IN VITRO AND IN VIVO MODIFICATION OF *NEISSERIA GONORRHOEAE* LIPOOLIGOSACCHARIDE EPITOPE STRUCTURE BY SIALYLATION

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Previous studies have shown that human serum, guinea pig and human red cells, and human white cells contain low and high M_r substances that induce gonococcal strains to become serum resistant (1–4) and change lipooligosaccharide (LOS)[1] pattern (5, 6). In more recent studies, the same investigators have shown that the low M_r substance in blood is cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA) or a related compound (7, 8). These studies suggest that in vivo, sufficient concentrations of CMP-NANA might induce serum resistance by sialylation of LOS. Because gonococci are not able to synthesize CMP-NANA and it is not present in the usual media, previous in vitro studies of gonococcal LOS may have dealt with different LOS structures than those that occur in vivo.

Each gonococcal strain makes multiple types of LOS (9–11), and the physical (M_r) and antigenic heterogeneity of a strain's LOS reflects physicochemical differences in their glycan moieties (10, 12). mAbs 3F11 and 06B4 identify epitopes on meningococcal and gonococcal LOS that are immunochemically similar to Galβ1→4GlcNAc-containing molecules present in human erythrocytes and on other human cells (13). These epitopes are conserved on gonococcal LOS (11, 14) and are variably expressed

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[1] *Abbreviations used in this report:* CMP-NANA, cytidine monophospho-*N*-acetylneuraminic acid; IEM, immunoelectronmicroscopy; LOS, lipooligosaccharide; OMC, outer membrane complex; SPRIA, solid phase RIA; STases, sialyltransferases.

on the LOS of meningococci and *Neisseria lactamica* (15). It is possible that Galβ1→4GlcNAc structures are potential targets of LOS sialylation, because these same structures also often are sialylated in mammalian glycoproteins (16) and in some glycosphingolipids (17).

In this report, we identify the LOS component and the epitope that is sialylated by CMP-NANA, show the effect of CMP-NANA on the antigenicity of these molecules, and show that LOS of gonococci incubated in human neutrophils (polymorphonuclear leukocytes [PMNs]) may be similarly sialylated.

Materials and Methods

Bacterial Strains. *N. gonorrhoeae* strains F62, DOV, and WR213 were kindly provided by Dr. Herman Schneider (Walter Reed Army Institute of Research, Washington, DC) (10, 11, 18). Strain 1291 and its pyocin-selected mutants 1291A and 1291C (19), strain 8551 (20), and strains 15253 and 15433, kindly provided by Dr. Peter A. Rice (Boston University, Boston, MA), have been described previously. Strains 15253 and 15433 have LOS components that are all <4.0 kD and do not bind mAbs 3F11 and 06B4 (21). A colony blot assay was done with mAb 3F11 to select from strains 15253 and 15433 population variants that made higher M_r LOS (22).

LOS and Outer Membrane Complex (OMC). LOS (18) and OMC (23) antigens were prepared as described.

mAbs. The preparation and characterization of mouse anti-LOS mAbs 3F11 (11, 13, 14), 06B4 (11, 13), 1-1-M (11), 6B7 (19), and 4C4 (24) have been described. mAb 2C3 was generated after immunization of mice with intact *N. gonorrhoeae*; it is an IgG2b isotype. It recognizes a surface-exposed H.8 protein epitope on >90% of gonococcal strains and is highly specific for pathogenic *Neisseria* (25, 26). mAbs were kindly provided by Dr. Peter A. Rice (2C3; Boston University), Dr. Julie Westerink (6B7 and 4C4; State University of New York at Buffalo, Buffalo, NY), Dr. Shinzo Isojima (H6-3C4; Hyogo Medical College, Hyogo, Japan), and Dr. Senitroh Hakomori (IB9 and NUH2; The Biomembrane Institute, Seattle, WA). mAb IB9 recognizes NeuNAcα2→6Gal residues on lacto series gangliosides and on glycoproteins (27). mAb NUH2 recognizes an epitope having two identical NeuNAcα2→3Galβ1→4GlcNAc structures, β1→3 and β1→6 linked to Galβ1→4GlcNAc (28, 29). mAb H6-3C4 is a human mAb that immobilizes human sperm and recognizes repetitive *N*-acetyllactosamine structures that are either sialylated or nonsialylated (28). mAb MC-813-70 was obtained from the Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD; it recognizes NeuNAcα2→3Galβ1→3GalNAcβ1→R found on mouse stage-specific embryonic antigen 4 (30). Each of the mAbs was tested in a TLC immunostaining assay (31) with mammalian glycosphingolipids to assure that the appropriate activity was present.

SDS-PAGE and Immunoblotting. LOS samples were separated through polyacrylamide slab gels by the method of Laemmli (32). In one experiment, the LOS of strain 1291 was separated using a tricine SDS-PAGE gel system in order to improve resolution of individual components (33). Immunoblotting (11) and silver staining (34) were done as described previously. The M_rs of LOS components were estimated by reference to the LOS of meningococcal strain 8032, which has six LOS components with different M_rs designated as 5.4, 5.1, 4.5, 4.0, 3.6, and 3.2 (24).

Solid Phase Radioimmunoassay (SPRIA). SPRIA was performed as described previously (11, 35). Stock solutions of LOSs or OMCs were diluted in Dulbecco PBS modified to contain 50 mM $MgCl_2$, and 50 mg/ml solution of the LOS or OMC was used to sensitize polyvinyl microtiter wells. In some assays, whole organisms were used as antigens (36).

