than the $(M - H)^-$ ion at m/z 1659, likely corresponds to the addition of an O-acetate moiety that was partially removed by dephosphorylation of the OS. The peak at m/z 998 is an X-type ion formed by ring cleavage of the heptose attached to the KDO moiety. The peak at m/z 1335, however, differs from the molecular ion peak at m/z 1659 by the loss of two hexose residues. Since no simple cleavage could result in the loss of two hexoses from the structure shown in Fig. 6B, we speculate that this ion may originate from an additional OS structure present in this preparation.

DISCUSSION

We have shown that some strains of *N. meningitidis* have sialic acid in their LOS and that this sialic acid alters the expression of the epitopes defined by MAb 3F11 and, to a lesser extent, MAb 06B4. The presence of sialic acid in some meningococcal LOS is indicated by the following observations. (i) LOS epitopes are exposed by neuraminidase (Table 1 and Fig. 1). (ii) Neuraminidase-treated LOS runs faster in SDS-PAGE than does untreated LOS (Fig. 2). (iii) LOS from organisms grown with CMP-NANA runs slower in SDS-PAGE than does LOS from untreated organisms (Fig. 3). (iv) Organisms grown with CMP- ^{14}C NANA incorporate radioactivity only in their LOS (Fig. 4). (v) The product released from neuraminidase-treated LOS is as sialic acid. (vi) LSIMS analysis of partially deacylated LOS reveals a fragmentation pattern characteristic of a sialylated molecule (Fig. 6).

Seven of eight group B and C prototype LOS strains (Table 1) have an LOS component in the range of 4.5 kDa (L2 to L8); except for strain M992 (L6), the LOS of these strains showed at least a partial increase in the binding of MAb 3F11 or 06B4 after treatment with neuraminidase. These results indicate that the 4.5-kDa component is strongly associated with 3F11 and 06B4 epitope expression. The MAb 3F11 binding-negative, MAb 06B4 binding-positive epitope expression on the L4 LOS before treatment with neuraminidase was consistent with our previous results for meningococcal LOS (30, 35). However, the lower expression of the 3F11 (on L5 and L7 LOS) and 06B4 (on L2 and L3 LOS) epitopes in this study than in the previous studies (30, 35) indicated that epitope expression could vary. We have noted differences in the expression of these two epitopes depending on whether whole organisms, OMCs, or purified LOS are used as antigens (data not shown), possibly reflecting the labile nature of the putatively sialylated epitopes during antigen preparation. From the results of this study and other studies (30, 35), we predict that many group B and C strains possess the potential to express the 3F11 and 06B4 epitopes but that, on some strains, the epitopes are covered by sialic acid and that, on other strains, the unsialylated epitopes are expressed only at very low levels (Table 1).

Careful inspection of the silver stain profiles of the pre-neuraminidase-treated LOS in Fig. 2 and 3 shows that they are composed of two major and closely migrating components. The high-mass component (approximately 4.8 kDa) is the one that contains sialic acid. Group B and C strains grown in medium supplemented with CMP-NANA, however, showed increased expression of this LOS component (Fig. 3) associated with decreased expression of the lower-mass component (approximately 4.5 kDa) and of the 3F11 and 06B4 epitopes. As noted, these results differ from the SPRIA results for some endogenously sialylated LOS, which showed an absence of 3F11 epitope expression with strong 06B4 epitope expression (Table 1) (30, 35). One explanation for this difference could be that since only endogenous (organism-synthesized) CMP-NANA is available in nonsupplemented medium, increased levels of exogenously sialylated LOS (Fig. 3, CMP-NANA+) as compared with endogenously sialylated LOS (Fig. 3, CMP-NANA-) may reflect a disruption or alteration in the regulation of sialylation of the capsule and/or LOS. The complexity of this regulation is reflected by the multiple enzymes required for the synthesis of the sialic acid capsule of group B meningococci ($\alpha 2 \rightarrow 8$ -linked sialic acid homopolymer). For capsule synthesis, group B meningococci have an *N*-acetylneuraminic acid-condensing enzyme, a CMP-NANA synthetase, a CMP-NANA hydrolase, and a chain-elongating sialyltransferase (40). It has been hypothesized that the CMP-NANA hydrolase regulates the capsular sialyltransferase and that the CMP-NANA hydrolase is regulated by the concentration of free CMP (40). Our studies suggest that group B and C organisms also have an LOS-specific sialyltransferase. Therefore, it will be important to determine whether the previously described sialic acid-associated enzyme activities and their regulation are related to the LOS-specific sialyltransferase activity.

