INFECTION AND IMMUNITY, Nov. 1997, p. 4436–4444 Vol. 65, No. 11
0019-9567/97/$04.00+0


# Sialylation of *Neisseria meningitidis* Lipooligosaccharide Inhibits Serum Bactericidal Activity by Masking Lacto-*N*-Neotetraose

MICHELE M. ESTABROOK,[1]* J. MCLEOD GRIFFISS,[2,3] AND GARY A. JARVIS[2]

*Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio,[1] and the Center for Immunochemistry, Department of Laboratory Medicine[2] and Department of Medicine,[3] University of California San Francisco School of Medicine, San Francisco, California*

Received 31 March 1997/Returned for modification 16 June 1997/Accepted 14 August 1997

**Exogenous sialylation of gonococcal lipooligosaccharide causes resistance to serum bactericidal activity. The aim of this study was to determine how lipooligosaccharide sialylation affects the serum sensitivities of group C *Neisseria meningitidis* strains. The relationship between the degree of sialylation or expression of the lipooligosaccharide sialic acid acceptor, lacto-*N*-neotetraose (LNnT), of nine meningococcal strains and their sensitivities to a pool of normal human sera was assessed. All strains expressed LNnT that was variously endogenously sialylated. Susceptibility to serum bactericidal activity ranged from extremely sensitive to resistant in 50% serum. For endogenously sialylated strains, the amount of killing correlated with the amount of free LNnT above a threshold of expression; strains that expressed less than the threshold survived in 25% serum. All strains added more sialic acid when they were grown in medium that contained cytidine monophospho-*N*-acetylneuraminic acid. Exogenous sialylation reduced the expression of free LNnT and significantly increased serum resistance. Exogenous sialylation affected killing through both classical and alternative complement pathways. The killing of exogenously sialylated strains also correlated with the amount of free LNnT. The amounts of endogenous, exogenous, and total sialic acid bound to LNnT did not correlate with the resistance of strains to serum bactericidal activity; rather, the loss of free LNnT expression by sialylation was associated with resistance. In conclusion, the expression of free LNnT by group C meningococcal strains is directly associated with the amount of killing of organisms in pooled human sera. Both endogenous and exogenous lipooligosaccharide sialylation are associated with increased serum resistance by masking LNnT.**

Disseminated meningococcal disease is relatively rare in light of the fact that 3 to 40% of healthy individuals are colonized with *Neisseria meningitidis* at any given time (3). When it does occur, this illness can be fulminant and devastating. Protection from disseminated disease is the result of serum bactericidal activity (SBA) and possibly opsonic activity. Bactericidal activity is due to complement-mediated cell lysis (12, 28, 32). Activation of complement can occur via the classical pathway (CP) or the alternative complement pathway (ACP). Both pathways result in the formation of C3 convertases on the bacterial surface that lead to the deposition of C3b. Both pathways result in a terminal sequence that forms the membrane attack complex comprised of C5b-C9 that is necessary for bacterial lysis. Complement activation on neisserial cell surfaces is regulated by glycoses and glycolipids (13, 36).

The majority of *N. meningitidis* disease in the United States is caused by serogroup B and C organisms. Most of these strains make various amounts of lipooligosaccharides (LOS) that contain a terminal lactosamine structure, lacto-*N*-neotetraose (LNnT). This carbohydrate serves as the major site for sialylation of meningococcal LOS (23, 25). Group B and C *N. meningitidis* strains can endogenously sialylate LOS (25) and do so to various degrees. Some strains express LOS with LNnT that is not endogenously sialylated, whereas others are heavily sialylated (7). Serogroup B and C *N. meningitidis* strains can also add sialic acid (exogenous sialylation) when they are grown in the presence of cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA). Monoclonal antibody (MAb) 1B2 binds to LNnT on the 4.5-kDa component of meningococcal LOS. The binding of this MAb is blocked by the addition of both endogenous and exogenous sialic acid to LNnT, and this loss of binding can serve as a surrogate marker for LOS sialylation (7, 23, 25, 42).

Exogenous sialylation of the LNnT structure of *Neisseria gonorrhoeae* LOS causes serum-sensitive organisms to become resistant to complement-mediated lysis (2, 26, 27, 30). We previously showed that the sialylated, serum-resistant variant of *N. gonorrhoeae* F62 bound significantly less total C3 than did the nonsialylated, serum-sensitive strain F62 (16). Much less is known about the role of LOS sialylation in the resistance of meningococci to SBA. A brief report by Fox et al. (10) concluded that growth in medium that contained exogenous CMP-NANA did not confer resistance to SBA on *N. meningitidis*, although the level of endogenous LOS sialylation was not reported. Of 21 strains tested, 13 were serogroup A, B, Y, or Z and 8 strains were nonencapsulated. To determine how sialylation affects the sensitivities of group C *N. meningitidis* strains to SBA, we assessed the relationship between the degree of LOS sialylation or expression of free LNnT and the sensitivities of nine well-characterized strains to a pool of normal human sera (PHS) from five people.

(This study was presented in part at the Tenth International Pathogenic Neisseria Conference, Baltimore, Md., 8 to 13 September 1996, and at the 96th General Meeting of the American Society for Microbiology, New Orleans, La. 19 to 23 May 1996.)

* Corresponding author. Mailing address: 11100 Euclid Ave., Mail Stop 8A, Cleveland, OH 44106. Phone: (216) 844-8918. Fax: (216) 844-8362. E-mail: mxe16@po.cwru.edu.

## MATERIALS AND METHODS

***N. meningitidis* strains.** The nine endemic, serogroup C meningococcal strains used were isolated from children and have been described previously (7). Five

were isolated from blood or cerebral spinal fluid samples, three were carrier isolates, and 1 was isolated from the middle ear fluid of a child with acute otitis media who did not develop disseminated disease. All strains were encapsulated, as evidenced by the binding of MAb to the group C polysaccharide by whole-cell enzyme-linked immunosorbent assay (ELISA), and all made LOS bearing the LNnT structure (7).

**OMC.** Outer membrane complex (OMC) antigens were purified from whole organisms as previously described (37, 43).

**MAbs.** MAb 1B2 is an immunoglobulin M (IgM) that was obtained from mice immunized against lacto-*N-nor*-hexaosylceramide and has previously been shown to bind to glycoconjugates that bear the terminal *N*-acetyllactosamine structure (Galβ1→4GlcNAcβ1→R) on human leukocytes (4, 42) and to neisserial LOS that bear the LNnT structure (Galβ1→4GlcNAcβ1→3GalB1→4Glc) (16, 23). MAb 1B2 has binding characteristics similar to those of MAb 3F11 (23). Sialic acid residue and MAbs 1B2 and 3F11 have previously been shown to bind to the same terminal galactose on the LNnT structure (23, 25, 26, 41, 42). The binding of MAb 1B2 is blocked by the addition of both endogenous and exogenous sialic acid to LNnT, and this loss of binding can serve as a surrogate marker for LOS sialylation (7, 23, 25, 42). The cell line (1B2-1B7) used for the production of this MAb was purchased from the American Type Culture Collection (Rockville, Md.).

**Serum.** Bactericidal assays were performed with PHS that was obtained from three men and two women who had no knowledge of neisserial infection, immunization with neisserial antigens, or concurrent use of antibiotics. PHS was divided into aliquots and stored at −70°C. Each aliquot was used only once. In some experiments, PHS was chelated with 10 mM Mg-EGTA (EGTA PHS). PHS depleted of factor B (FBD PHS) was purchased from Quidel Q (San Diego, Calif.) and was obtained from a different pool of donors.