Neuraminidase Treatment of Immobilized LOS. We used two different preparations of neuraminidase for these studies. For initial SPRIA studies of the effect of neuraminidase on LOS antigens, we used *Clostridium perfringens* neuraminidase (type V; Sigma Chemical, St. Louis, MO). For confirmation of results, we used a purified streptococcal neuraminidase (Genzyme, Boston, MA). This preparation contains other relevant glycosidases at levels <0.033% by activity.

After sensitization of polyvinyl microtiter wells with LOS (25 μl of 25-μg/ml concentration) for 1 h, the wells were washed with PBS, pH 6, and then 25 μl of various concentrations of neuraminidase (0.01–10 mU/ml, diluted in PBS, pH 6) were 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 treated with filler to block nonspecific binding. Mouse mAb and ^{125}I-labeled goat anti-mouse IgG or IgM were added as described previously (11).

Growth of Organisms in Media with CMP-NANA. *N. gonorrhoeae* strain 1291, and its pyocin-resistant mutant strains 1291A and 1291C, were grown overnight on GC agar base (Difco Laboratories Inc., Detroit, MI) containing 1% supplement (37), then used to inoculate the defined liquid GC media of Morse and Bartenstein (38) or liquid or solid GC media (18, 37) with or without CMP-NANA (Sigma Chemical Co.) (8). Organisms were removed at various time points during growth. Proteinase K (Sigma Chemical Co.) lysates were prepared from these samples (39) for SDS-PAGE, silver staining, and immunoblot analyses. For strains 1291 and F62, we found that concentrations of CMP-NANA >50 μg/ml in both media resulted in maximal modification of the mAb-defined epitopes on the 4.5-kD LOS component. For strains grown on solid GC agar, organisms were collected, washed once in PBS, and diluted to a concentration of ~0.2×10^6 CFU/ml. A 0.5-ml volume of diluted organisms was mixed with an equal volume of a 500-μg/ml solution of CMP-NANA in PBS, pH 7.4, and spread on GC agar plates. After the liquid evaporated, the plates were incubated in 5% CO_2 for 18 h at 37°C. Organisms were removed from the surface of the medium and suspended in Dulbecco PBS (OD_{620} = 0.2) for whole cell SPRIA (36) or in SDS-PAGE sample buffer for treatment with proteinase K (39).

Immunoelectronmicroscopy (IEM). For in vitro studies, IEM was as described (40) on *N. gonorrhoeae* strain 1291 that had been grown for 6 h in defined medium with and without 100 μg/ml CMP-NANA. Before IEM, an aliquot of these cells was tested by SDS-PAGE and electroblot studies to insure that CMP-NANA had reacted with the LOS. The specimen was suspended in 1–2% glutaraldehyde and 0.1 M cacodylate buffer, pH 7.4, then incubated for 2 h, then embedded in LR-white embedding solution (London Resin Co., Ltd., London, UK) as described (41). Sections were incubated sequentially with various combinations of mAb 06B4 (10 μg/ml), colloidal gold conjugated to a goat anti-mouse IgM antibody (Janssen Biotechnology, Piscataway, NJ), and neuraminidase (2.0 U/ml, type VI; Sigma Chemical Co.; 1 h at 37°C). The sections were washed between each step. The specimens were examined with a transmission electron microscope (1A; Siemens Elmskop) at 80-kV accelerating voltage.

Strain 1291 also was incubated with human PMN, and LOS epitope expression was examined by IEM. Freshly obtained whole human blood was collected in siliconized, heparinized blood tubes. Dextran in 0.15 M NaCl was added to a final concentration of 1%. After sedimentation for 45–60 min at room temperature, the upper plasma layer was transferred to a siliconized tube and 2 vol of 0.87% ammonium chloride was added to remove residual erythrocytes. The PMNs were sedimented at 100 *g* for 10 min and washed twice with sterile Dulbecco's PBS without Ca^{2+} or Mg^{2+}, but containing 0.1% glucose, pH 7.4. *N. gonorrhoeae* strain 1291 (p^-, op^+) was grown for 18 h on GC agar medium (18), suspended in PBS (OD_{660} = 0.2, ~10^8 cells/ml), added to suspended PMNs at a ratio of 10 gonococci/1 PMN, and then gently rotated at 37°C. PMNs were fixed either before addition of strain 1291 or at various times up to 1 h after addition of 1291. Glutaraldehyde was added to 1% and the fixed specimens were kept at 4°C until IEM studies were done as described above.

Treatment of Purified Gonococcal Strain F62 LOS with a 0.5% Triton X-100 Extract of the Organisms. Gonococcal strain F62 was added to liquid GC medium (18) to an OD_{620} of 0.1 and grown for 4 h at 37°C. Organisms were pelleted and washed once with PBS. The pelleted organisms were suspended in 0.5% Triton X-100, the suspension was sonicated (Branson Sonic Power Co., Danbury, CT) for 15 s, then centrifuged in a microfuge. The supernatant was collected and stored at −20°C until further use. The reaction mixture consisted of 20 μl of F62 LOS (1 mg/ml in distilled water), 10 μl 0.5M sodium phosphate buffer (pH 6.8), 10 μl cytidine 5'-monophospho-[^{14}C]-*N*-acetylneuraminic acid (0.25 μCi, ~0.6 μg; Amersham Corp., Arlington Heights, IL), and 50 μl of 0.5% Triton X-100 extract (~25 μg protein). For negative controls, 50 μl of 0.5% Triton X-100 buffer was added in place of the extract.

The samples were added to microfuge tubes and incubated for 14 h at 37°C. After incubation, 50 μl of SDS-PAGE gel sample buffer (see SDS-PAGE section) was added, the samples were incubated at 37°C for 10 min, then the samples were run in SDS-PAGE. The gel was silver stained (34), dried, then incubated in Kodak X-Omatic cassettes with regular intensifying screens and Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) at −70°C for 7 d. The exposed film was processed for viewing.

