

Human Lipooligosaccharide IgG That Prevents Endemic Meningococcal Disease Recognizes an Internal Lacto-*N*-neotetraose Structure*

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Hui Cheng[‡], Zhijie Yang[‡], Michele M. Estabrook^{‡§1}, Constance M. John[‡], Gary A. Jarvis^{‡¶}, Stephanie McLaughlin[‡], and J. McLeod Griffiss^{‡¶12}

From the [‡]Department of Veterans Affairs Medical Center, San Francisco, California 94121 and the Departments of [§]Pediatrics and [¶]Laboratory Medicine, University of California, San Francisco, California 94121

Background: IgG that protects children from meningococcal disease binds lipooligosaccharides, but the antigen was not known.

Results: The lacto-*N*-neotetraose α chain conforms at least four antigens; IgG that killed strains with a lacto-*N*-neotetraose α chain bound internal antigens.

Conclusion: Only the internal lacto-*N*-neotetraose antigens induce broadly protective IgG.

Significance: Induction of IgG that recognizes internal lacto-*N*-neotetraose antigens by vaccination would prevent endemic meningococcal disease.

Antibodies that initiate complement-mediated killing of *Neisseria meningitidis* as they enter the bloodstream from the oropharynx protect against disseminated disease. Human IgGs that bind the neisserial L7 lipooligosaccharide (LOS) are bactericidal for L3,7 and L2,4 meningococci in the presence of human complement. These strains share a lacto-*N*-neotetraose (nLc₄) LOS α chain. We used a set of mutants that have successive saccharide deletions from the nLc₄ α chain to characterize further the binding and bactericidal activity of nLc₄ LOS IgG. We found that the nLc₄ α chain conforms at least four different antigens. We separately purified IgG that required the nLc₄ (non-reducing) terminal galactose (Gal) for binding and IgG that bound the truncated nLc₃ α chain that lacks this Gal residue. IgG that bound the internal nLc₃ α chain killed both L3,7 and L2,4 strains, whereas IgG that required the nLc₄ terminal Gal residue for binding killed L2,4 strains but not L3,7 strains. These results show that the diversity of LOS antibodies in human serum is as much a function of the conformation of multiple antigens by a single glycoform as of the production of multiple glycoforms. Differences in sensitivity to killing by human nLc₄ LOS IgG may account for the fact that fully two-thirds of endemic group B meningococcal disease in infants and children is caused by L3,7 strains, but only 20% is caused by L2,4 strains.

Neisseria meningitidis is a unique commensal of the human pharynx that occasionally causes sepsis and meningitis that can

be fulminate and rapidly fatal (1). The asymptomatic commensal relationship is maintained by circulating antibodies that initiate complement-mediated killing of the organisms as they enter the bloodstream from the pharynx (2–4). Bactericidal antibodies in the newborn's serum are IgGs of maternal origin (2), but they are rapidly catabolized and only slowly and irregularly replaced by antibodies induced, first, by colonization of the pharynx by *Neisseria lactamica*, a related bacterium that can split lactose, the disaccharide in milk (5), and then by unencapsulated and therefore essentially avirulent strains of *N. meningitidis* (5–7). By age 10, 45–75% of children have regained serum bactericidal activity (2). The window of susceptibility between the loss of maternal IgG and the autonomous acquisition of bactericidal antibodies correlates with the peak incidence of meningococcal disease (2). The antigenic specificity of protective antibodies has long been sought (8), because their induction by vaccination in infancy would be the most straightforward approach to the prevention of endemic disease.

N. lactamica share lipooligosaccharide (LOS)³ epitopes with *N. meningitidis* but lack common capsular and class 1, 2, and 3 protein epitopes (9). This suggests that the earliest bactericidal antibodies induced in infants may be LOS-specific. However, because each LOS glycoform molecule conforms multiple different epitopes (10, 11), and each meningococcal strain makes several different LOS glycoforms (12), it has been difficult to sort out just which LOS antigens induce protective antibodies (10, 11, 13).

We recently reported a means of affinity-purifying human IgG that binds different LOS structures and showed that these antibodies can kill group B endemic case isolates in the presence of human complement (14). One IgG pool bound LOS

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¹ Present address: Dept. of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110.

² To whom correspondence should be addressed: VAMC (111W1), 4150 Clement St., San Francisco, CA 94121. Fax: 415-221-7542; E-mail: crapaud@loursage.org.

³ The abbreviations used are: LOS, lipooligosaccharide(s); nLc₄, lacto-*N*-neotetraose; nLc₃, lacto-*N*-neotriaose; Lc₂, lactose; Gb₃, globotriaose; Hex, hexose; HexNAc, *N*-acetylhexosamine; PEA, phosphoethanolamine; Hep, heptose; NeuAc, *N*-acetylneuraminic (sialic) acid; SPRIA, solid phase radioimmunoassay; ANOVA, analysis of variance; NHS, normal human serum; ACP, alternative complement pathway; Kdo, 3-deoxy-D-manno-octulosonic acid.

made by the *Neisseria gonorrhoeae* strain, 1291, which have the paraglobosyl lacto-*N*-neotetraose (nLc₄)⁴ structure as their α chain (15). These IgGs were bactericidal for meningococci that have nLc₄ α chains (16–18), as adduced by their LOS immunotypes (19), and that account for ~85% of endemic meningococcal cases (12). *N. lactamica* also make LOS with the nLc₄ α chain (9).

Those data supported the hypothesis that LOS antibodies contribute to natural immunity, but because the strains were characterized only by LOS immunotype, which incompletely captures LOS diversity (12, 21, 22), the exact LOS structures to which the antibodies bind could not be determined. Such detailed information is needed before the question of how best to induce protective LOS IgG in infants can be addressed.

In order to define the specificity of LOS IgG and the breadth of protection that they might afford, we used LOS from pyocin C-selected mutants of strain 1291 that have successive saccharide deletions from the nLc₄ α chain to affinity-purify LOS IgG (14, 15). We then performed genetic analyses of endemic group B case strains and mass spectrometry of intact LOS molecules to provide structural information beyond immunotype. We report that the full-length nLc₄ LOS presents at least four antigens. Our data show that IgG that requires the nLc₄ terminal galactose (Gal) for binding is not bactericidal for L3,7 strains that account for roughly two-thirds of endemic cases but readily kills L2,4 strains that account for about 20% of endemic disease (10–13). IgG that efficiently kills L3,7 strains in the presence of human complement bind one or more internal antigens within the truncated nLc₃ glycolipid. These antibodies also efficiently kill L2,4 strains. Their induction during infancy would protect infants and young children from 85% of endemic group B meningococcal disease.

EXPERIMENTAL PROCEDURES

Bacteria—Strains are described in Tables 1 and 2. *N. gonorrhoeae* strain 1291 is the Gc₂ prototype strain in the Apicella serogrouping system (23). Its LOS has an nLc₄ α chain that lacks the terminal *N*-acetylgalactosamine (GalNAc) substitution common to most gonococcal LOS (24, 25), no β chain, and an *O*-acetylated, *N*-acetylglucosamine (GlcNAc) γ chain (15). There is a single phosphoethanolamine (PEA) substitution of the second heptose (Hep2) at C₃ (15). This is the same structure as that of the L7 meningococcal LOS (16). The 1291 mutants are denominated 1291a–e, based on the electrophoretic mobility of their LOS through SDS-PAGE (26). For this study, we used LOS made by 1291wt and 1291a to affinity-purify LOS IgG.