**Bactericidal assay.** A modification of the method of Ross et al. (31) was used to assess the sensitivities of meningococcal strains to the SBA of PHS. For bactericidal assays, bacteria were prepared as follows. Organisms from frozen stock cultures were grown overnight in 5% $CO_2$ on GC agar with 1% supplement. For each strain, a stock culture was made the next morning and divided into aliquots to be stored at −70°C. Each aliquot was used only once to inoculate modified Frantz liquid medium (19) that did or did not contain 200 μg of CMP-NANA (Sigma) per ml. Bacteria were grown to mid-log phase by end-over-end rotation at 37°C in 12- by 75-mm polystyrene tubes (7, 21). Bacteria were washed twice in gonococcal buffer (GB) as previously described (33) and suspended in GB to an optical density (OD) at 580 nm of 0.10 ($10^8$ organisms/ml) (34). Mid-log-phase organisms were used in bactericidal assays and were also resuspended in phosphate-buffered saline (PBS; pH 7.4) for whole-cell ELISA.

The reaction tubes contained 10 μl of bacteria ($10^6$ CFU), 100 μl of PHS or PHS appropriately diluted in GB to a final volume of 100 μl, and 90 μl of GB. Control tubes were identical except that heat-inactivated PHS (56°C for 30 min) was substituted for PHS. Tubes were incubated for 60 min in a 37°C shaking water bath (140 rpm). At time zero and 60 min, duplicate aliquots were removed and diluted and each was plated twice. After overnight growth in 5% $CO_2$, the number of CFU was counted. Survival was expressed as the percentage of organisms at time zero that survived to 60 min. The percent survival of each strain (grown with and without CMP-NANA) in serial twofold dilutions of serum was obtained. For each strain, organisms that were and were not grown in exogenous CMP-NANA were always analyzed together in the bactericidal assay and whole-cell ELISA. A maximum of five serum dilutions was done for each bactericidal assay, and the maximum serum concentration that could be used was 50%. Each strain was tested twice.

**Whole-cell ELISA.** We used MAb 1B2 and a modification of the whole-cell ELISA method of Abdillahi and Poolman (1, 7, 39) to test meningococcal strains for the expression of LNnT. Briefly, mid-log-phase meningococci grown with and without exogenous CMP-NANA were washed and used in the bactericidal assay as described above. Remaining bacteria were also immediately pelleted and resuspended in PBS to an OD at 640 nm of 0.1. This suspension was used to coat microtiter wells (Immulon 1; Dynatech Laboratories, Chantilly, Va.), which were then reacted with MAb 1B2. After incubation with alkaline phosphatase-conjugated secondary antibody, wells were developed for exactly 15 min and the absorbance was read at 410 nm with a Dynatech MR5000 microplate reader. The binding of MAb 1B2 was reported in OD units (ODU) (16).

Because a MAb that binds meningococcal LOS sialic acid is not available, the loss of MAb 1B2 binding by sialylation of LNnT was used to assess the semiquantitative amount of sialic acid bound to that structure. The amount of endogenous sialic acid bound to LNnT (strain grown in broth without CMP-NANA) was determined for each strain in each trial by subtracting the binding of MAb 1B2 (by whole-cell ELISA and reported in ODU) before the removal of sialic acid from LOS by *Clostridium perfringens* neuraminidase (type V; Sigma) from the binding of MAb 1B2 after neuraminidase treatment (7, 25). The percentage of LNnT endogenously sialylated was calculated as follows: 100 − (MAb 1B2 binding before neuraminidase treatment/MAb 1B2 binding after neuraminidase treatment)(100). The amount of exogenous sialic acid added to LNnT was determined for each strain in each trial by subtracting the binding of MAb 1B2 (by whole-cell ELISA) to organisms after growth with CMP-NANA from the binding of MAb 1B2 after growth without CMP-NANA (7, 26). The amount of total sialic acid bound to LNnT (endogenous plus exogenous) was determined for each strain in each trial by subtracting the binding of MAb 1B2 after growth in CMP-NANA from the binding of MAb 1B2 after treatment with neuraminidase. For each strain grown without and with CMP-NANA, whole-cell ELISAs were always run in parallel.

TABLE 1. Characteristics of nine endemic serogroup C meningococcal strains isolated from children[a]

| Strain | Proteins[b] | % Sialylation[c] | Source[d] |
|---|---|---|---|
| 8026 | NT:NT | 4 | Case, 15 yr old |
| 15021 | NT:NT | 16 | CSF, 5 mo old |
| 7954 | NT:1.15 | 48 | CSF, 4 yr old |
| 7970 | NT:NT | 74 | CSF, 16 yr old |
| 7973 | NT:1.2 | 86 | CSF, 9 yr old |
| 15030 | NT:NT | 8 | Throat, 12 yr old |
| 15029 | NT:1.15 | 11 | Throat, 10 yr old |
| 15031 | NT:NT | 19 | Throat, 10 yr old |
| 15700 | NT:NT | 2 | Middle ear aspirate, 5 yr old |

[a] All strains expressed LNnT (L3,7) that was variously endogenously sialylated, as judged by the binding of MAb 1B2 before and after the removal of sialic acid from LOS by neuraminidase.

[b] Class 2/3:class 1 proteins. Strains were tested for the expression of serotype proteins 2a, 2b, and 2c (class 2), 15 (class 3), and p1.2, p1.16, and p1.15 (class 1).

[c] Percent endogenous sialylation of LOS was calculated as follows: 100 − (MAb 1B2 binding before neuraminidase treatment/MAb 1B2 binding after neuraminidase treatment)(100). Data are the means of two trials.

[d] Each source is identified by the type of isolate and the age of the child. CSF, cerebrospinal fluid.

**HIMELISA.** Human inhibition monoclonal ELISA (HIMELISA) was performed as previously described (6, 8) and was used to measure the antibody in PHS capable of inhibiting the binding of MAb 1B2 to the LNnT component of the LOS of strain 15029. Our previous work with this assay showed that HIMELISA permits epitope-specific discrimination among LOS antibodies in human sera (6, 8).

**SDS-PAGE and immunoblot analysis.** The proteins and LOS in purified OMC (20) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by a previously described modification of the method of Laemmli (7, 38). LOS molecules were visualized by silver staining (40), and proteins were stained with Coomassie R-250 (Sigma). SDS-PAGE-separated LOS and proteins of nine meningococcal strains were transferred to nitrocellulose and immunoblotted with a 1:10 dilution of PHS (7). After being washed with PBS, the blot was incubated in a mixture of alkaline phosphatase-conjugated goat antibodies to human IgG, IgA, and IgM and developed in substrate as previously described (7). SDS-PAGE-separated proteins and LOS of strain 15029 were also immunoblotted with MAb 1B2 to indicate the location of the 4.5-kDa LOS that expressed LNnT.

**Analysis of data.** The percent survival of each strain grown with or without CMP-NANA in serial twofold dilutions of serum was determined. For each strain in each trial, the linear portion of the percent survival curve was graphed and fitted to a linear equation by the least square error curve fit method. The linear equation was used to calculate the $\log_2$ serum dilution in which survival reached 100%. This was correlated with the binding of MAb 1B2 to LNnT for each strain in each assay, as measured by whole-cell ELISA. Each strain was tested twice. Differences in the $\log_2$ serum dilution in which survival reached 100% before and after experimental modification were analyzed by the Wilcoxon paired-sample test. For this analysis, the means of the results from two trials for each strain were used to avoid the use of multiple samples from the same experimental source.

## RESULTS

***N. meningitidis* strains.** Characteristics of the nine serogroup C isolates used are shown in Table 1. All of the strains expressed L3,7 LOS bearing LNnT that bound MAb 1B2 by whole-cell ELISA. The percent endogenous sialylation of LNnT, as assessed by using neuraminidase to remove sialic acid from LOS, ranged from very little (2%) to nearly complete (86%) endogenous sialylation of the LNnT structure when strains were grown to mid-log phase in broth without exogenous CMP-NANA.