Treatment of Purified Gonococcal LOS with α2→3 and α2→6 Sialyltransferases (STases). Rat liver CMP-NANA:Galβ1→4GlcNAcα2→6 STase and porcine submaxillary gland CMP-NANA:βGalα2→3 STase enzymes (Genzyme) (no detectable α2→6 STase activity in the α2→3 STase and no detectable α2→3 STase activity in the α2→6 STase, according to the manufacturer) were assayed with F62 LOS. The reaction mixture for the assay consisted of 10 μl of a 4-mg/ml suspension of F62 LOS, 20 μl BSA (2.5 mg/ml), 10 μl detergent buffer (2.5% Triton X-100 in 0.25 M sodium cacodylate, pH 6.0), 5 μl cytidine 5'-monophospho-[^{14}C]-*N*-acetylneuraminic acid (0.125 μCi, ~0.3 μg; Amersham Corp.), and 5 μl enzyme (120–170 mU/ml). For negative controls, 5 μl of detergent buffer was added in place of enzyme; for positive controls, 10 μl of asialofetuin (0.8 mg/ml) was added in place of LOS. The samples were added to microfuge tubes and incubated for 14 h at 37°C. The samples were run in SDS-PAGE and the dried gels autoradiographed (8 d) as described above.

Results

Identification of the Component Modified by CMP-NANA. To define the LOS component that was modified by the addition of NANA, gonococcal strains 1291 and 1291A were grown in a defined medium in the presence and absence of CMP-NANA. Fig. 1 shows the silver-stained tricine SDS-PAGE gel of strain 1291 LOS (lanes *a* and *b*) and the LOS of its pyocin-resistant mutant, strain 1291A (lanes *c* and *d*), grown in the presence (lanes *b* and *d*) and absence (lanes *a* and *c*) of CMP-NANA. A 4.5-kD LOS component of strain 1291 expresses the 3F11 and 06B4 epitopes, whereas strain 1291A does not have this component (19). Fig. 1, *b*, shows the shift in the M_r of the 4.5-kD band of 1291 to an M_r of ~4.9 kD. There was no shift in the major band of 1291A in the presence of CMP-NANA (Fig. 1, lane *d*). Note that cultures of strain 1291A contain a small population of revertant bacteria of the parent 1291; this is seen as lightly stained LOS at the same location as seen with strain 1291 LOS (Fig. 1, lane *c*). This band (Fig. 1, lane *d*) behaves identically to the band of parental 1291 LOS, confirming the ability of the revertants to use the active CMP-NANA in the medium.

We also tested another pyocin-resistant mutant of strain 1291, 1291C, which, due to reversion, had approximately equal amounts of 1291 parental strain 4.5-kD LOS and the 3.6-kD LOS characteristic of strain 1291 C. When this strain was grown in the presence of CMP-NANA, only the 4.5-kD band shifted M_r (data not shown). Electroblot analysis with mAbs 4C4 and 6B7, which recognize epitopes on the 3.6-kD LOS of 1291 C, indicated that binding to these epitopes was not modified by the CMP-NANA. Similar immunoblots with mAb 06B4 showed that it reacted with the 4.5-kD LOS of 1291 while the 1291 LOS from the CMP-NANA-treated organisms ran at a higher M_r and showed decreased binding of mAb 06B4.

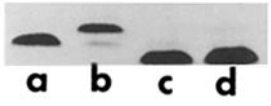

FIGURE 1. Effect of CMP-NANA on the migration of gonococcal strains 1291 and 1291A LOS. Proteinase K lysates of strains 1291 (lanes *A* and *B*) and 1291A (lanes *C* and *D*) grown in the presence (lanes *B* and *D*) and absence (lanes *A* and *C*) of CMP-NANA were separated by tricine SDS-PAGE then silver stained as described (see Materials and Methods).

TABLE I
Expression of mAb-defined LOS Epitopes on CMP-NANA-treated Gonococci

Strain	Neuraminidase	CPM bound with mAb:* 3F11	1-1-M
		cpm	*cpm*
F62	–	3,841 (16)‡	83,443 (100)
F62	+	23,071	76,723
WR213	–	17,839 (31)	77,041 (95)
WR213	+	55,963	81,019
DOV	–	9,577 (18)	78,487 (94)
DOV	+	51,103	83,053

* A SPRIA was done using as antigen whole organisms grown on GC agar plates with CMP-NANA. After antigen sensitization, neuraminidase (Genzyme) in PBS (pH 6) or PBS alone was added and the plates were incubated for 2 h at 37°C. The wells then were washed and incubated sequentially with mAb and secondary antibody (see Materials and Methods). Data are presented as the cpm of ^{125}I secondary antibody bound.

‡ The numbers in parentheses indicate the percentage of cpm bound by the PBS-treated antigen compared with the neuraminidase-treated antigen.

Reversal of CMP-NANA Modification of LOS by Treatment with Neuraminidase. We used a SPRIA to test the expression of an epitope present on the 4.5-kD component of gonococcal LOS (defined by mAbs 3F11 or 06B4) and an epitope on a higher M_r component (defined by mAb 1-1-M). The data in Table I show decreased binding of mAb 3F11 to each of the three CMP-NANA-treated gonococcal strains. F62 and DOV LOS from CMP-NANA-treated organisms had similar increases in 3F11 expression after treatment with neuraminidase. In contrast, the binding of mAb 1-1-M to the LOS was not decreased by growth in the presence of CMP-NANA, nor was it modified by treatment with neuraminidase.