We used the SPRIA inhibition technique of Zollinger and Mandrell (19), with their original polyclonal rabbit antisera, to determine the LOS immunotype(s) (L-types) of 34 consecutive and unique *N. meningitidis* isolates from the blood or cerebrospinal fluid of epidemiologically unrelated infants and children

TABLE 1
LOS of *N. gonorrhoeae* 1291 and its mutants

Strain	α chain	Structure of LOS α and β Chains ^a (14)
1291wt	LNNt or nLc ₄	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2
1291a	nLc ₃	GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2
1291b	Gb ₃	Galα1→4Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2
1291c	Lc ₂	Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2
1291d	β-Glc	^b Glcβ1→4Hep1 ↑ PEA→3Hep2
1291e	None	Hep1 ↑ ^b PEA→3Hep2

^a Gal, galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Hep, heptose; PEA, phosphoethanolamine.

^b 1291d and 1291e each can make various amounts of both of the LOS glycoforms shown.

TABLE 2
N. meningitidis strains, LOS immunotypes and LOS structures (16–18, 20)

L-type (Strains)	α Chain Designation	Structure of LOS α and β Chains ^a
L2 (8024, 7993)	NeuAc-nLc ₄	^b NeuAc→Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ Glcα1→3Hep2 6 ↑ PEA
L3 (7946, 8024)	NeuAc-nLc ₄	^b NeuAc→Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2
L4 (7993)	NeuAc-nLc ₄	^b NeuAc→Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ Hep2 6 ↑ PEA
L6 (7993)	nLc ₃	GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ Hep2 6 ↑ PEA
L7 (7946Δ <i>lst</i>)	LNNt or nLc ₄	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2
7946Δ <i>igtB</i>	nLc ₃	GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 ↑ PEA→3Hep2

^a NeuAc, *N*-acetylneuraminic (sialic) acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Hep, heptose; PEA, phosphoethanolamine.

^b Only a portion of the nLc₄ glycoforms are sialylated.

⁴ Designations for LOS glycoses follow the recommendations of the International Union of Pure and Applied Chemistry/International Union of Biochemistry and Molecular Biology (IUPAC-IUB) Joint Commission on Biochemical Nomenclature, Nomenclature of Glycolipids (1997). These recommendations can be found at the IUPAC-IUB Web site.

hospitalized during a 15-month period of endemic disease in Houston, Texas (10–13) and selected three group B strains, 7946 (L3,7), 8024 (L2,3), and 7993 (L2,4,6,7), for this study. The Zollinger and Mandrell system arbitrarily sets 35% inhibition

TABLE 3
Oligonucleotide primers used in this study

Primer	Sequence	Source or reference
CB-5	GCCGGCATCGAGGACGTGGAACCTGA	Ref. 28
JL-12	AGCGGCCATCCCAGATACGGA	Ref. 30
JL-50	GGCCGACATCGCGCTTTTGGGCG	Ref. 29
JL-51	GGGGCGATTTTACCTAGCAGATGAA	Ref. 29
LgtB F	GCTTCCGCCGACAGAACGCG	This study
LgtB R	CTGTTCCGCGCTTTTCCGCG	This study
Lst F	CGGCATATTTTCGGTGAAAT	This study
Lst R	CCTTATTTTACCCACGCA	This study
KanF	TTTATGGACAGCAAGCGAAC	This study
KanR	GGAGAAAATACCGCATCAGG	This study
lpt3 F	CGCTGACATTTGTGATTGCT	This study
lpt3 R	TATCGTAGCCCAACGCTGTGA	This study
lpt6 F	ATTTTGTGTTGTAACGGCGG	This study
lpt6 R	GTCTTTGGCTTCCAGACCAG	This study
lgtGin F	ACGCTCATTCTCTTTTCAA	This study
lgtGin R	CCATATGGTAAAGTCGCGG	This study
6GF F	GAAACCCTGTCCAAATTC	This study
6GF R	GAACCAGATGCCGTTGATT	This study

by a test strain of the binding of a type-specific antiserum to its homologous prototype strain as the threshold criterion for a serotype designation; inhibition of prototype-antiserum pairs by <35% is ignored (19). Structures assigned to the serotypes of the strains (16–18, 20) are in Table 2. All organisms were cultured as described previously (12–14).

Sequencing the 1291 *lgt* Operon—Although the structures of LOS made by 1291wt and its pyocin-resistant mutants were known (15), the only known mutations were those of 1291d and 1291e, which both have mutations in *pgm*, which encodes the neisserial phosphoglucomutase (27). We used PCR to amplify *lgtABCDE* from 1291wt and the 1291a–c mutants with the use of primers in Table 3 (28, 29) and sequenced the resulting amplicons. We used the DNA sequence numbering for the *lgtABCDE* region of gonococcal strain, F62, as described in the National Center for Biotechnology Information database under accession number U14554 to orient the sequences to the literature (29).

PCR mixtures contained 2 μ M primer pairs (Integrated DNA Technologies, Coralville, IA), 1 mM dNTPs (Invitrogen), 1% DMSO (Finzymes, Espoo, Finland), 1 \times GC buffer (Finzymes), and 0.5 ml of Phusion High Fidelity Polymerase (Finzymes). The PCR program used was as follows: denaturation at 99 $^{\circ}$ C for 10 min, followed by 10 cycles of 30 s at 98 $^{\circ}$ C, 30 s at 64 $^{\circ}$ C, and 5 min 20 s at 72 $^{\circ}$ C and then 30 cycles during which the annealing temperature was 68 $^{\circ}$ C. Final extension was for 7 min at 72 $^{\circ}$ C.

Amplicons were isolated from a preparative 1.4% agarose gel with use of the Qiagen gel extraction kit (Mountain View, CA), according to the manufacturer's instructions, and then cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen). One Shot TOP10 chemically competent cells provided with the kit were used to generate transformants, which were plated onto Luria-Bertani plates that contained 50 μ g/ml kanamycin. Plasmid minipreps of transformants were analyzed by restriction digestion with EcoRI (New England Biolabs, Ipswich, MA). Digestion products were visualized in 1.4% agarose gels with SBRY Safe stain (Invitrogen). Plasmids from transformants with correct insert size were purified using the QIAprep Spin Miniprep Kit (Qiagen) according

to the manufacturer's instructions and sequenced by Sequetech (Valencia, CA).

LOS—The late Herman Schneider (Walter Reed Army Institute of Research) purified LOS from 1291wt and 1291a (30). LOS were purified from other strains with the use of the same modified hot phenol/water method (30, 31).

Genotyping *N. meningitidis* LOS—We determined the LOS genotypes of 7946, 8024, and 7993 by sequencing *lgtA*, *lgtB*, *lgtE* (28, 32), *lgtG* (33), *lpt3* (34), and *lpt6* (35) with the use of primers in Table 3. The 6GF primers amplify both *lpt6* and *lgtG*, which are on opposite strands, along with short flanking regions. *lgtGin* amplifies an internal *lgtG* segment; *lpt6* amplifies *lpt6*.