**SBA.** The nine group C strains used varied markedly in sensitivity to the SBA of PHS (Fig. 1). Strain 8026, a case isolate,

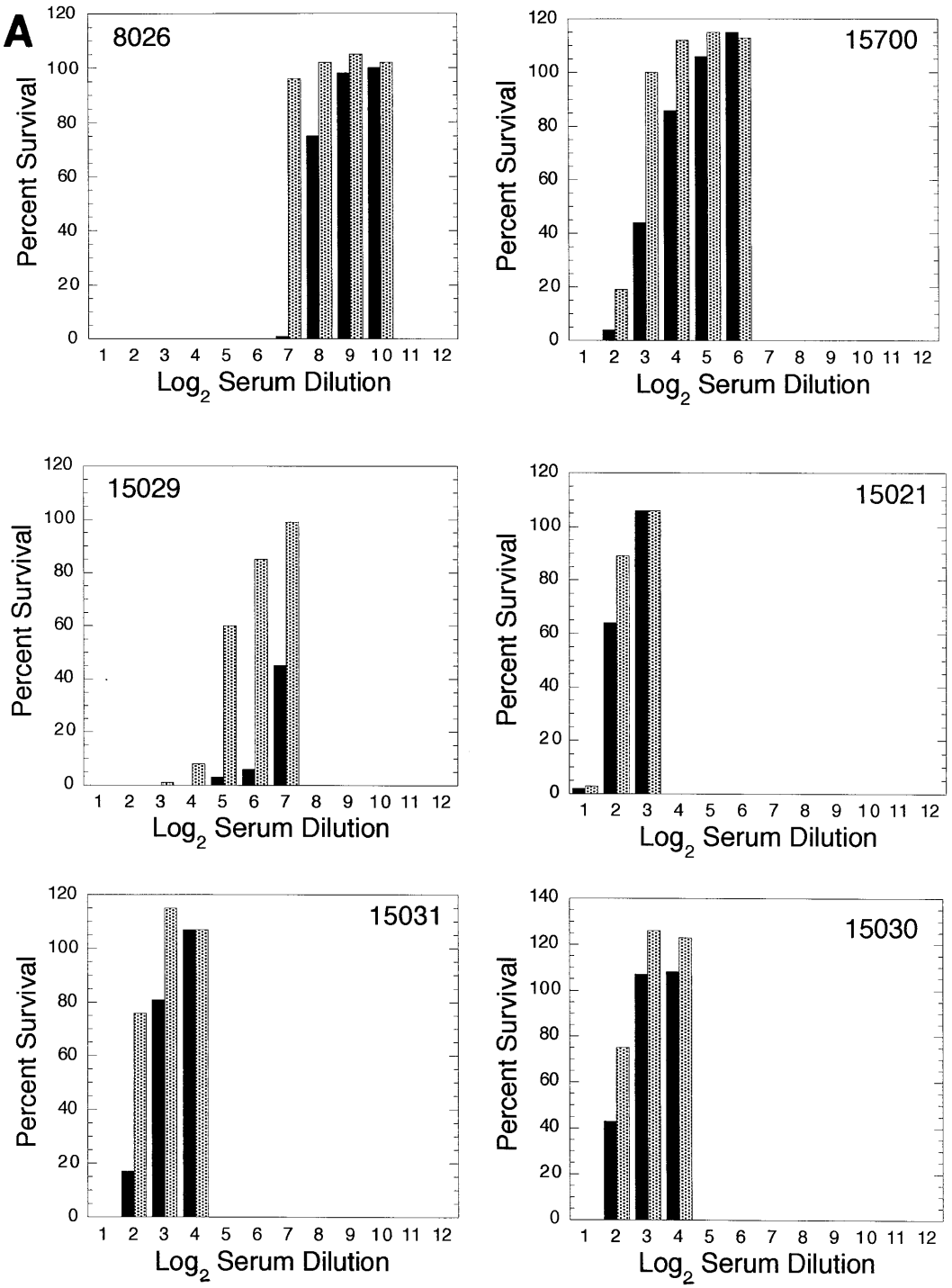


FIG. 1. Percent survival of nine meningococcal strains in serial twofold dilutions of PHS after growth to mid-log phase in broth without (black bars) and with (gray bars) exogenous CMP-NANA. (A) Exogenous sialylation inhibited SBA for six strains. The amount of inhibition correlated directly with sensitivity to SBA. (B) The three most resistant strains were heavily endogenously sialylated and did not become more resistant after growth in CMP-NANA. The results from one representative trial for each strain are shown. Strains were tested twice.

was extremely sensitive, with no survival in as little as 0.8% PHS (Fig. 1A). The three most resistant strains (7973, 7954, and 7970) were also case isolates (Fig. 1B). Strains 7973 and 7954 did not survive in 50% PHS but grew (>100% survival) in 25% PHS. Strain 7970 had nearly 100% survival in 50% PHS. The remaining five strains had intermediate sensitivities to the SBA of PHS (Fig. 1A).

**Exogenous sialylation of LOS.** To examine the effect of adding sialic acid to LOS on sensitivity to SBA, strains were grown to mid-log phase in the presence of an exogenous sia-

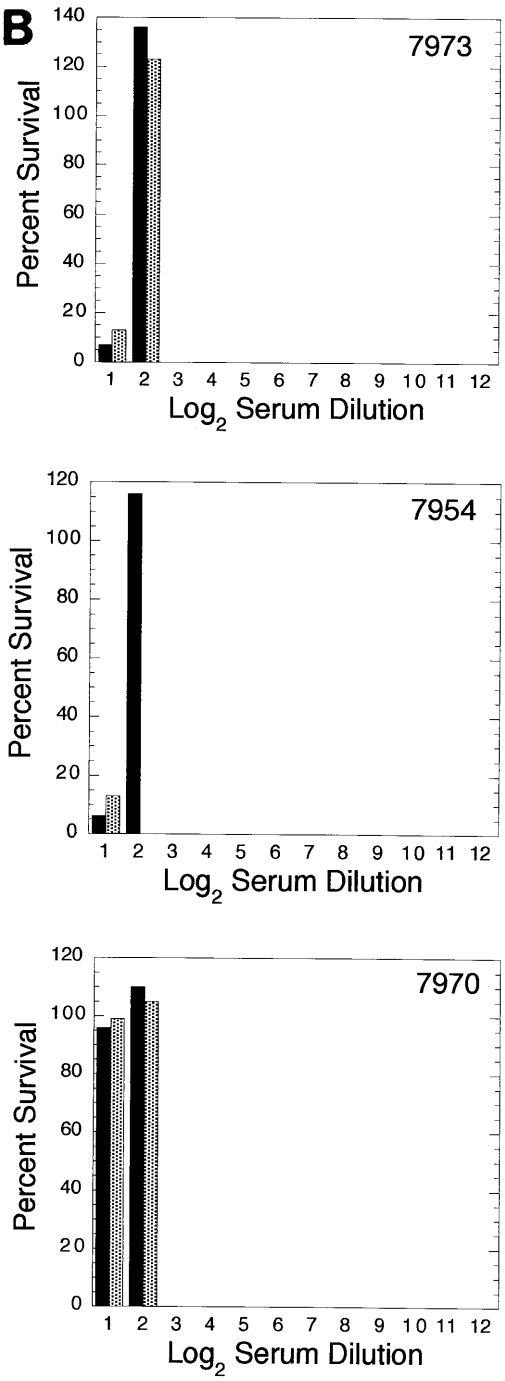


FIG. 1—*Continued.*

lylating nucleotide, CMP-NANA. All strains added sialic acid to LOS bearing LNnT, as evidenced by decreased MAb 1B2 binding by whole-cell ELISA. Exogenous sialylation inhibited SBA for the six most sensitive strains (Fig. 1A) but did not affect the three most resistant strains (7973, 7954, and 7970), which were heavily sialylated endogenously (Fig. 1B). Strains 7973 and 7954 did not become more resistant to the SBA of 50% PHS when they were grown in CMP-NANA. Strain 7970 was already resistant in 50% PHS (maximum concentration); therefore, the effect of exogenous sialylation on sensitivity to SBA could not be assessed.

The $\log_2$ serum dilutions in which survival reached 100% were compared for the six sensitive strains grown without and with exogenous CMP-NANA. Exogenous sialylation significantly increased the survival ($P = 0.028$) of these serogroup C meningococcal strains. This effect of exogenous sialylation was most pronounced at lower PHS concentrations and was lost at greater PHS concentrations (the 10th, 50th, and 90th percentiles for strains grown without CMP-NANA were 2.4, 4.0, and 8.6, respectively, and those for strains grown with CMP-NANA were 2.3, 3.0, and 7.5, respectively).