Modification of LOS at Various Times during Growth of F62 in CMP-NANA. To determine the rate of the change in CMP-NANA-altered epitope expression, we tested organisms grown for various times in liquid medium with 500 μg CMP-NANA/ml. We used strain F62 because it has two major components of ~4.5 and ~5.4 kD M_r that are well resolved by SDS-PAGE and because we have mAbs that bind separately to epitopes in the high M_r component (mAb 1-1-M) and to those in the low M_r component (mAbs 3F11 and 06B4). The results are shown in Fig. 2. The loss of the 4.5-

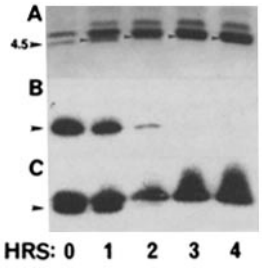

FIGURE 2. Modification of F62 LOS at various time intervals during growth in CMP-NANA. Gonococcal strain F62 was grown in GC liquid media containing 500 μg/ml CMP-NANA. Samples extracted at 0, 1, 2, 3, and 4 h were treated with proteinase K, then lysates were separated by SDS-PAGE and one gel was silver stained (*A*) and a duplicate gel was electroblotted and probed with mAb 3F11 and ^{125}I-labeled secondary antibody (*B*). After autoradiography, the immunoblot shown in *B* was probed with mAb 1-1-M and ^{125}I-labeled secondary antibody (*C*). The large arrows point to the 4.5-kD component of F62 LOS that binds mAb 3F11 and is modified by the CMP-NANA (*A–C*, 0 h), and the small arrows point to the modified component (*A*, 1–4 h). mAb 1-1-M binds to the higher component.

kD component (Fig. 2 *A*, see arrow) was almost complete at 2 h, and this loss was accompanied by both an increase in the size of the 4.5-kD component and the appearance of a component higher than the 5.4-kD component. In a subsequent experiment using organisms grown in media with 100 μg/ml CMP-NANA, the 4.5-kD component of CMP-NANA-modified LOS migrated just below the 5.4-kD component (data not shown). Thus, the increased density of staining below the major silver-stained band in Fig. 2 (5.4 kD at 0 h, ~4.9 kD at 1–4 h, see arrows) probably is a result of two closely migrating bands that are unresolved in this experiment and possibly due to the higher concentration of CMP-NANA in the growth medium. The binding of mAb 3F11 to the 4.5-kD component (Fig. 2 *B*) was decreased markedly by 2 h and was absent by 3 h, while the binding of mAb 1-1-M to the 5.4-kD component (Fig. 2, *C*) was not negatively affected by growth in the presence of CMP-NANA. In contrast to the 4.5-kD component, the 5.4-kD component exhibited an increased binding of mAb 1-1-M seen as a diffuse band in the 3- and 4-h samples.

Effect of CMP-NANA on 4.5-kD$^+$ (mAb 3F11$^+$) and 4.5-kD$^-$ (mAb 3F11$^-$) Gonococcal Strains. To determine whether the effect of CMP-NANA on LOS occurred with other gonococcal strains, eight 3F11$^+$ and four 3F11$^-$ strains were grown on GC agar containing CMP-[^{14}C]-NANA. The results are shown in Fig. 3. The silver stain showed that most of the LOS samples had multiple components (Fig. 3 *A*) and that the M_r of bands from 3F11$^-$ strains were lower than those of the 3F11$^+$ strains. However, only the 3F11$^+$ organisms incorporated any of the radioactivity, and they incorporated it into the 4.5-kD LOS component (Fig. 3 *B*, lanes *1* and *6–12*). In contrast, the 3F11$^-$ strains (Fig. 3, lanes *2–5*) did not incorporate the radioactivity. Although strains 15253v and 15433v are population variants of strains 15253 and 15433, both strains are designated as 3F11$^-$ strains because they reverted from 3F11$^+$ to 3F11$^-$ during passage. In a few of the 4.5-kD$^+$ LOSs, radioactivity also was incorporated into a higher or lower M_r component (Fig. 3 *B*, lanes *7*, *8*, and *12*).

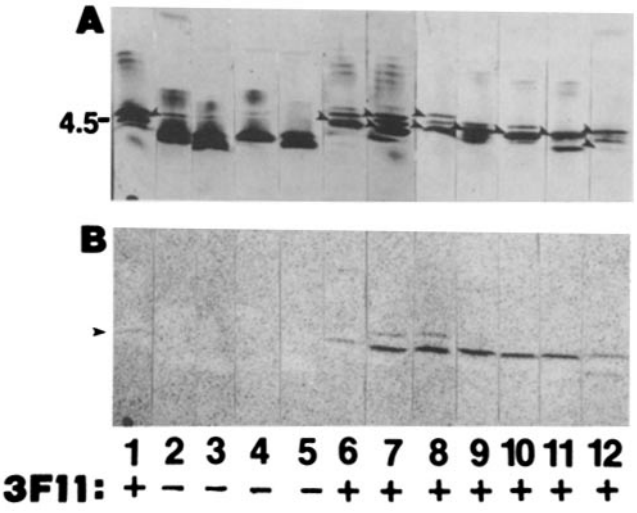

FIGURE 3. Effect of CMP-NANA on the LOS of gonococcal strains that are 3F11$^+$ or 3F11$^-$. 12 strains were grown on solid GC media in the presence of CMP-[^{14}C]-NANA. Proteinase K lysates were separated by SDS-PAGE and silver stained (*A*). The gel was dried, then autoradiographed. Lanes were for (*A*) F62, (*2*) 15253, (*3*) 15253v, (*4*) 15433, (*5*) 15433v, (*6*) DOV, (*7*) WR213, (*8*) 15254, (*9*) 15255, (*10*) 15219, (*11*) 15220, and (*12*) 15221. Strains 15253v and 15433v are mAb-selected population variants of strains 15253 and 15433, respectively. The 4.5-kD component of F62 is designated in *A*. The arrow points to the components that have radioactivity incorporated. The 3F11$^+$ and 3F11$^-$ strains are indicated below *B*.