Construction of LOS Mutants—Group B *N. meningitidis* endogenously sialylate a portion of their LOS molecules (12, 36–37). LOS sialylation is catalyzed by an α -2,3-sialyltransferase (*Lst*) (38). We inactivated *lst* (38) and *lgtB* (28) in 7946 by insertion of antibiotic resistance cassettes to generate mutant strains, 7946 Δ *lst* and 7946 Δ *lgtB*. *lst* and *lgtB* were amplified by PCR with the use of primers in Table 3. The PCR products were cloned into EcoRI restriction sites of pUC19. The kanamycin resistance gene was amplified by PCR (Table 3) and inserted into the AgeI site of *lst* or the BssHII site of *lgtB*. The plasmids carrying the inactivated genes then were transformed into 7946. Transformants were selected on GC agar plates that contained 50 μ g/ml kanamycin, and the mutants were confirmed by PCR, SDS-PAGE of proteinase K (PK) lysates (30, 39), and mass spectrometry of *O*-deacylated LOS (14, 40).

SDS-PAGE—We screened LOS made by the mutants by SDS-PAGE (30) of PK-treated whole cell lysates from overnight growth (39) with use of a Mini-Protean 3 cell (Bio-Rad) (14). For optimal resolution, LOS were electrophoresed through 27 cm of a 13.1% polyacrylamide gel with an SE600 series vertical slab gel unit (Hoefer, San Francisco, CA) (41). LOS were visualized with silver stain (12, 30).

Mass Spectrometry—Intact 7946 and 8024 LOS and *O*-deacylated LOS of 7946 and its Δ *lst* and Δ *lgtB* mutants were analyzed by negative ion matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), as described (40, 42, 43). MALDI-TOF MS was performed in the linear as well as the reflectron mode on a Voyager-DE STR instrument equipped with a 337-nm nitrogen laser and delayed extraction. Spectra were processed with digital smoothing and base-line correction using Data Explorer software. The spectra of the intact LOS were externally calibrated using the average fragment and molecular ion masses of the intact 1291wt LOS at *m/z* 1836.3, *m/z* 1881.6, and *m/z* 3718.9; a one-point internal calibration using the mass of the lipid A fragment ions at *m/z* 1836.3 also was used for the 8024 spectrum. The spectra of the *O*-deacylated 7946, 7946 Δ *lst*, and 7946 Δ *lgtB* LOS were acquired and externally calibrated using ions for intact 7889 LOS at *m/z* 1836.3 and *m/z* 3676.6 or 891 LOS at *m/z* 1738.3 and *m/z* 4090.1 (42, 43), and then internally calibrated using either one or two calculated masses for peaks at *m/z* 952.0, *m/z* 1677.4, *m/z* 1839.9, and *m/z* 2792.6. Structures were assigned to molecular ions on the basis of composition, previously published structures (12, 15–18, 20, 42–44), L-type (19), and the genetics of LOS synthesis (45).

Affinity Purification of Human LOS Antibodies—Human LOS IgGs were isolated as described (14). Briefly, purified LOS from strains 1291wt and 1291a were disaggregated in 3% deoxycholic acid (46) in 0.2 M carbonate buffer (pH 10.2) and then coupled to epoxy-activated Sepharose 6B (Amersham Biosciences) through the epoxy linkage. Immune Globulin Intravenous (Human) (IGIV) (CSL Behring AG, Bern, Switzerland) was used as the antibody source. 1 g diluted in 10 ml of phosphate-buffered saline (PBS) was loaded onto a column and allowed to bind to LOS. After washing, the bound IgG was eluted with glycine-HCl (pH 2.3), and the pH of the eluate was immediately adjusted to pH 7–8 with 1 M Tris, followed by dialysis against PBS.

To isolate IgG that bound nLc₄ LOS, IGIV was passed through the 1291wt column, and IgG was eluted from the column. To isolate IgG that bound nLc₃ LOS, IGIV was passed through the 1291a column, and IgG was eluted. To isolate IgG that required the nLc₄ terminal Gal for binding, IGIV was passed through the 1291a column multiple times to ensure complete removal of nLc₃ IgG and then passed through the 1291wt gel, after which the IgG was eluted; this IgG is denominated nLc₄-nLc₃ IgG.

Whole-cell ELISA—A whole-cell ELISA (47) was used to analyze binding of IgG. Briefly, organisms were harvested from overnight growth on GC agar plates, washed, and suspended in PBS to an optical density (OD) at 640 nm of 0.1. The suspension was heated at 56 °C for 1 h and kept at 4 °C overnight. 100 μl of the suspension was used to coat microtiter wells, which then were blocked with 1× Roche Applied Science blocker, washed with PBS, and incubated with either 10 μg/ml or serial dilutions of IgG. After washing, goat anti-human IgG conjugated with alkaline phosphatase (Sigma-Aldrich) was added into each well as secondary antibody. After incubation, the wells were washed and developed with 4-nitrophenyl phosphate (Sigma-Aldrich). The absorbance at 405 nm was determined with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). Negative control wells received everything except IgG.

Bactericidal Activity—The bactericidal assay has been described (14). Briefly, bacteria were harvested after overnight growth on GC agar and grown to mid-log phase in GC broth with 1% IsoVitaleX. Bacteria were washed, suspended in gonococcal buffer, and adjusted to an OD at 580 nm of 0.1 (10⁵ cell/ml). The reaction mixture contained 10⁴ organisms/ml, 10–40% NHS, and various concentrations of human IgG. Control reactions contain only NHS or heated inactivated NHS. The tubes were incubated with end-over-end rotation at 37 °C. At time 0 and 60 min, duplicate 10-μl aliquots were removed and plated. Colony-forming units (cfu) were determined after overnight growth. Survival was expressed as the percentage of cfu present at time 0 that survived after 60 min.

NHS was obtained from one of the authors (J. M. G.) who has no history of neisserial infection or meningococcal carriage, and the concentration was adjusted to maximize the complement concentration while minimizing any intrinsic bactericidal activity against each strain. Each assay was repeated in duplicate at least twice.

Statistical Analysis—Statistical analyses of the binding of IgG to the 1291 mutants were performed using SigmaStat for Win-

dows version 3.11 (Systat Software, San Jose, CA). When ANOVA found significant differences among multiple measurements, groups of data were analyzed by the Student-Newman-Keuls method for multiple pairwise comparisons. Analyses of paired data were done using the Excel Student's *t* test program. Values of *p* < 0.05 were considered significant for all comparisons.

RESULTS

1291 LOS—1291wt has a +2 frameshift in its *lgtD* poly(G) tract that results in early termination of *lgtD* transcription and that accounts for the absence of a terminal α chain GalNAc residue. 1291a has a 2867-bp deletion from bp 213 of *lgtB* to bp 223 of *lgtE* that results in deletion of *lgtC* and *lgtD* and translation of an LgtB/E fusion protein.

1291b has a +1 frameshift in its *lgtA* poly(G) tract (15 G residues) that results in early termination of *lgtA* transcription and that accounts for the absence of the terminal *N*-acetylglucosamine of the nLc₄ α chain. Instead, it has the LgtC-catalyzed globosyl Gb₃ α chain, which is not made by the parent strain.

1291c has a +2 frameshift in its *lgtA* poly(G) tract (13 G residues) and a 2875-bp deletion from the end of the *lgtA* poly(G) tract through the *lgtD* poly(G) tract that results in deletion of *lgtB* and *lgtC* and in early termination of translation of an LgtA/D fusion protein. These mutations are consistent with the structures in Table 1.