**CP and ACP.** The contributions of the classical pathway of complement and the ACP to SBA were examined with CP-inactivated or ACP-depleted PHS. Inactivation of the CP resulted in a marked decrease in the SBA of PHS against a relatively sensitive strain (15029; Fig. 2). Figure 2B shows that even 50% PHS with inactivated CP allowed >20% survival of strain 15029. Exogenous sialylation of LOS led to a modest

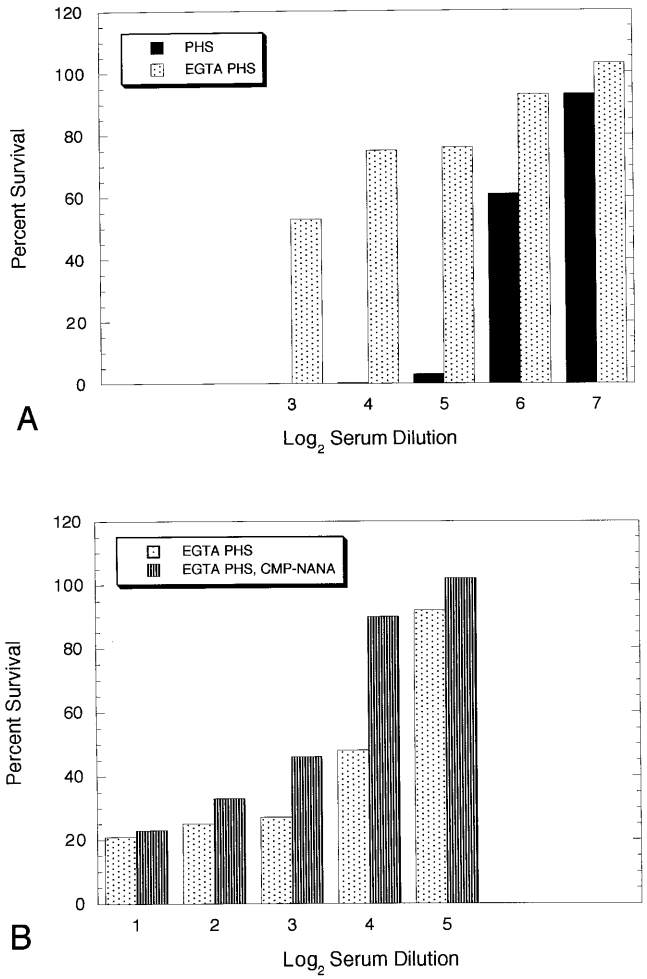


FIG. 2. Bactericidal assays comparing the SBAs of PHS and EGTA PHS to inactivate the CP of complement. (A) Percent survival of strain 15029 grown in medium without exogenous CMP-NANA in serial twofold dilutions of PHS or EGTA PHS; (B) percent survival of strain 15029 grown without or with CMP-NANA in serial twofold dilutions of EGTA PHS. For strain 15029, inactivation of the CP resulted in a marked decrease in the SBA of PHS against this relatively sensitive strain. Panel B shows that the ACP alone could not completely kill the strain even in 50% serum. Exogenous LOS sialylation resulted in a modest increase in resistance to ACP activity. Because of the increased resistance of strain 15029 in PHS with the CP inactivated, the assay for panel B was begun at a serum concentration of 50% rather than 12.5%, as used for the panel A assay.

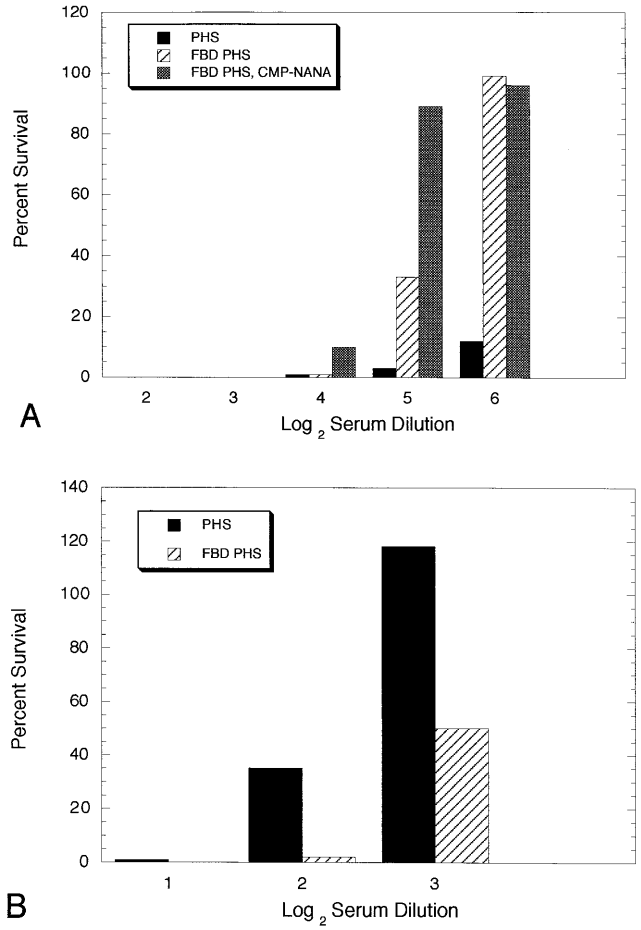


FIG. 3. Bactericidal assays comparing the SBAs of PHS and FBD PHS to inactivate the ACP. (A) Percent survival of strain 15029 grown in medium without exogenous CMP-NANA in serial twofold dilutions of PHS or grown in medium without or with CMP-NANA in FBD PHS. Depletion of the ACP led to a decrease in the SBA of PHS against this strain but to a lesser degree than did inactivation of the CP. Exogenous LOS sialylation resulted in a modest increase in resistance to CP activity. (B) Percent survival of strain 15030 grown in medium without exogenous CMP-NANA in serial twofold dilutions of PHS or FBD PHS. Inactivation of the ACP did not lead to increased survival for this more resistant strain. Note that FBD PHS was made from a different donor pool than was intact PHS and that strain 15030 survived slightly better in intact PHS than it did in FBD PHS.

increase in survival at low serum concentrations when the SBA was due to the ACP alone (Fig. 2B).

Depletion of the ACP led to a decrease in the SBA of PHS against strain 15029 but to a lesser degree than did inactivation of the CP (Fig. 3A). Exogenous LOS sialylation resulted in a modest increase in resistance to CP activity (Fig. 3A). Figure 3B shows that for a more resistant strain (15030), inactivation of the ACP did not lead to increased survival.

**Expression of free LNnT.** For each trial, the $\log_2$ dilution of PHS in which survival of each strain reached 100% was plotted against that strain's expression of the free LNnT structure of LOS, as determined by the binding of MAb 1B2 by whole-cell ELISA at 410 nm and reported in ODU. Figure 4A shows the results for strains grown without CMP-NANA, and Figure 4B shows the results for strains grown with CMP-NANA. Figure 5 depicts the linear relationship between free LNnT expression and sensitivity to PHS for these strains (only datum points on the linear portion of the curve in Fig. 4A are included, whereas all datum points in Fig. 4B are included).

For endogenously sialylated group C meningococcal strains (Fig. 4A and 5), the amount of killing directly correlated with the amount of free LNnT ($r^2 = 0.81$) above a threshold of LNnT expression. Strains that expressed less than the threshold survived in 25% PHS. Case isolates were not more resistant than were nondisseminated isolates that expressed equivalent amounts of free LNnT (Fig. 4A).