IEM of N. gonorrhoeae Strain 1291 Grown in Liquid Medium Containing CMP-NANA. To reveal sialylation of the LOS on individual organisms, we performed immunoelectronmicroscopy on strain 1291 grown in the presence or absence of CMP-NANA. We used mAb 06B4 as the probe for the expression of the epitope before and after sialylation. The results are shown in Fig. 4. mAb 06B4 bound circumferentially on strain 1291 grown in the absence of CMP-NANA (Fig. 4 *A*), but bound minimally to strain 1291 grown in the presence of CMP-NANA (Fig. 4 *B*). The CMP-NANA-treated organisms (same sample as shown in Fig. 4 *B*) were treated with neuraminidase and incubated with mAb 06B4, then with 15-nm colloidal gold-conjugated anti-mouse IgM. The circumferential binding of 15-nm gold secondary antibody (Fig. 4 *C*) indicated that neuraminidase treatment resulted in reexpression of the 06B4 epitope.

IEM of Gonococci in Normal Human Polymorphonuclear Leukocytes (PMNs). Strain 1291, which was positive for mAb 06B4 (and 3F11) in an in vitro binding assay (Fig. 4) (14) was incubated with PMNs for 1 h, and the mixture was incubated sequentially with mAb and gold-conjugated secondary antibody, neuraminidase, mAb, and sec-

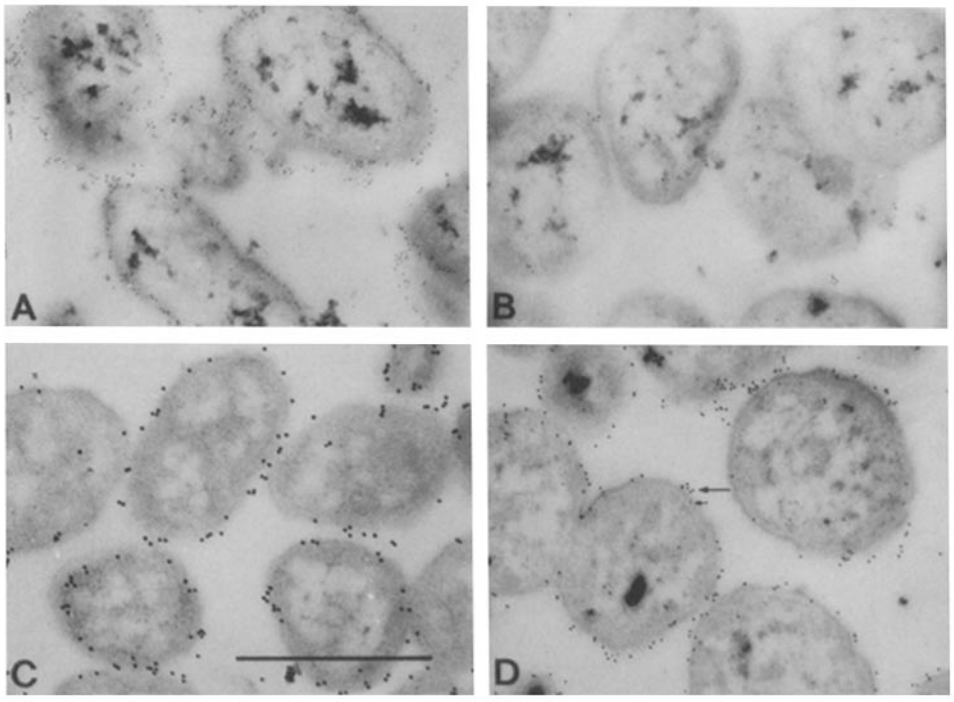

FIGURE 4. Colloidal gold IEM of gonococcal strain 1291 grown in the presence and absence of CMP-NANA. Strain 1291 was grown in liquid GC media with or without CMP-NANA, treated sequentially with mAb 06B4 and colloidal gold-conjugated secondary antibody, then examined with an electron microscope. (*A*) No CMP-NANA, mAb, 5-nM gold secondary antibody; (*B*) CMP-NANA, mAb, 5-nM gold secondary antibody; (*C*) CMP-NANA, treatment with neuraminidase, mAb, 15-nM gold secondary antibody; (*D*) CMP-NANA, mAb, 5-nm gold secondary antibody (same as *B*), treatment with neuraminidase, mAb, 10-nm gold secondary antibody. The small arrow points to 5-nm gold particles (few) and the large arrow points to 10-nm gold particles (predominate). The bar equals 1 μm.

ondary antibody with gold particles of a size larger than the first. Fig. 5 shows that strain 1291 in PMNs only slightly bound mAb 06B4 before neuraminidase treatment (Fig. 5 *A*, 10 nm; *B*, 5 nm), but showed increased binding after neuraminidase treatment (Fig. 5 *A*, 5 nm; *B*, 10 nm). Each of two neuraminidases tested (*Clostridium perfringens* and *Streptococcus*, see Materials and Methods) had the same effect. Some antibody binding occurred with organisms before they were treated with neuraminidase, and antibody bound both to organisms inside and outside the PMNs (data not shown), indicating that they were not completely modified during a 1-h incubation with PMNs. Neuraminidase-treated and -nontreated sections of the organisms in PMNs tested with anti-H.8 (mAb 2C3) showed that anti-H.8 bound circumferentially to >90% of the organisms (data not shown), indicating that blockage of the 4.5-kD LOS component was not due to a general effect on outer membrane surface antigens.