SDS-PAGE of the LOS of the 1291 mutants is shown in Fig. 1A; the LOS demonstrated the sequential loss of non-reducing terminal sugars shown in Table 1. 1291a made a very small amount of 1291wt LOS, and 1291e LOS was composed of two closely migrating species that together migrated more slowly than 1291d LOS. The second 1291e LOS, made in low abundance, would be the basal phosphoethanolaminyl diheptoside glycolipid plus the β-Glc substitution that initiates the α chain (Table 1 and Ref. 27).

Antigenic Structure of 1291 LOS—Fig. 1 also depicts the binding of 1291wt (nLc₄) LOS IgG to each of the 1291 mutants, as compared with its binding to 1291wt, and as determined by whole-cell ELISA (Fig. 1B), and the binding of 1291a (nLc₃) LOS IgG to 1291wt and each of the 1291b–e mutants, as determined by whole-cell ELISA and as compared with its binding to 1291a (Fig. 1C).

Binding of nLc₄ LOS IgG to the mutants (Fig. 1B) was not uniform (*p* = 0.002; ANOVA); the 1291b–e mutants bound significantly less nLc₄ LOS IgG than 1291a (*p* = 0.007 for 1291b and 1291c versus 1291a; *p* = 0.002 for 1291d and 1291e versus 1291a), which showed that the nLc₄ LOS IgG was composed of more than one specificity. Because 1291wt does not make the 1291b LOS, IgG affinity-purified by its LOS would not contain 1291b specificity, and binding to this mutant would be to the internal 1291c structure that is proximal to the (non-reducing) terminal α-galactose of the 1291b Gb₃ α chain.

Binding of nLc₃ LOS IgG to 1291wt and the 1291b–e mutants (Fig. 1C) partially clarified the multiple specificities within nLc₄ LOS IgG. Again, binding was not uniform (*p* = 0.001; ANOVA); not only did all four of the 1291b–e mutants bind significantly less nLc₃ LOS IgG than 1291wt (*p* < 0.001), but both 1291d (*p* = 0.001) and 1291e (*p* = 0.007) bound less

Bactericidal Human Meningococcal LOS IgG

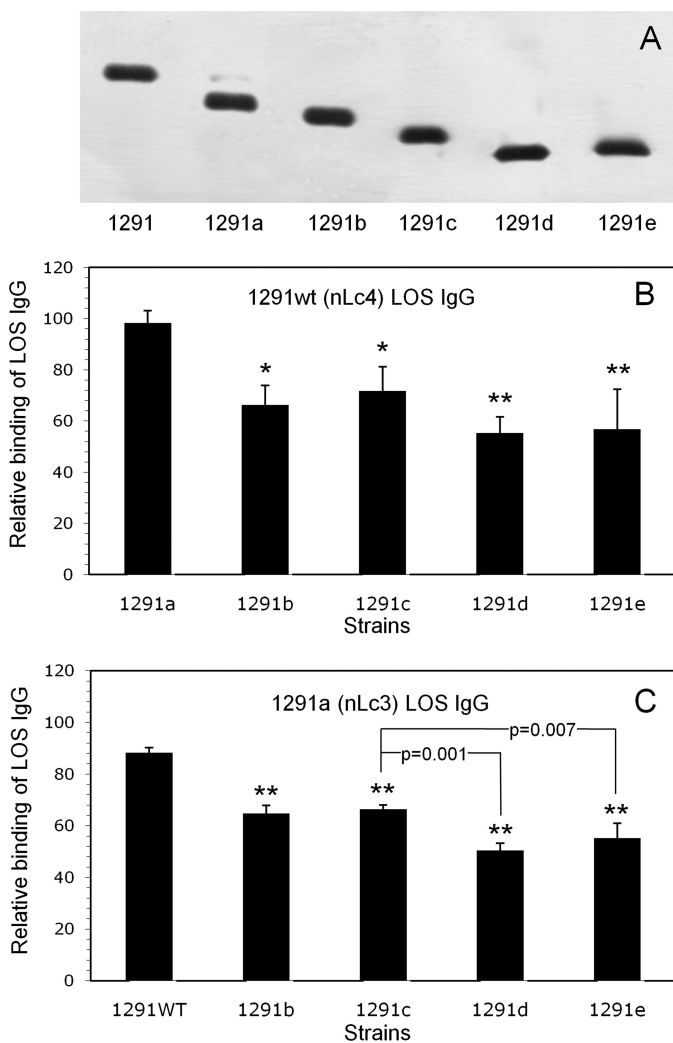


FIGURE 1. Binding of affinity-purified 1291wt (nLc₄) LOS IgG and 1291a (nLc₃) LOS IgG to 1291 and its pyocin-selected mutants. *A*, SDS-PAGE separated LOS from 1291 and each of the 1291 mutants. LOS were electrophoresed through 27 cm of 13.1% polyacrylamide gel and visualized with silver stain. The LOS α chain structures are in Table 1. Note that 1291a makes a small amount of 1291wt LOS and that 1291e LOS is composed of two closely migrating species that, together, migrate more slowly than 1291d. *B*, whole-cell ELISA of 1291wt (nLc₄) LOS IgG. Microtiter wells were coated with the strains and then reacted with 10 μ g/ml IgG. Binding is expressed as the percentage of the binding of the IgG to 1291wt (100%). Error bars, S.D. of three assays. Differences in binding to the strains were statistically significant ($p = 0.002$; ANOVA). Pairwise multiple comparisons (Student-Newman-Keuls method) revealed that the 1291b–e mutants bound significantly less nLc₄ LOS IgG than 1291a (**, $p = 0.002$; *, $p = 0.007$). *C*, whole-cell ELISA of 1291a (nLc₃) LOS IgG. Microtiter wells were coated with the strains and then reacted with 10 μ g/ml IgG. Binding is expressed as a percentage of the binding of the IgG to 1291a (100%). Error bars, S.D. of three assays. Differences in binding to the strains were statistically significant ($p < 0.001$; ANOVA). Pairwise multiple comparisons revealed not only that the 1291b–e mutants bound significantly less nLc₃ LOS IgG than 1291wt (**, $p < 0.001$) but that both 1291d ($p = 0.001$) and 1291e ($p = 0.007$) bound less than 1291c or 1291b. The difference in binding between 1291d and 1291e was not significant ($p = 0.12$).

than 1291c or 1291b. Because 1291a does not make the 1291b LOS, IgG affinity-purified by its LOS would not contain 1291b specificity, and binding to 1291b would be to the internal 1291c structure. Thus, nLc₃ LOS IgG was shown to contain at least three different specificities: itself, 1291c, and 1291d–e.

Binding of nLc₃ LOS IgG to 1291wt was 12% less than to itself, which showed that 1291wt LOS conformed an antigen

that was missing from 1291a. Because 1291a made a small amount of 1291wt LOS (Fig. 1A), the nLc₄ LOS IgG bound 1291a as well as 1291wt, but the amount of 1291wt LOS made by 1291a did not appear to be sufficient to affinity-purify much 1291wt LOS IgG.