As can be seen in Fig. 4B and 5, exogenous sialylation decreased free LNnT expression and increased resistance to SBA. Again, killing directly correlated with the amount of free LNnT ($r^2 = 0.92$). However, with exogenous sialylation, the association of free LNnT expression with killing was evident for all strains, not just for those whose LNnT expression was above the threshold. Note also that the linear components of the curves depicted in Fig. 5 are nearly parallel. This suggests that the associations between killing and the expression of free LNnT are similar for both endogenous and exogenous sialylation of LOS of these group C *N. meningitidis* strains. Of interest, however, is the observation that even when the strains (Fig. 4A) that expressed more than the threshold amount of LNnT were exogenously sialylated (Fig. 4B) so that their expression of free LNnT was decreased to less than the threshold, they did not all show complete survival in 25% serum. This indicates that these strains may possess additional characteristics that render them more sensitive to SBA than would be predicted by free LNnT expression alone.

**LOS sialic acid.** To examine whether the amount of sialic acid bound to meningococcal LOS or the loss of expression of free LNnT by this sialylation was associated with increased resistance to SBA, the amounts of endogenous, exogenous, and total (endogenous plus exogenous) LOS sialic acid for these nine strains were determined. The change in the binding of MAb 1B2, as measured by whole-cell ELISA, showed that neither the amount of endogenous sialic acid (Fig. 6A) nor the percent endogenous sialylation of LNnT (data not shown) correlated with resistance to SBA. Likewise, when strains were grown in CMP-NANA, the amounts of exogenous sialic acid (Fig. 6B) and total sialic acid (Fig. 6C) were not directly associated with resistance to the SBA of PHS. This indicates that the loss of LNnT expression by sialylation rather than the amount of sialic acid is associated with increased resistance to SBA.

**Antibodies in PHS to noncapsular meningococcal antigens.** To determine what meningococcal LOS and proteins bound antibodies in PHS, the SDS-PAGE-separated molecules in purified OMC of these nine strains were immunoblotted with 10% PHS. While human antibodies bound to several proteins and faster-migrating LOS, no binding was detected to the 4.5-kDa LOS molecule that bound MAb 1B2 on any strain. The lack of significant antibody in PHS that bound LNnT was confirmed by HIMELISA. Preincubation of purified OMC of strain 15029 with 50% PHS decreased the subsequent binding of MAb 1B2 by only 35%. Preincubation with 25 and 12.5% PHS decreased the binding of MAb 1B2 by 14 and 7%, respectively. The association between LNnT expression and sensitivity to the SBA of PHS did not appear to be due to bactericidal antibodies that bound to this structure on LOS.

## DISCUSSION

Of nine serogroup C meningococcal strains isolated from children, all expressed LOS bearing LNnT that was variously endogenously sialylated. Susceptibility to the SBA of PHS ranged from extremely sensitive to resistant in 50% serum. Both the CP and ACP contributed to the SBAs against rela-

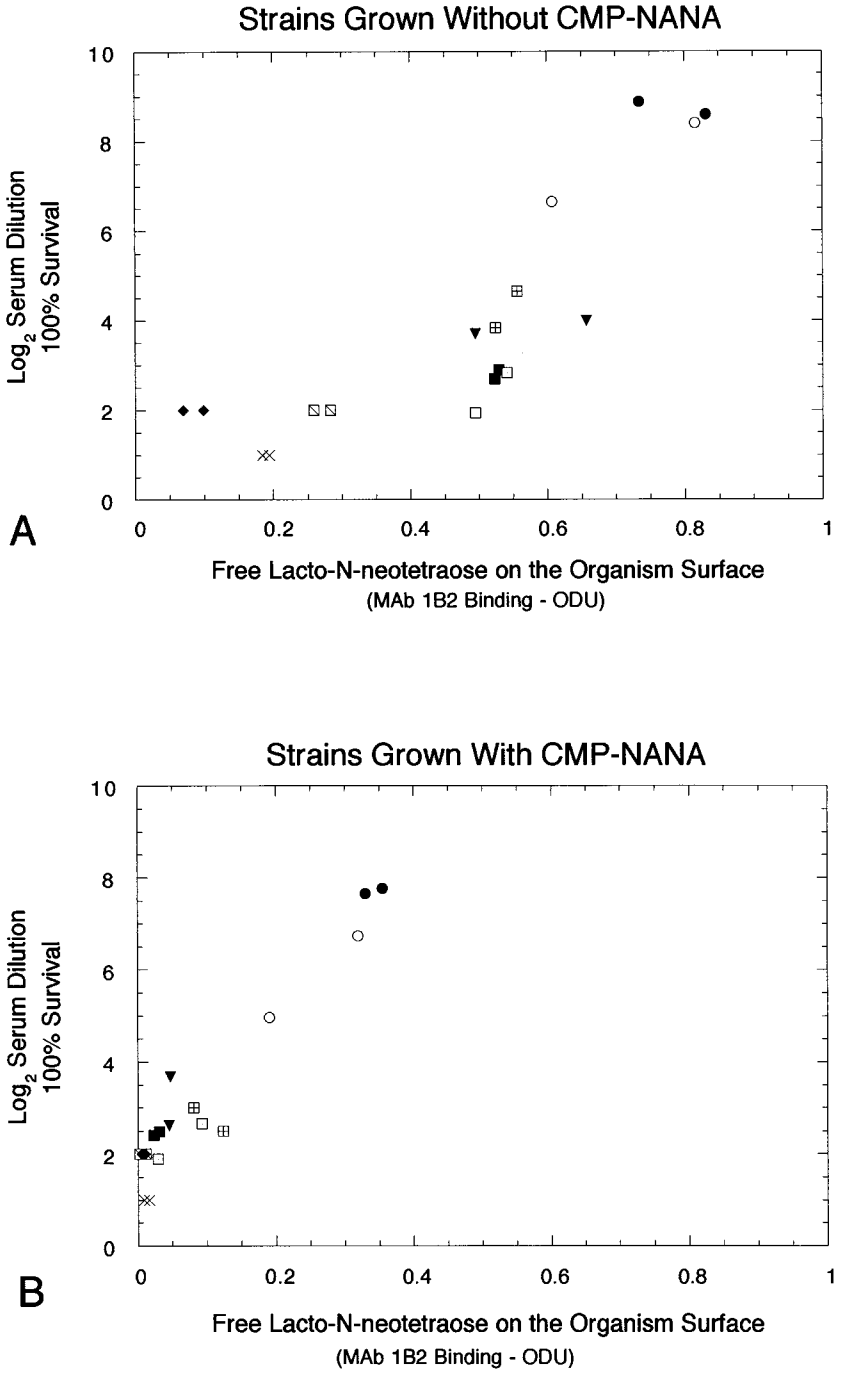


FIG. 4. Serum bactericidal assays of nine meningococcal strains grown to mid-log phase without (A) and with (B) exogenous CMP-NANA. For each trial, the $\log_2$ dilution of PHS in which survival of each strain reached 100% was plotted against that strain's expression of free LNnT of LOS, as determined by the binding of MAb 1B2 by whole-cell ELISA and reported in ODU. Each point represents an individual trial for each strain. Strain 7970 was resistant in the maximum serum concentration used in assays (50% PHS), and strains 7954 and 7973 were resistant in 25% PHS so these strains were assigned values of 1, 2, and 2, respectively, for the $\log_2$ dilution of PHS in which survival reached 100%. ●, 8026; ○, 15029*; ■, 15030*; ⊡, 15021; ▼, 15031*; ⊞, 15700*; ⧅, 7954; ×, 7970; ◆, 7973. (Asterisks indicate nondisseminated strains.)

tively sensitive strains, but only the CP appeared to be important for lysis of more serum-resistant strains. For endogenously sialylated meningococcal strains, the amount of killing directly correlated with the amount of free LNnT above a threshold of LNnT expression; strains that expressed less than the threshold survived in 25% PHS. Case isolates were not more resistant than were nondisseminated isolates that expressed equivalent amounts of free LNnT.