Transfer of Sialic Acid to LOS by a Gonococcal Detergent Extract. The rapid modification of the LOS of strains grown in the presence of CMP-NANA (Fig. 2) indicated that an enzymatic step may be involved. To test this hypothesis, purified LOS from *N. gonorrhoeae* strain F62 was incubated with CMP-[^{14}C]-NANA and a detergent extract of F62. The results of this experiment are shown in Fig. 6. The autoradiographs of SDS-PAGE-separated samples indicated that the extract resulted in incorporation of the label in an LOS component that migrated at an M_r slightly above the 4.5-

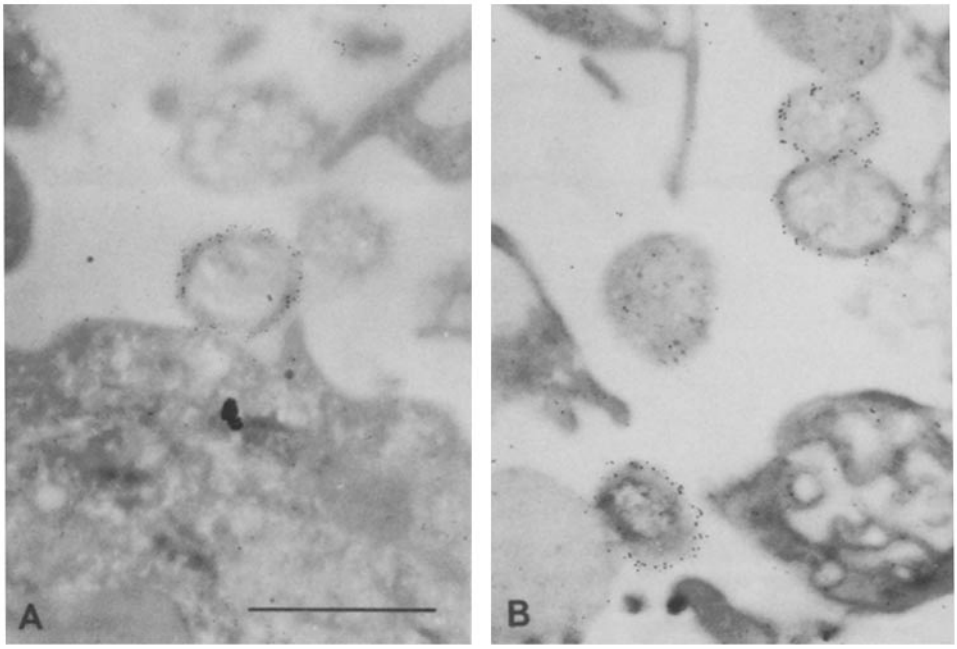

FIGURE 5. IEM of gonococcal strain 1291 incubated with human PMNs. Strain 1291 was grown on solid GC media, then washed and prepared as described in Materials and Methods. (*A*) mAb 06B4, 10-nm gold secondary antibody; neuraminidase, mAb, 5-nm gold secondary antibody. (*B*) mAb 06B4, 5-nm gold secondary antibody, neuraminidase, mAb, 10-nm gold secondary antibody. The bar equals 1 μm.

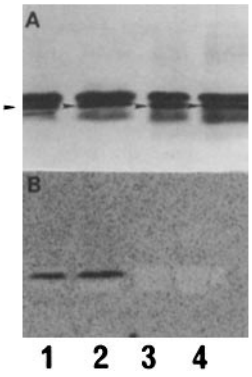

FIGURE 6. Modification of gonococcal strain F62 LOS by a detergent extract of the organism. Purified F62 LOS and CMP-[^{14}C]-NANA were incubated separately with and without a 0.5% Triton X-100 extract of F62 gonococci. Samples were separated by SDS-PAGE, silver stained, then the gel was dried and autoradiographed. (*A*) Silver stain; (*B*) autoradiograph. Lanes were for (*1*) LOS, extract, 10-μl sample (1 μg LOS); (*2*) LOS, extract, 20-μl sample (2 μg LOS); (*3*) LOS, no extract, 10-μl sample (1 μg LOS); and (*4*) LOS, no extract, 20-μl sample (2 μg LOS). For samples tested without extract, all other reagents were added except the extract. The arrows in *A* point to the position of the silver stained band that is radioactive.

kD LOS component (Fig. 6, lanes *1* and *2*). Negative control samples containing all reagents, except the extract, did not incorporate CMP-NANA (Fig. 6, lanes *3* and *4*). These results suggested that strain F62 has a STase that catalyzes the transfer of sialic acid to LOS.

Transfer of Sialic Acid to LOS by α2,3 and α2,6 STase. The immunochemical similarity between structures on human glycoconjugates and on the 4.5-kD component of gonococcal LOS suggested that the LOS might act as an acceptor for mammalin STase specific for terminal β-Gal or Galβ1→4GlcNAc. To test this hypothesis, purified LOS from *N. gonorrhoeae* strain F62 was incubated with CMP-[^{14}C]-NANA and either rat liver CMP-NANA:Galβ1→4GlcNAcα2→6 STase or porcine submaxillary gland CMP-NANA:βGalα2→3 STase. The autoradiographs of the SDS-PAGE-separated samples (Fig. 7) indicated that the two STases acted on different LOS components. Treatment of F62 LOS with α2→6 STase resulted in the label being incorporated in a component that migrated slightly below the predominate low M_r component of F62 LOS (Fig. 7, *2-6ST*), while treatment with the α2→3 STase resulted in the incorporation of label into a component that migrated at the top of the high M_r component (Fig. 7, *2-3ST*). As a positive control, asialofetuin was used in place of LOS as acceptor, and the presence of radioactivity in the SDS-PAGE-separated protein confirmed that the two enzymes were active (data not shown). The silver-stained components of F62 LOS after enzyme treatment appeared identical to the nontreated components (Fig. 7, lanes *1* and *2*). These results indicated that, although some radioactivity was incorporated in F62 LOS, only partial modification of the components occurred.