Although the presence of sialic acid in meningococcal glycolipids has been reported by numerous investigators (19, 23, 27, 28, 41), it should be noted that the results of these and other studies (10, 20, 42, 45, 46, 49, 51, 58) were derived from material cleaved under acidic conditions that could degrade possible constituents of LOS (e.g., sialic acid, fucose, and phosphate) (Fig. 6B). Therefore, alternative methods of chemical analysis may be needed to obtain a more complete picture of the native LOS structure. Two alternatives that we used for the studies reported here and that may prove useful for the chemical analysis of other neisserial LOS were anion-exchange chromatography of neuraminidase-treated LOS and mass spectrometric analysis of partially intact LOS.

Analysis of a neuraminidase-treated LOS by strong anion-exchange chromatography indicated that strain 6275 LOS was composed of approximately 1.5% sialic acid. The approximately 300- to 400-Da difference in the migration of the LOS after neuraminidase treatment and/or because of growth in CMP-NANA (Fig. 2 and 3) suggests that there is one sialic acid per sialylated LOS molecule. Because the

FIG. 6. LSIMS analysis of de-O-acylated and dephosphorylated LOS from *N. meningitidis* 6275. (A) De-O-acylated and dephosphorylated LOS (partial negative-ion spectrum). (B) Acid-hydrolyzed and dephosphorylated OS derivatized with 4-BPH (negative-ion spectrum). The tentative structures shown are those most consistent with the data and structures reported for meningococcal LOS (29, 42, 53). Exact masses are reported for the ^{12}C -containing isotopically pure component of the molecular ion or fragment ion. In panel A, the mass of the de-O-acylated lipid A moiety agrees with that of the corresponding lipid found in *N. gonorrhoeae*, as does that of the off-scale molecular ion peak at m/z 871 (see inset) for the de-O-acylated lipid A containing one phosphate (53). In panel B, the loss of 4-BPH (147 Da) from the OS is responsible for the ion at m/z 1512. The nomenclature used in the assignment of the Y-type ions is from Domon and Costello (12).

strain used as the source of the LOS (group B strain 6275) also produced a sialic acid capsule, it was important to confirm that the neuraminidase-sensitive sialic acid in the LOS preparation was covalently bound to the LOS. LSIMS of partially deacylated LOS allowed identification of the peaks for the molecular ion $[(M - H)^-]$ ion of m/z 2799, for a lower-mass ion (m/z 2508) corresponding to the release of sialic acid, and for a third ion (m/z 2346) corresponding to the release of sialic acid and hexose (Fig. 6A). Although mass spectrometric analysis of intact (nonhydrolyzed and nonderivatized) LOS has been relatively unsuccessful, partial deacylation of LOS by hydrazinolysis has been used for nuclear magnetic resonance analysis (24). Hydrazinolysis of LOS releases O-linked fatty acids from the lipid A moiety (24). For strain 6275 LOS, this treatment resulted in a decrease in the hydrophobicity of the LOS as compared with that of the untreated LOS (data not shown) and an increase in the sputtering efficiency of the molecular ion and other fragments (Fig. 6A).