All strains added sialic acid when they were grown to mid-log phase in medium that contained CMP-NANA. Exogenous sialylation reduced the expression of free LNnT. Again, the amount of killing was associated with the amount of free

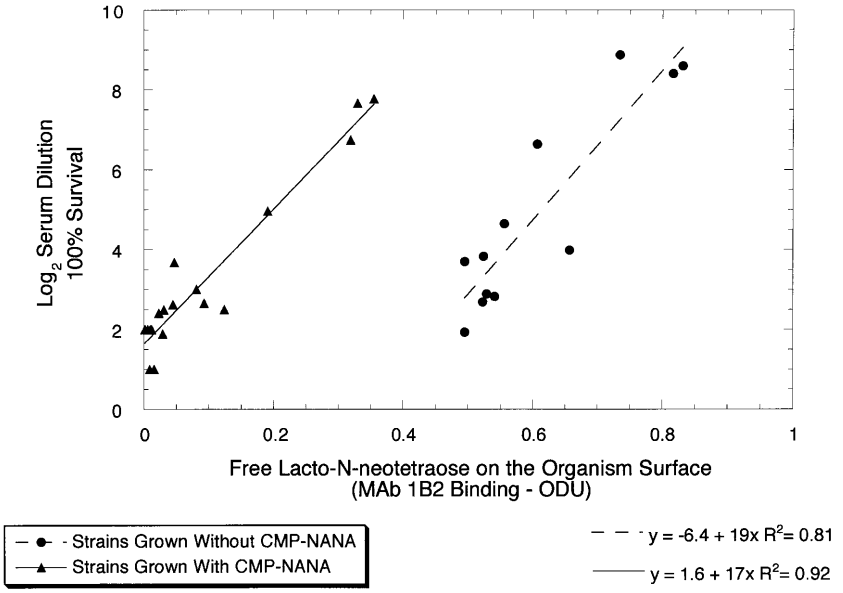


FIG. 5. Correlation between the expression of LNnT structure of LOS, as determined by the binding of MAb 1B2 by whole-cell ELISA and reported in ODU, and sensitivity to the SBA of PHS for nine strains grown without and with exogenous CMP-NANA. When strains were grown without CMP-NANA, SBA susceptibility correlated with the amount of unsialylated LNnT above a threshold of LNnT expression; strains that expressed less than the threshold survived in 25% PHS. Exogenous sialylation reduced the expression of free LNnT. Susceptibility to SBA still correlated with expression of LNnT. Only datum points on the linear portion of the curve in Fig. 4A are included, whereas all datum points in Fig. 4B are included.

LNnT. Exogenous sialylation also significantly increased resistance to SBA. The strains that were least resistant to SBA when they were not exogenously sialylated had the greatest increase in resistance when they were exogenously sialylated. The resistance-inducing effect of exogenous sialylation could be overcome by increasing the concentration of PHS. The masking of LNnT expression by sialylation affected killing through both the CP and ACP. The amounts of endogenous, exogenous, and total sialic acid bound to LNnT did not correlate with the resistance of strains to SBA; rather, the loss of free LNnT expression by sialylation was associated with resistance. Because strains make different amounts of LOS bearing LNnT, strains with similar amounts of sialic acid bound to LNnT can vary in the expression of free LNnT (7, 23, 25). It appears that the expression of free LNnT, not the amount of LOS sialic acid, correlates with the amount of killing of organisms in PHS.

The dependence of a neisserial strain's sensitivity to SBA on the concentration of serum has previously been shown for gonococcal strains (13, 36) but remains poorly understood. The SBA depends on the activation of complement on the bacterial surface in such a way that it results in cell lysis. C3 plays a central role in complement-mediated host defense, as it is the site of convergence of the two pathways of complement activation, the CP and ACP. The amount of C3 deposition and the extent of its degradation determine the subsequent fate of complement-opsonized bacteria. C3 binds covalently through a reactive intramolecular thiolester in the C3d domain of its $\alpha$ chain to hydroxyl groups via an ester linkage or to primary amino groups via an amide bond (15). The fate of surface-bound C3b is regulated by factors H and I, found in plasma. The binding of factor H to C3b prevents the formation of C3bBb, the ACP-derived surface-phase C3 convertase, and augments the degradation of C3b by factor I (29). C3b, which binds in a protected molecular environment where factor H is excluded or binds C3b with low affinity, is refractory to cleavage by factor I (11). It can then bind activated factor B to serve as a positive feedback amplifier of its own deposition through the ACP. C3 also participates in the formation of the C5 convertase that cleaves C5 and initiates sequential cleavage of the remaining complement components to form the C5b-C9 membrane attack complex, which results in bacterial lysis (9).

The activated thioester site of metastable C3b has previously been shown to primarily form ester linkages with hydroxyl groups rather than amide linkages with primary amino groups (5, 22). The work of Sahu et al. indicates that the reactivity of metastable C3 exhibits a strong preference for terminal sugar residues (35). Galactose, which is the terminal sugar of unsialylated LNnT, has a strong reactivity with C3, and in particular the six-position hydroxyl group is considerably more reactive than is the average hydroxyl group in sugars. Sialylation of the terminal galactose of LNnT may affect the attachment of C3b to the terminal galactose and to other sugars of the LOS by creating either steric or electrostatic constraints. Previous work from one of our laboratories (16) showed that the deposition of both ester- and amide-linked iC3b as well as total C3 exhibited strain variability for 10 group C LNnT-bearing meningococcal strains but was not influenced by the percentage of LNnT that was sialylated or the amount of group C capsule. This work did not examine the association of free LNnT expression and C3 deposition, however. The results of the present study suggest that the sensitivity of group C organisms to complement-mediated killing is associated with free LNnT expression and may be dependent on the binding of C3 to LNnT.

Previous work (16) also showed that total C3 deposition did not correlate with the sensitivities of group C meningococcal strains to the SBA of 40% human serum. This suggests that a decrease in C3 attachment to LNnT or other LOS structures by sialylation affects sensitivity to SBA by altering the site rather than the total amount of C3 deposition. Several previous studies of the mechanism of stable serum resistance by *N. gonorrhoeae* support this proposal (14, 18). The qualitative rather