Activity of Anti-sialic Acid mAbs with CMP-NANA-treated LOS. Because of the activity of mammalian STases with LOS and the immunochemical similarity of epitopes on LOS and human glycosphingolipids, we tested strain F62 grown in the presence and absence of CMP-NANA with mAbs that recognize sialylated structures (α2→3- and α2→6-Gal-R) on mammalian glycosphingolipids and glycoproteins. Although the mAb 3F11-defined epitope was blocked (>95% reduction in counts) by the CMP-NANA treatment, indicating significant incorporation of sialic acid in the LOS, none of the anti-sialic acid mAbs (H6-3C4, IB9, NUH2, MC-813-70; see Materials and Methods for specificities) recognized either the CMP-NANA-treated or -untreated F62 gonococci.

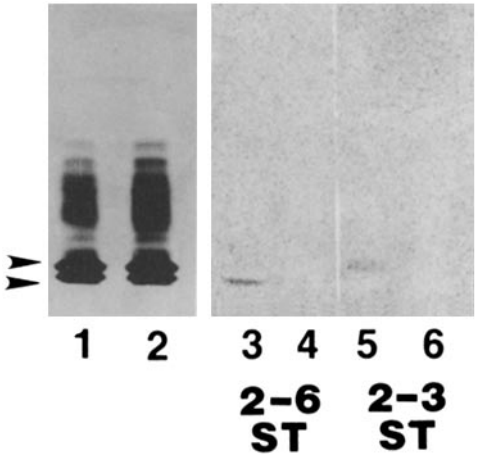

FIGURE 7. Modification of gonococcal strain F62 LOS by mammalian α2→3 and α2→6 STases. Purified F62 LOS and CMP-[^{14}C]-NANA were incubated separately with and without porcine submaxillary gland α2→3 STase or rat kidney α2→6 STase. Samples were treated as above (see legend, Fig. 6). Lanes were for (*1*) silver stain, α2→6 STase; (*2*) silver stain, no enzyme; (*3*) autoradiograph, α2→6 STase; (*4*) autoradiograph, no enzyme; (*5*) autoradiograph, α2→3 STase; (*6*) autoradiograph, no enzyme. For samples tested without enzyme, all other reagents were added except enzyme. The arrows in *A*, lanes *1* and *2*, point to the position of the silver-stained bands that are radioactive; the arrows in lanes *3* and *4* pointing to the same component are provided as a reference.

Discussion

Our studies demonstrate that a 4.5-kD component of gonococcal LOS is altered when organisms are grown in the presence of CMP-NANA (Fig. 1), and that there is a concomitant decrease in the expression of mAb-defined epitopes (Fig. 2 and Table I). The results of these studies extended those of Nairn et al. (7) and Parsons et al. (8), who showed that CMP-NANA probably is the low M_r factor extracted from human RBC that induces gonococcal resistance to killing by human serum, and that CMP-NANA sialylates gonococcal LOS, decreasing its reaction with bactericidal antibody (42).

With cells grown both in liquid and solid medium, the component with incorporated radioactivity was of an M_r larger than the 4.5-kD component, and the incorporation of radioactivity occurred only with strains having the 4.5-kD$^+$ (3F11$^+$) LOS, not with strains lacking the 4.5-kD component (Figs. 1–3). These results suggested that the 4.5-kD component is the minimal structure required for modification by CMP-NANA. Furthermore, the epitope recognized by the mAb is possibly the site of sialylation. However, the levels of CMP-NANA required for blocking the 4.5-kD LOS component (100–500 μg/ml) are relatively high compared with the levels reported for inducing gonococci to serum resistance (5×10^7 serum-sensitive gonococci induced to serum resistance in defined medium containing ~12.3 μg CMP-NANA) (8). The difference in concentrations may be due to our use of a nondefined medium for CMP-NANA treatment and/or to slightly higher concentrations of organisms (10^9) in the liquid medium. In subsequent studies, we have used lower concentrations of inoculum in lower concentrations of CMP-NANA and retained blockage of the 4.5-kD epitope (data not shown).

Gold particle IEM studies with mAb 06B4 and gonococci in human PMNs were suggestive that modification of the 4.5-kD LOS component by addition of sialic acid may occur in vivo (Fig. 5). For gonococci incubated with PMNs, a 1-h incubation was sufficient to modify binding of mAb. In subsequent experiments, we have noted that even a 10-min incubation resulted in a 60–90% decrease in mAb 06B4 binding (data not shown), indicating this was a rapid process. Since we could not detect which

organisms previously had been inside a PMN during processing, we could not make any conclusions by IEM regarding differences in 3F11 epitope expression (and sialylation of the 4.5-kD component) on organisms inside or outside the PMNs. These results are supported by experiments with gonococci in human urethral exudates where we found little reaction of the urethral gonococci with mAb 06B4 before neuraminidase treatment, but, similar to the experiments with CMP-NANA in vitro (Fig. 4), there was an increased binding of the mAb after treatment (43). These results are relevant in two respects. Gonorrhoea is an inflammatory disease, so many PMNs are found in urethral exudates, and PMNs have been shown to contain high resistance-inducing activity (4) that may be due to CMP-NANA or a related compound. Again, the binding of mAb 06B4 to the gonococci was decreased after incubation for 1 h with the PMNs and restored by treatment with neuraminidase (Fig. 5).