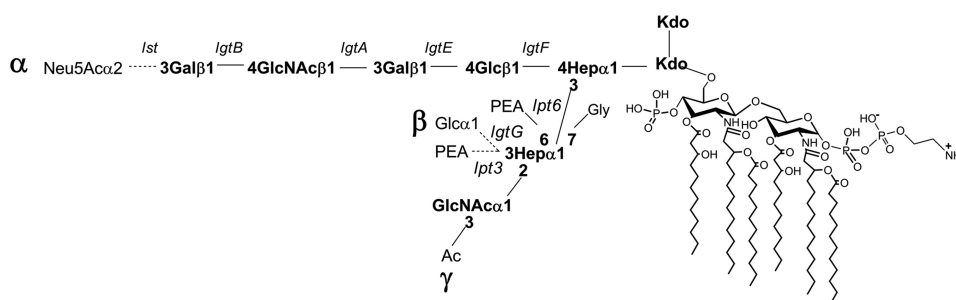
That the nLc₄ LOS α chain conformed an antigen that was not conformed by the nLc₃ α chain was confirmed by passage of IGIV first through the 1291a gel to remove nLc₃ IgG and then through the 1291wt gel, followed by elution of nLc₄ IgG. The yield of nLc₄ LOS IgG from 1 g of IGIV was about 1185 μ g, that of nLc₃ LOS IgG was about 838 μ g, and that of nLc₄-nLc₃ LOS IgG was about 206 μ g. Thus, the yield of nLc₄-nLc₃ IgG from 1 g of IGIV was 17% of that of nLc₄ IgG, a percentage reasonably close to the 12% estimated from the binding of nLc₃ LOS IgG to 1291wt. Thus, nLc₄ LOS IgG was shown to contain at least four different specificities: itself, 1291a, 1291c, and 1291d–e.

Taken together, these analyses enabled estimates of the four specificities within nLc₄ LOS IgG. Roughly 15% of the IgG was specific for the nLc₄ (non-reducing) terminal β -galactose, another 20% was specific for the 1291a nLc₃ α chain, and 15% was specific for the 1291c Lc₂ glycoside. The remaining 50% of the IgG bound one or more of the antigens formed by the basal phosphoethanolaminy diheptoside glycolipid and/or the γ chain GlcNAc. 1291e, which made a small amount of the β -Glc-substituted phosphoethanolaminy diheptoside glycolipid, bound more of both IgG preparations than 1291d, which suggested that this basal glycoside expressed an antigen not formed by the 1291d glycolipid, but the difference between the triplicate measurements was not significant ($p = 0.12$).

Antigenic Structures of Meningococcal LOS—The immunotype glycoside structures are in Table 2; each strain made at least two different immunotypes. Based on SPRIA inhibition, ~30–40% of the LOS molecules made by 8024 or 7993 have the L3 or L7 glycoside, respectively. The L7 glycoside is identical to that of 1291wt, and the L6 glycoside is identical to that of 1291a, except for the location of the PEA substitution.

LOS Genotypes—The LOS genotypes were as expected from their L-types (Fig. 2). 7946 had an active *lpt3*, but *lpt6* was missing, and *lgtG* was mutated and out of frame. 8024 and 7993 had active *lpt6* and *lgtG* genes. 8024 also had an active *lpt3*, as would be needed for it to make the L3 glycoform, but *lpt3* was missing from 7993.

Mass Spectrometry of 7946 and 8024 LOS—MALDI-TOF MS spectra of intact 7946 and 8024 LOS revealed that each strain made multiple lipid moieties to which a common glycoside was linked (Table 4). The lipids differed in the number and positions of phosphate and PEA substitutions (phosphoforms). With one exception, masses of the glycoside moieties were as expected from their immunotypes and genotypes; the compositions assigned to molecular ion peaks are shown in Table 4. The intact 7946 spectrum found two molecular ion peaks that had the same glycoside compositions but differed in their lipid components. The highest mass peak, denominated LOS #1, was observed at m/z 3842.8 and was assigned the composition of Glycoside #1 and Lipid #1. A second molecular ion peak, denominated LOS #2, was found at m/z 3799.4 and was assigned the composition of Glycoside #1 and Lipid #2. Prompt fragment ions observed at m/z 1880.7 (Glycoside #1), m/z 1938.4 (Glycoside #2),



Strain	<i>lgtA</i>	<i>lgtB</i>	<i>lgtE</i>	<i>lpt3</i>	<i>lpt6</i>	<i>lgtG</i>
7946	(+)	(+)	(+)	(+)	(-)	(-)†
8024	(+)	(+)	(+)	(+)	(+)	(+)
7993	(+)	(+)	(+)	(-)	(+)	(+)

FIGURE 2. Schematic presentation of the biosynthetic genes responsible for *N. meningitidis* LOS structures and the genotypes of the study strains. (+), the gene is both present and in frame; (-), the gene is missing from a strain; (-)†, *lgtG* is present in 7946 but inactive because it has a mutation and is out of frame.

TABLE 4

Composition of molecular ion peaks in MALDI-TOF MS spectra of intact LOS

	7946				8024		
	LOS #1	LOS #2	LOS #3	LOS #4	LOS #1	LOS #2	LOS #3
Experimental (M-H)	3842.8	3799.4	3717.3	3777.7	4254.2	4211.5	4088.3
Calculated (M-H)	3842.0	3798.9	3719.0	3776.0	4253.4	4210.3	4087.2
Glycose (Mass)	#1 (L7) (1881.6)	#1 (L7) (1881.6)	#1 (L7) (1881.6)	#2 (1938.6)	#3 (L2) (2293.0)	#3 (L2) (2293.0)	#3 (L2) (2293.0)
Kdo	2	2	2	2	2	2	2
Hep	2	2	2	2	2	2	2
Hex	3	3	3	3	4	4	4
HexNAc	2	2	2	2	2	2	2
O-Acetate	1	1	1	1	0	0	0
PEA	1	1	1	1	1	1	1
NeuAc	0 ^a	0 ^a	0 ^a	0 ^a	1	1	1
Glycine	0	0	0	1	0	0	0
Lipid (Mass)	#1 (1959.4)	#2 (1916.3)	#3 (1836.4)	#3 (1836.4)	#1 (1959.4)	#2 (1916.3)	#4 (1793.3)
HexN	2	2	2	2	2	2	2
C ₁₂	2	2	2	2	2	2	2
3OHC ₁₂	2	2	2	2	2	2	2
3OHC ₁₄	2	2	2	2	2	2	2
Phosphate	2	3	2	2	2	3	3
PEA	2	1	1	1	2	1	0

^a Although not observed in MALDI-TOF MS spectra of intact LOS, (M-H⁻) peaks that resulted from the addition of *N*-acetylneuraminic (sialic) acid were seen in spectra of *O*-deacylated LOS.

m/z 1958.2 (Lipid #1), and *m/z* 1915.4 (Lipid #2) confirmed the assigned compositions.

An additional prompt fragment ion observed at *m/z* 1836.4 was assigned the composition of Lipid #3. A molecular ion peak consistent with Glycose #1 in combination with Lipid #3 was observed at *m/z* 3717.3 and denominated LOS #3. This ion peak was accompanied by an *m/z* 3777.7 molecular ion peak, denominated LOS #4, that would be consistent with the composition of Glycose #2 in combination with Lipid #3. Glycose #2 differs from Glycose #1 by the addition of glycine (*m/z* +57). This assignment was confirmed by the presence of a prompt fragmentation ion at *m/z* 1938.4. Molecular ion peaks that would be consistent with Glycose #2 in combination with either Lipid #1 or Lipid #2 were not seen.