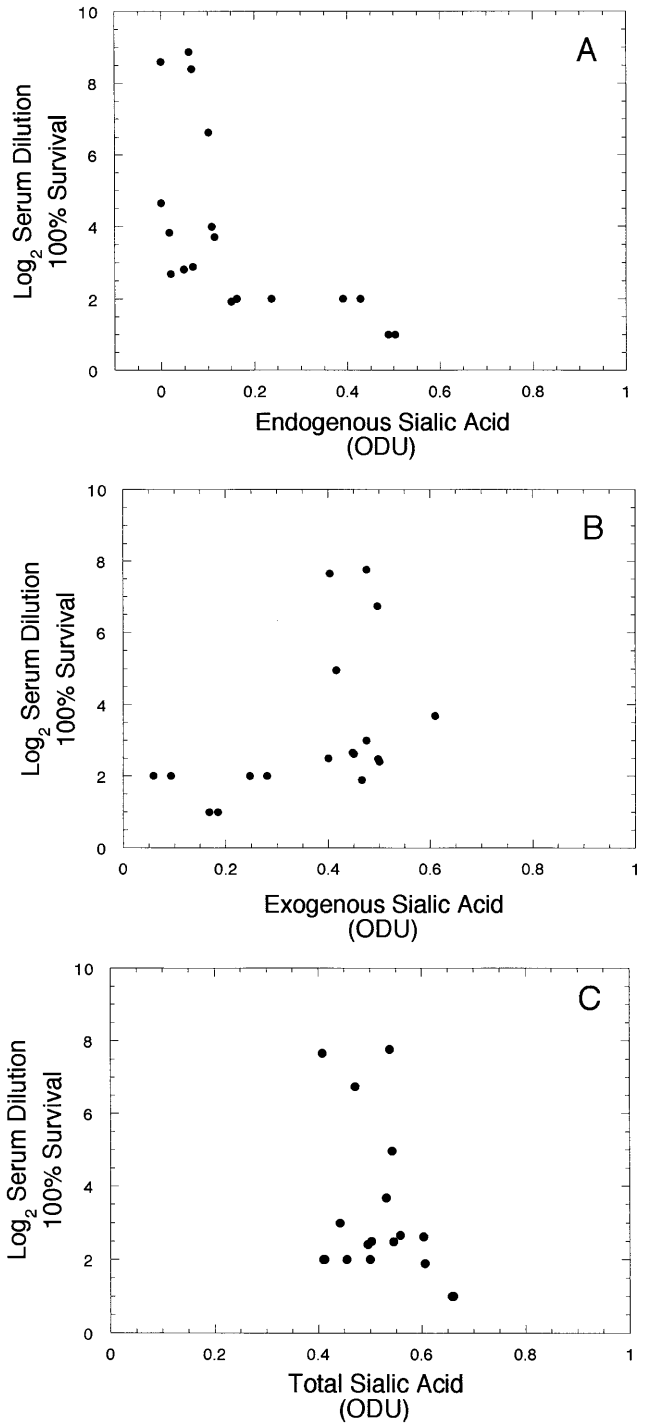


FIG. 6. Serum bactericidal assays of nine meningococcal strains grown to mid-log phase without (A) and with (B and C) exogenous CMP-NANA. For each of two trials per strain, the $\log_2$ dilution of PHS in which survival of each strain reached 100% was plotted against that strain's amount of sialic acid on the LNnT structure of LOS. (A) Because a MAb that binds meningococcal LOS sialic acid is not available, the amount of endogenous sialic acid bound to LNnT (strain grown in broth without CMP-NANA) was determined for each strain in each trial by subtracting the binding of MAb 1B2 (by whole-cell ELISA and reported in ODU) before the removal of sialic acid from LOS with neuraminidase from the binding of MAb 1B2 after neuraminidase treatment. (B) The decrease in the binding of MAb 1B2 (by whole-cell ELISA) to LOS on strains grown in broth with CMP-NANA was used to monitor exogenous LOS sialylation. The amount of exogenous sialic acid added to LNnT was determined for each strain in each trial by subtracting the binding of MAb 1B2 after growth with CMP-NANA from the binding of MAb 1B2 after growth without CMP-NANA. (C) The amount of total sialic acid bound to LNnT (endogenous plus exogenous) was determined for each strain in each trial by subtracting the binding of MAb 1B2 after growth in CMP-NANA from the binding of MAb 1B2 after treatment with neuraminidase (by whole-cell ELISA). The amounts of endogenous, exogenous, and total sialic acid bound to LNnT did not correlate with resistance to the SBA of PHS.

than the quantitative binding of C3 and terminal complement components to the bacterial surface accounts for the stable resistance of gonococci to complement-mediated lysis. The membrane attack complex which mediates efficient bacterial lysis is in a different molecular configuration on the surfaces of sensitive organisms compared to that on the surfaces of resistant organisms. This in turn may influence the covalent linkage of nascent C3 molecules to specific outer membrane components via noncovalent association with these components (18). Whether sialylation of LNnT and the loss of LNnT expression result in redirection of C3 and membrane attack complex deposition on group C meningococci remains to be tested, but if C3 and membrane attack complex deposition is restricted to alternative sites by sialylation of LNnT, these sites may be relatively inaccessible and require higher concentrations of C3 for the deposition of sufficient complexes in close proximity to initiate lysis.

The association of LNnT expression and sensitivity to SBA is not due to bactericidal antibody binding to LNnT. The PHS used in our study was unable to significantly inhibit the binding of MAb 1B2 to LNnT and did not contain antibodies that bound to this LOS on immunoblots. The PHS used did contain antibodies that bound to faster-migrating LOS, which was consistent with our previous work (6, 8). The absence of LNnT antibodies of the same specificity as that of MAb 1B2 was not surprising, as we have previously reported that such antibodies are not found in human sera except after disseminated neisserial infections (24, 36). Although these studies of over 50 individual serum samples were done with gonococcal LOS, the structures of the LNnT on the LOS of these two species are identical; there is no reason to believe that the specificity of MAb 1B2 differs between the two species. In addition, such antibody would not be expected, as neisserial LOS share LNnT structures with human glycosphingolipids (23, 25, 26). Whether sialylation of LNnT structures would interfere with the binding of other bactericidal antibodies to proteins or other LOS structures is unknown. We have previously shown, however, that LOS sialylation of the serum-sensitive *N. gonorrhoeae* strain F62 rendered it serum resistant but did not reduce the total amount of bactericidal IgM antibody bound to the gonococcal surface (16). Meningococci do not make the *lgtD*-dependent structure that binds bactericidal IgM antibody to *N. gonorrhoeae* F62 (13, 17), but they do bind LOS bactericidal antibodies that are directed to structures other than LNnT (6, 8). In the absence of C3 binding to the terminal galactose of LNnT, the killing of meningococci may be dependent on the binding of these antibodies to other LOS sites. The binding of sufficient antibody to provide lysis would require increased concentrations of serum, and therefore of antibody, in the absence of antibody-independent C3 binding.

In conclusion, the expression of free LNnT is directly associated with the amount of killing of group C meningococci in PHS. Both endogenous LOS sialylation and exogenous LOS sialylation are associated with the increased serum resistance of group C meningococcal strains by masking free LNnT. The loss of LNnT expression by sialylation rather than the amount of sialic acid is associated with increased resistance to SBA.

## ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant A132944 to G.A.J.

We thank Ronda Brady for excellent technical assistance.