If it is assumed that the LOS of strains 6275 and 89I are composed of a single component with a mass of 4.8 kDa (4.5-kDa component plus approximately 0.3 kDa for *N*-acetylneuraminic acid) and that each LOS molecule has one sialic acid, then the sialic acid in 1 mg of LOS would be approximately 64 μ g (6.4%). This value is somewhat higher than the approximately 4% sialic acid that we measured for strain 6275 LOS by a resorcinol assay (52) (data not shown) and the 1.5% sialic acid that we measured by anion-exchange chromatography. This lower experimental value could reflect heterogeneous sialylation of LOS (Fig. 2), incomplete sialylation of individual LOS components, and/or incomplete neuraminidase cleavage of sialic acid from LOS.

More complete chemical studies will be required to characterize the exact structure of the sialylated LOS. Jennings and co-workers used nuclear magnetic resonance to study acid-released OS of LOS and reported that the terminal tetrasaccharide of the nonreducing end of the LOS of an L2 strain (17, 26), an L3, 7, 9 strain (29), and an L5 strain (42) is composed of lacto-*N*-neotetraose (Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc). Also consistent with this structure are a common structure deduced by fast-atom-bombardment spectrometry analysis of acid-released OS of meningococcal strains 89I (L4) and M981 (L5) (10) and part of the preliminary structure described for the L3 LOS in this study (Fig. 6: Hex-HexNAc-Hex-Hex-). Also, the presence of an O-acetate moiety on the nonreducing terminal HexNAc of strain 6275 LOS (Fig. 6B) is in agreement with the results of previous studies of *N. meningitidis* (10, 11) and *N. gonorrhoeae* (47) LOS.

The presence of lacto-*N*-neotetraose in neisserial LOS is notable because it is also present in paragloboside, a glycosphingolipid precursor of the major human blood group antigens (22) and of some sialylated glycosphingolipids (gangliosides) in human cells (16, 33). However, in preliminary studies done to determine the nature of the sialic acid linkage on LOS, we found that MAbs specific for sialylated carbohydrates of mammalian glycoconjugates, such as sialic acid, bound α 2 $\rightarrow$ 3 or α 2 $\rightarrow$ 6 to lactosamine (Gal β 1 $\rightarrow$ 4GlcNAc) or α 2 $\rightarrow$ 3 to Gal β 1 $\rightarrow$ 3GalNAc, did not bind to sialylated LOS of either meningococci (data not shown) or gonococci (36). These results suggested that the sialylated LOS have a structure different from those recognized by these MAbs or that the LOS have the same structure but not in a recognizable conformation (59).

What might be the functions of sialylated LOS for meningococci in vivo? One function could be to increase the serum

resistance of the strains. Although it has been shown that gonococci grown in the presence of CMP-NANA become more resistant to human serum (43, 44), in contrast, recent studies have shown no effect of CMP-NANA on the serum resistance of group A, B, Y, and Z and noncapsulated meningococci (14). This difference may be related to the fact that group B, C, W, and Y meningococci synthesize CMP-NANA, but gonococci do not (7, 15, 55). In light of the results that we have described, it would be interesting to know whether the meningococcal strains that were tested in the serum resistance studies had sialylated LOS. Although serum-sensitive meningococcal strains having "completely" sialylated LOS would suggest a limited role for sialylation in meningococcal serum sensitivity, it is possible that sialylated neisserial LOS are involved in mechanisms of pathogenesis other than serum resistance. For example, gonococci present in male urethral exudates also appear to have sialylated LOS (4), possibly reflecting the availability of sufficient CMP-NANA in the environment of the urethral epithelium and associated neutrophils and at this phase of the infectious process, in contrast to the absence of free CMP-NANA in human plasma (8). Similarly, it will be important to determine whether meningococcal LOS also are sialylated in vivo and, if so, where the sialylated LOS exist (nasopharynx, blood, cerebrospinal fluid). Other biological functions of sialic acid that are relevant to concepts of meningococcal pathogenesis and host and tissue specificity are lack of recognition by mucosal antibody, complement, phagocytes (48), and terminal galactose-specific lectins (6) and, conversely, recognition by sialic acid-specific lectins (2). Whether the differences observed for in vitro LOS expression accurately reflect any in vivo mechanisms of meningococcal pathogenesis will be an area for future studies.

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