The intact 8024 spectrum was more complex. Two glycoses that differed by the presence or absence of sialic acid (292.3 Da) were each attached to one of the four lipids in Table 4. These four sialylated LOS molecular ion peaks were at *m/z* 4254.2, *m/z* 4211.5, *m/z* 4131.5 (not shown in Table 4), and *m/z* 4088.3; the respective *m/z* -291.3 ion peaks were observed at *m/z* 3964.6, *m/z* 3919.6, *m/z* 3839.8, and *m/z* 3796.4. Prompt fragment ions consistent with the masses of the four respective lipid moieties were observed at *m/z* 1958.2, *m/z* 1915.4, *m/z* 1836.4, and *m/z* 1794.3, and a fragment ion consistent with Glycose #3 was seen at low abundance at *m/z* 2292.5. The mass of Glycose #3 was consistent with the structure of the L2 glycose in Table 2 but lacked the γ GlcNAc *O*-acetate.

As expected from its antigenic structure, a molecular ion peak consistent with the 7946 LOS #3 was seen at *m/z* 3717.3 along with a prompt fragment ion at *m/z* 1880.7 that would be consistent with the 7946 Glycose #1. Fragment ion peaks that represented various combinations of losses of Kdo (-220 Da), phosphate (-80 Da), and hexose (-162 Da) from the various molecules also were present.

Binding of LOS IgG to 7946 and 8024—Both 7946 and 8024 cells bound nLc₄-nLc₃ IgG and nLc₃ IgG (Fig. 3), but both strains bound significantly less nLc₄-nLc₃ IgG than nLc₃ IgG, as would be expected, because the nLc₃ IgG contained at least three specificities absent from the nLc₄-nLc₃ IgG. 8024 sialylates its LOS much more than does 7946, but this did not appear to affect the binding of nLc₄-nLc₃ IgG, because the decrease in binding of nLc₄-nLc₃ IgG, relative to that of nLc₃ IgG, was the same for both strains.

Bactericidal Activity of Human LOS IgG—Both nLc₄ IgG and nLc₃ IgG killed 7946, but nLc₄-nLc₃ IgG did not kill at concentrations of 20–100 μ g/ml (Fig. 4). Thus, all of the bactericidal activity in the nLc₄ IgG was initiated by its nLc₃ component. Increasing the concentration of nLc₄-nLc₃ IgG up to 100 μ g/ml did not result in a significant reduction in survival of 7946 (Fig. 4).

Bactericidal Human Meningococcal LOS IgG

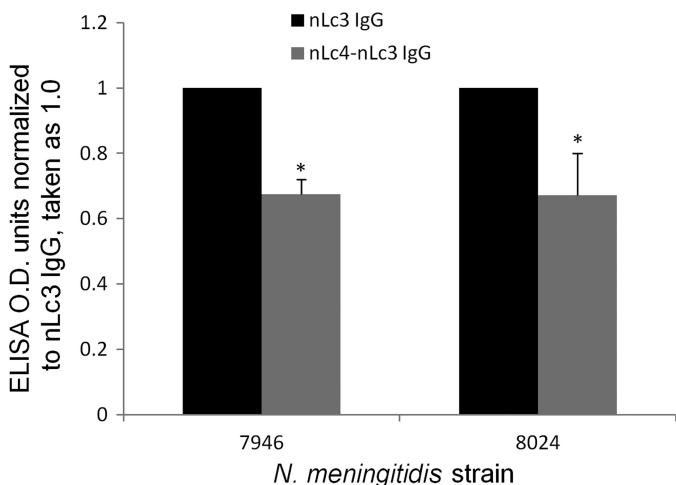


FIGURE 3. Binding of nLc₃ (black bars) and nLc₄-nLc₃ LOS IgG to *N. meningitidis* strains, 7946 and 8024. Each IgG was tested at a concentration of 10 $\mu\text{g}/\text{ml}$. y axes are ELISA OD units normalized to the binding of nLc₃ LOS IgG, which was assigned a value of 1.0. Error bars, S.D.; *, difference between the binding of nLc₄-nLc₃ IgG and nLc₃ IgG to each strain was significant ($p < 0.05$; *t* test).

In contrast, nLc₄-nLc₃ IgG reduced survival of 8024 and 7993 by $\sim 60\%$ at both 50 and 100 $\mu\text{g}/\text{ml}$ IgG concentrations (Fig. 5). nLc₃ IgG was somewhat more bactericidal, reducing survival by about 80% at a concentration of 100 $\mu\text{g}/\text{ml}$.

7946 LOS Mutants—The 7946 Δlst and 7946 ΔltB mutants were designed to have the same LOS glucose as 1291wt and 1291a, respectively, but with the heterogeneous meningococcal lipids. PCR of the mutants with the primers in Table 3 confirmed the deletions, and as expected, migration of 7946 Δlst and 7946 ΔltB LOS in SDS-PAGE was equivalent to that of 1291 and 1291a, respectively (Fig. 6).

Negative ion MALDI-TOF MS of *O*-deacylated 7946wt, 7946 Δlst and 7946 ΔltB LOS confirmed the absence of the Lst- and LgtB-catalyzed glycoses, respectively, without additional structural alterations (Fig. 7). Prominent molecular ion peaks in the 7946wt LOS spectrum at m/z 3084.7 and m/z 2792.6 are in accordance with calculated masses (m/z 3083.8 and m/z 2792.6) for an *O*-deacylated LOS with a diphosphorylated lipid with one PEA and an nLc₄ α chain, with and without sialic acid, respectively. These ions confirm the presence of the sialylated