## REFERENCES

1. **Abdillahi, H., and J. T. Poolman.** 1987. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. FEMS Microbiol. Lett. **48:**367–371.
2. **Apicella, M. A., R. E. Mandrell, M. Shero, et al.** 1990. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: an immunoelectron microscopic analysis. J. Infect. Dis. **162:**506–512.
3. **Artenstein, M. S., B. L. Brandt, E. C. Tramont, J. W. C. Branch, H. C. Fleet, and R. L. Cohen.** 1971. Serologic studies of meningococcal infection and polysaccharide vaccination. J. Infect. Dis. **124:**277–288.
4. **Campos, L., J. Portoukalian, S. Bonnier, et al.** 1992. Specific binding of anti-N-acetyllactosamine monoclonal antibody 1B2 to acute myeloid leudemia cells. Eur. J. Cancer **28:**37–41.
5. **Dodds, A. W., and S. K. Law.** 1988. Structural basis of the binding specificity of the thioester-containing proteins, C4, C3, and alpha-2-macroglobulin. Complement **5:**89–97.
6. **Estabrook, M. M., C. J. Baker, and J. M. Griffiss.** 1993. The immune response of children to meningococcal lipooligosaccharides during disseminated disease is directed primarily against two monoclonal antibody-defined epitopes. J. Infect. Dis. **167:**966–970.
7. **Estabrook, M. M., N. C. Christopher, J. M. Griffiss, C. J. Baker, and R. E. Mandrell.** 1992. Sialylation and human neutrophil killing of group C *Neisseria meningitidis*. J. Infect. Dis. **166:**1079–1088.
8. **Estabrook, M. M., R. E. Mandrell, M. A. Apicella, and J. M. Griffiss.** 1990. Measurement of the human immune response to meningococcal lipooligosaccharide antigens by using serum to inhibit monoclonal antibody binding to purified lipooligosaccharide. Infect. Immun. **58:**2204–2213.
9. **Fearon, D. T., and K. F. Austen.** 1980. The alternative pathway of complement—a system for host resistance to microbial infection. N. Engl. J. Med. **303:** 259–263.
10. **Fox, A. J., D. M. Jones, S. M. Scotland, et al.** 1989. Serum killing of meningococci and several other gram-negative bacterial species is not decreased by incubating them with cytidine-5′-monophospho-N-acetyl acid. Microb. Pathog. **7:**317–318.
11. **Gordon, D. L., J. Rice, J. J. Finlay-Jones, P. J. McDonald, and M. K. Hostetter.** 1988. Analysis of C3 deposition and degradation on bacterial surfaces after opsonization. J. Infect. Dis. **157:**697–704.
12. **Griffiss, J. M.** 1982. Epidemic meningococcal disease: synthesis of a hypothetical immunoepidemiologic model. Rev. Infect. Dis. **4:**159–172. (Review.)
13. **Griffiss, J. M., G. A. Jarvis, J. P. O'Brien, M. M. Eads, and H. Schneider.** 1991. Lysis of *Neisseria gonorrhoeae* initiated by binding of normal human IgM to a hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain-specific, properdin-binding-dependent alternative complement pathway activation. J. Immunol. **147:**298–305.
14. **Harriman, G. R., E. R. Podack, A. I. Braude, L. C. Corbeil, A. F. Esser, and J. G. Curd.** 1982. Activation of complement by serum-resistant *Neisseria gonorrhoeae*. Assembly of the membrane attack complex without subsequent cell death. J. Exp. Med. **156:**1235–1249.
15. **Hostetter, M. K., and D. L. Gordon.** 1987. Biochemistry of C3 and related thiolester proteins in infection and inflammation. Rev. Infect. Dis. **9:**97–109.
16. **Jarvis, G. A.** 1994. Analysis of C3 deposition and degradation on *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Infect. Immun. **62:**1755–1760.
17. **Jennings, M. P., D. W. Hood, I. R. A. Peak, M. Virji, and E. R. Moxon.** 1995. Molecular analysis of a locus for the biosynthesis and phase-variable expression of the lacto-*N*-neotetraose terminal lipooligosaccharide structure in *Neisseria meningitidis*. Mol. Microbiol. **18:**729–740.
18. **Joiner, K. A., K. A. Warren, C. Hammer, and M. M. Frank.** 1985. Bactericidal but not nonbactericidal C5b-9 is associated with distinctive outer membrane proteins in *Neisseria gonorrhoeae*. J. Immunol. **134:**1920–1925.
19. **Kim, J. J., R. E. Mandrell, and J. M. Griffiss.** 1989. *Neisseria lactamica* and *Neisseria meningitidis* share lipooligosaccharide epitopes but lack common capsular and class 1, 2, and 3 protein epitopes. Infect. Immun. **57:**602–608.
20. **Kim, J. J., R. E. Mandrell, Z. Hu, M. A. Westerink, J. T. Poolman, and J. M. Griffiss.** 1988. Electromorphic characterization and description of conserved epitopes of the lipooligosaccharides of group A *Neisseria meningitidis*. Infect. Immun. **56:**2631–2638.
21. **Kim, J. J., D. Zhou, R. E. Mandrell, and J. M. Griffiss.** 1992. Effect of exogenous sialylation of the lipooligosaccharide of *Neisseria gonorrhoeae* on opsonophagocytosis. Infect. Immun. **60:**4439–4442.
22. **Law, S. K., N. A. Lichtenberg, and R. P. Levine.** 1979. Evidence for an ester linkage between the labile binding site of C3b and receptive surfaces. J. Immunol. **123:**1388–1394.
23. **Mandrell, R. E., J. M. Griffiss, and B. A. Macher.** 1988. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. J. Exp. Med. **168:**107–126.
24. **Mandrell, R. E., M. Apicella, J. Boslego, R. Chung, P. Rice, and J. M. Griffiss.** 1988. Human immune response to monoclonal antibody-defined epitopes of *Neisseria gonorrhoeae* lipooligosaccharides, p. 569–574. *In* J. T. Poolman et al. (ed.), Gonococci and meningococci. Kluwer Academic Publishers, Dordrecht, The Netherlands.
25. **Mandrell, R. E., J. J. Kim, C. M. John, B. W. Gibson, J. V. Sugai, M. A. Apicella, J. M. Griffiss, and R. Yaurasaki.** 1991. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J. Bacteriol. **173:**2823–2832.
26. **Mandrell, R. E., A. J. Lesse, J. V. Sugai, et al.** 1990. In vitro and in vivo modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. J. Exp. Med. **171:**1649–1664.
27. **Nairn, C. A., J. A. Cole, P. V. Patel, N. J. Parsons, J. E. Fox, and H. Smith.** 1988. Cytidine 5′-monophospho-*N*-acetylneuraminic acid or a related compound is the low $M_r$ factor from human red blood cells which induces gonococcal resistance to killing by human serum. J. Gen. Microbiol. **134:** 3295–3306.
28. **Nicholson, A., and I. H. Lepow.** 1979. Host defense against *Neisseria meningitidis* requires a complement-dependent bactericidal activity. Science **205:**298–299.
29. **Pangburn, M. K., and H. J. Muller-Eberhard.** 1978. Complement C3 convertase: cell surface restriction of B1H control and generation of restriction on neuraminidase-treated cells. Proc. Natl. Acad. Sci. USA **75:**2416–2420.
30. **Parsons, N. J., J. R. C. Andrade, P. V. Patel, J. A. Cole, and H. Smith.** 1989. Sialylation of lipopolysaccharide and loss of absorption of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5′-monophospho-*N*-acetyl neuraminic acid. Microb. Pathog. **7:**63–72.
31. **Ross, S. C., H. M. Berberich, and P. Densen.** 1985. Natural serum bactericidal activity against *Neisseria meningitidis* isolates from disseminated infections in normal and complement-deficient hosts. J. Infect. Dis. **152:**1332–1335.
32. **Ross, S. C., and P. Densen.** 1984. Complement deficiency states and infection: epidemiology, pathogenesis and consequences of neisserial and other infections in an immune deficiency. Medicine **63:**243–273.
33. **Ross, S. C., and P. Densen.** 1985. Opsonophagocytosis of *Neisseria gonorrhoeae*: interaction of local and disseminated isolates with complement and neutrophils. J. Infect. Dis. **151:**33–41.
34. **Ross, S. C., P. J. Rosenthal, H. M. Berberich, and P. Densen.** 1987. Killing of *Neisseria meningitidis* by human neutrophils: implications for normal and complement-deficient individuals. J. Infect. Dis. **155:**1266–1275.
35. **Sahu, A., T. R. Kozel, and M. K. Pangburn.** 1994. Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation. Biochem. J. **302:**429–436.
36. **Schneider, H., J. M. Griffiss, R. E. Mandrell, and G. A. Jarvis.** 1985. Elaboration of a 3.6-kilodalton lipooligosaccharide, antibody against which is absent from human sera, is associated with serum resistance of *Neisseria gonorrhoeae*. Infect. Immun. **50:**672–677.
37. **Schneider, H., J. M. Griffiss, G. D. Williams, and G. B. Pier.** 1982. Immunological basis of serum resistance of *Neisseria gonorrhoeae*. J. Gen. Microbiol. **128:**13–22.
38. **Schneider, H., T. L. Hale, W. D. Zollinger, R. Seid, Jr., C. A. Hammack, and J. M. Griffiss.** 1984. Heterogeneity of molecular size and antigenic expression within lipooligosaccharides of individual strains of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Infect. Immun. **45:**544–549.
39. **Scholten, R. J. P. M., B. Kuipers, H. A. Valkenburg, J. Dankert, W. D. Zollinger, and J. T. Poolman.** 1994. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. J. Med. Microbiol. **41:**236–243.
40. **Tsai, C., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
41. **Yamasaki, R., J. M. Griffiss, K. P. Quinn, and R. E. Mandrell.** 1993. Neuraminic acid is $\alpha 2 \rightarrow 3$ linked in the lipooligosaccharide of *Neisseria meningitidis* serogroup B strain 6275. J. Biochem. **175:**4565–4568.
42. **Young, W. W., J. Portoukalian, and S. Hakomori.** 1981. Two monoclonal anticarbohydrate antibodies directed to glycosphingolipids with a lacto-*N*-glycosyl type II chain. J. Biol. Chem. **21:**10967–10972.
43. **Zollinger, W. D., R. E. Mandrell, J. M. Griffiss, P. Altieri, and S. Berman.** 1979. Complex of meningococcal group B polysaccharide and type 2 outer membrane protein immunogenic in man. J. Clin. Invest. **63:**836–848.

*Editor:* J. G. Cannon