Using *Salmonella* rough mutant LPSs of known structure as our reference (10), we estimated the shift in the M_r of the 4.5-kD component of 1291 and F62 (Figs. 1 and 2) to be ~400 daltons. This suggests that the newly expressed 4.9-kD LOS component of these strains has at least one sialic acid residue present. In addition, the loss of epitope expression on CMP-NANA-treated gonococci could be reversed if the organisms were treated with neuraminidase (Table I and Fig. 4), suggesting that the alteration in epitope expression was due to the addition of sialic acid, as concluded from other studies (42). However, the silver-stain pattern of the higher M_r component of F62 LOS at hours 3 and 4 (Fig. 2 *A*) did not correspond with the pattern of 1-1-M binding at these time points (Fig. 2 *C*). Some possible explanations for these results are: (*a*) a 5.4-kD component negative for mAb 1-1-M incorporates some CMP-NANA when CMP-NANA is present in high concentrations (500 μg/ml); (*b*) a 5.4-kD- and 1-1-M-positive component incorporates CMP-NANA, but sialylation does not block 1-1-M binding as it does for the 06B4 epitope on the 4.5-kD component; (*c*) some 4.5-kD components polysialylated in high concentrations of CMP-NANA migrate at an M_r of 5.4 kD. Each of these cases could account for the presence of a component that does not stain well with silver and is altered in migration, but not in binding of 1-1-M.

The relative stability of 1-1-M epitope expression on CMP-NANA-treated gonococci (Table I, Fig. 2) indicates that the higher M_r LOS component of F62 does not possess the terminal sugar needed for modification by CMP-NANA. Chemical analysis of the oligosaccharide components of F62 LOS by liquid secondary ion mass spectroscopy has revealed that, except for an additional sugar on their nonreducing end, the higher and lower M_r components of F62 LOS are similar, if not identical, and the terminal sugar on the nonreducing end of the longest branch of these 4.5-kD, lower M_r molecules is galactose (44). If modification of the 4.5-kD component by CMP-NANA is dependent on the terminal sugar at the nonreducing end of the molecule, then the additional sugar on the higher M_r component may prevent modification.

The data presented so far suggest that gonococcal strains have STase that is active with exogenous CMP-NANA, although, as yet, no STase has been purified. However, detergent extracts of strain F62 were able to transfer label from CMP-[^{14}C]-NANA into a component of F62 LOS that migrated at a slightly higher M_r than the 4.5-kD component (Fig. 6). This result shows that the purified LOS can act as acceptor for sialic acid and that viable gonococci are not necessary for sialylation

to occur. Rat and pig STases also were able to use F62 LOS as an acceptor, but they incorporated radioactivity from CMP-NANA into different locations in the LOS, and <10% of the total label added to the reaction mixture was incorporated (Fig. 7). The later results may be due to the use of STases from species other than human. For example, STases are present in human cervical epithelium (45, 46) and human seminal plasma (47), and they vary in activity depending on the hormonal state (46) or sperm concentration (47). In future studies it will be important to locate where the putative gonococcal STase activity resides (inner or outer membrane, intracytoplasmic), whether human STases can modify gonococcal LOS, and whether these STases have any role in vivo.

The phenotypic conversion (sialylation of LOS) of in vitro grown organisms after their incubation with in vivo substances (CMP-NANA) (7, 42) may be one explanation for the paradox of organisms having in vitro sensitivity to the serum in which they caused infection (48). Since the 4.5-kD component of gonococcal LOS was the only outer membrane molecule that showed any change with CMP-NANA (Fig. 2), it is likely that it is associated with the lysis of serum-sensitive gonococci. It should be noted, however, that many serum-resistant strains also have the 4.5-kD component (and bind mAb 3F11) (10, 11, 14), indicating other, unidentified factors may be involved in the shift to serum resistance.

Although the presence of sialic acid in LOS has not been directly measured, a number of observations support this conclusion: (*a*) the NANA moiety of CMP-NANA is incorporated into LOS (7, 8) (Figs. 1 and 2); (*b*) growth of organisms in media containing CMP-NANA results in an increase in the M_r of the LOS, a result less likely to occur if modification of LOS is due simply to incorporation of sugars already part of the LOS (e.g., *N*-acetylglucosamine, *N*-acetylgalactosamine (49, 50); (*c*) the LOS component modified by CMP-NANA is sensitive to neuraminidase (Table I and Figs. 4 and 5) with a concurrent restoration to serum sensitivity (42); and (*d*) gonococcal detergent extracts and mammalian STases can use gonococcal LOS as acceptors with CMP-NANA (Figs. 6 and 7).

The biologic functions described for sialic acid most relevant to gonococcal pathogenesis are those of recognition and antirecognition (51–53). In future studies of LOS sialylation, we will study the effects of sialylation on recognition of human anti-LOS antibodies and complement (52), and of galactose-specific human cell lectins (54) that may be involved in bacterial-host cell interaction.

Summary

After growth of gonococci in the presence of cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA), their 4.5-kD lipooligosaccharide (LOS) component was increased by ~400 daltons, whereas the LOS of strains lacking the 4.5-kD component were unaffected. Expression of mAb-defined epitopes on the 4.5-kD component was decreased on LOS of strains grown in CMP-NANA, and treatment of the LOS with neuraminidase reversed this affect. Gonococci incubated with human PMNs also had decreased expression of the 4.5-kD$^+$ epitopes. A detergent extract of gonococci incorporated radiolabeled NANA in the LOS, suggesting the presence of a sialyltransferase in gonococci. Exogenous sialyltransferases also could use LOS as an acceptor.

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