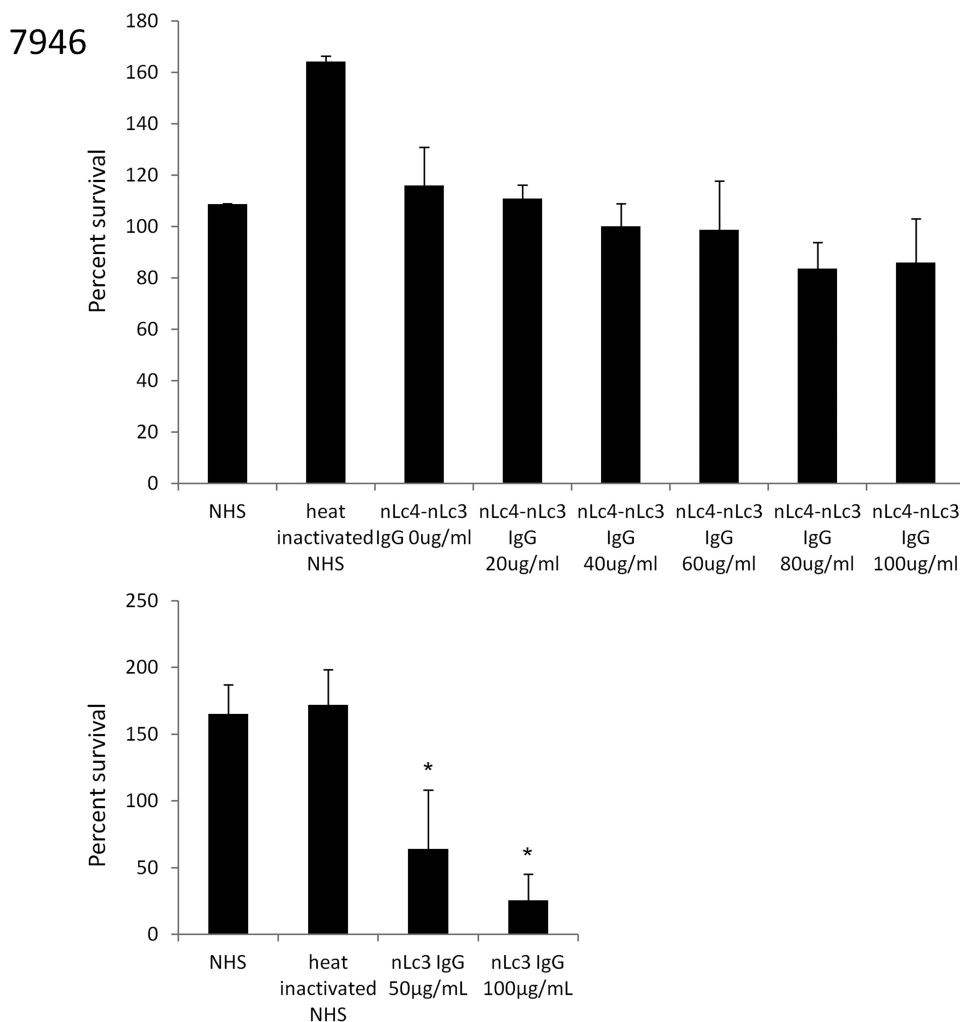


FIGURE 4. Bactericidal activity of nLc₄-nLc₃ IgG (top) and nLc₃ IgG (bottom) for the L3,7 *N. meningitidis* strain, 7946. y axis, percentage of cfu present at time 0 that survived after 60 min in the presence of increasing concentrations of each IgG added to a human serum (NHS) used as the complement source. The NHS added 2.7 μg of nLc₃ IgG and a negligible amount of nLc₄-nLc₃ IgG to each reaction. Error bars, S.D. values; *, $p < 0.05$ (*t* test; one tail) as compared with NHS. The nLc₄-nLc₃ IgG did not significantly reduce survival at any IgG concentration, whereas 50 $\mu\text{g}/\text{ml}$ of the nLc₃ IgG reduced survival by $\sim 50\%$, and 100 $\mu\text{g}/\text{ml}$ of this IgG reduced survival by $\sim 75\%$.

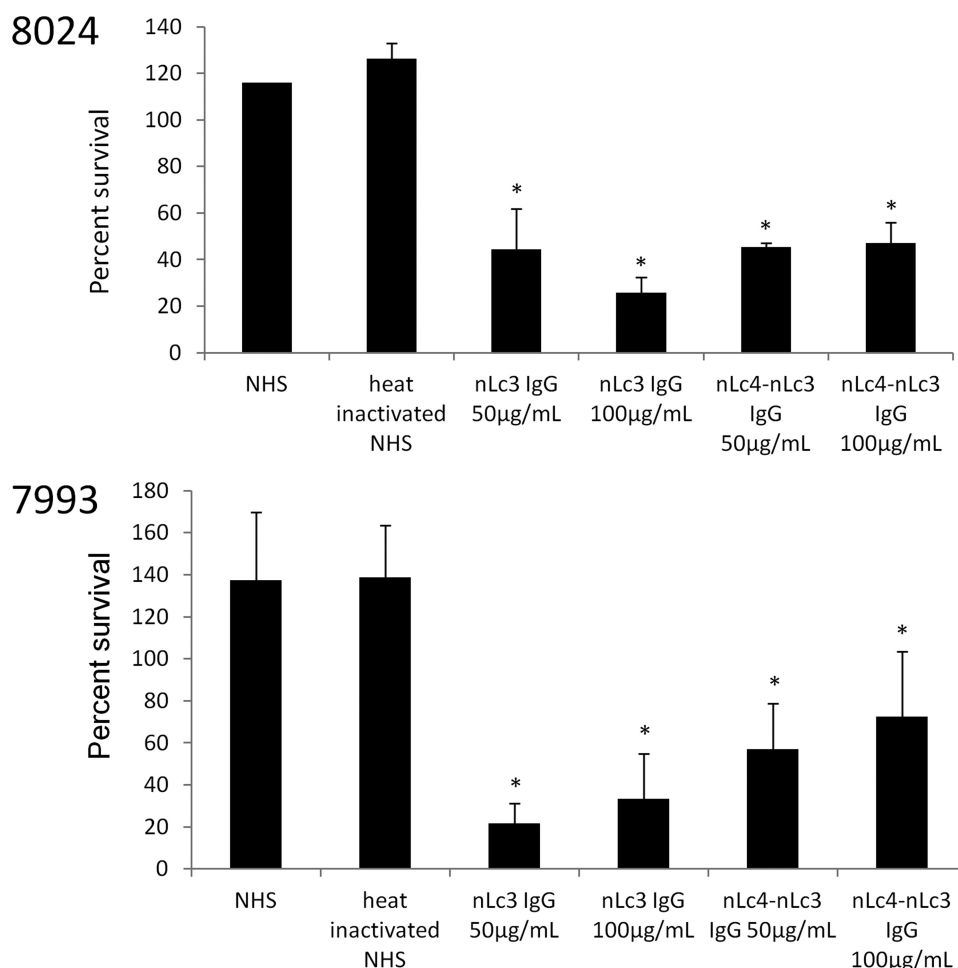


FIGURE 5. Bactericidal activity of nLc₄-nLc₃ IgG and nLc₃ IgG for *N. meningitidis* strains 8024 (L2,3) and 7993 (L2,4,6,7). *y* axis, percentage of cfu present at time 0 that survived after 60 min in the presence of 50 or 100 µg/ml of each IgG added to a human serum (NHS) used as the complement source. The NHS added 2.7 µg of nLc₃ IgG to each of the 7993 reactions and 0.9 µg to each of the 8024 reactions but only a negligible amount of nLc₄-nLc₃ IgG to each reaction. Error bars, S.D. values; *, $p < 0.05$ (t test; one tail) as compared with NHS. The nLc₄-nLc₃ IgG at a concentration of 50 µg/ml significantly reduced survival of both strains, whereas it was unable to reduce survival of strain 7946 at even higher IgG concentrations. Increasing the IgG concentration to 100 µg/ml did not increase the killing of either strain. The nLc₃ IgG also significantly reduced survival of both strains and was more bactericidal than the nLc₄-nLc₃ IgG at concentrations of 100 µg/ml.

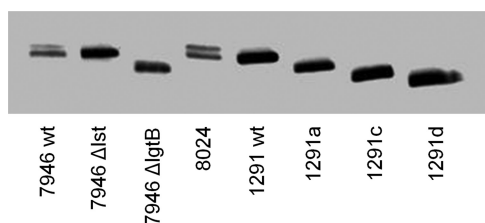


FIGURE 6. SDS-PAGE separated LOS from 7946 and its Δlst and $\Delta lgtB$ mutants compared with LOS from 8026 and 1291wt, 1291a, 1291c, and 1291d. LOS were electrophoresed through 27 cm of 13.1% polyacrylamide gel and visualized with silver stain. 7946 and 8026 both make two LOS; the slower migrating molecule (upper band) is the sialylated LOS, and this glycoform is absent from 7946 Δlst , which has the same electrophoretic mobility as 1291wt. 7946 $\Delta lgtB$ has the same electrophoretic mobility as 1291a and migrates more slowly than 1291c.

L3 and unsialylated L7 LOS glycoforms; the absence of the m/z 3084.7 ion in the spectrum of the 7946 Δlst LOS confirms that *lst* is inactive in the mutant.

7946 $\Delta lgtB$ LOS was expected to differ from 7946wt LOS by the absence of the nLc₄ non-reducing terminal Gal (162 Da), as shown in Table 1. This was confirmed by the mass difference of 163.1 Da between the molecular ions at m/z 2630.8 in the

7946 $\Delta lgtB$ spectrum and the molecular ions at m/z 2793.9 in the 7946 Δlst spectrum. The loss of hexose was supported further by the oligosaccharide fragment ions in the 7946 $\Delta lgtB$ LOS spectrum at m/z 1677.4 and m/z 1456.3 that differ from the analogous ions in the 7946 Δlst LOS spectrum at m/z 1839.9 and m/z 1618.9, respectively, by 162 Da.

Use of the reflectron mass analyzer enabled assignment of oligosaccharide fragment ion masses with greater mass accuracy. For example, using external calibration, the m/z 1618.9 ion in Fig. 7 was calculated at m/z 1618.410 in the 7946 Δlst LOS spectrum; this differs from the calculated mass of 1618.503 by ~60 ppm. The 1618.5 Da ion represents the L7 glucose minus one Kdo (220 Da) and the γ chain GlcNAc *O*-acetate (42 Da). Higher mass ions were not analyzed.

Binding of LOS IgG to 7946 $\Delta lgtB$ —Because the 7946 $\Delta lgtB$ mutant lacks the nLc₄ non-reducing terminal Gal residue, it should not bind nLc₄-nLc₃ IgG, and it did not (Fig. 8). As expected, nLc₃ IgG, which should not require this nLc₄ Gal residue for binding, bound the 7946 $\Delta lgtB$ mutant (Fig. 8). This confirmed the specificity of the nLc₄-nLc₃ IgG for the nLc₄ terminal Gal.

Bactericidal Human Meningococcal LOS IgG

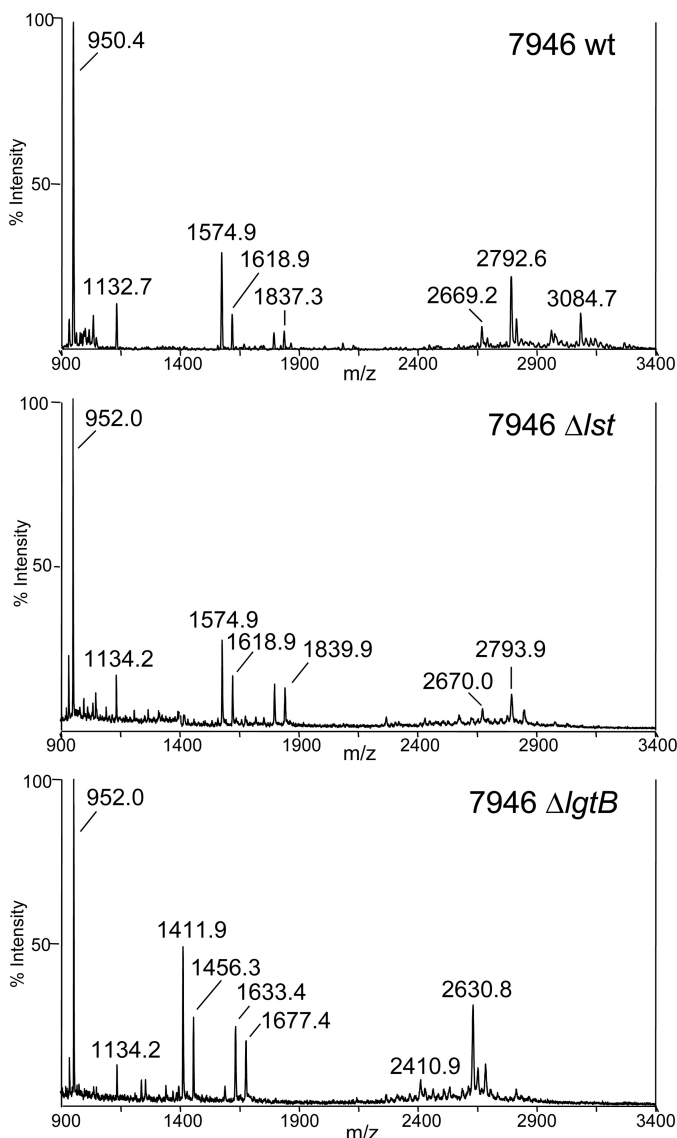


FIGURE 7. MALDI-TOF mass spectra of O-deacylated LOS of 7946, 7946 Δ lst, and 7946 Δ lgtB. Two prominent molecular ion peaks in the 7946wt LOS spectrum (top) at m/z 3084.7 and 2792.6 are in accordance with calculated masses (m/z 3083.8 and m/z 2792.6) for a diphosphorylated O-deacylated phosphoethanolaminylated LOS with an nLc₄ α chain, with and without sialic acid substitution, respectively. The m/z 3083.8 peak is absent from the 7946 Δ lst spectrum. The m/z 2630.8 ion peak in the 7946 Δ lgtB spectrum represents the loss of hexose (162 Da).

Bactericidal Activity of LOS IgG for 7946 Δ lst and 7946 Δ lgtB—nLc₃ IgG, at concentrations of 50 and 100 μ g/ml, reduced survival of 7946 Δ lst by 39 and 76%, respectively (Fig. 9). The corresponding values for nLc₄-nLc₃ IgG were 23 and 58%. The Δ lgtB 7946 mutant did not survive in the serum used as a complement source.

DISCUSSION

These experiments provide new insights into the immunochemistry of neisserial LOS. Each LOS glycoform was thought to present a single antigen centered on the non-reducing terminal glucose, the “immunodominant glucose,” as modified by the various substitutions of Hep2. This paradigm informs the LOS serotyping system (16–20) and many previous studies,

including some of our own (12–14). The diversity of LOS antibodies, according to this notion, was provided by the diversity of LOS glycoforms. The finding that the nLc₄ LOS α chain conforms four or more different antigens was not expected and leads us to conclude that the diversity of LOS antibodies is as much a function of the presentation of multiple antigens within the α chain as it is of the presence of multiple different glycoforms.

Both 1291d and 1291e have mutations in *pgm*, which encodes the neisserial phosphoglucomutase, and neither can convert glucose 6-phosphate to glucose 1-phosphate (27). Because the LOS α chain is initiated by β -Glc, *pgm* mutations would be expected to result in the absence of an α chain. However, both the 1291d and 1291e *pgm* mutations occur outside the active site involved in phosphate binding, both mutants are thought to be able to take up and use glucose 1-phosphate from the growth medium (27), and both have been shown to be able to make some LOS molecules with an α chain β -Glc (15). As can be seen in Fig. 1, the 1291e mutant used in this study made two LOS species that migrated closely together in a slower and broader band than the 1291d LOS. We conclude that this 1291e mutant, but not the 1291d mutant, did make a small amount of LOS with an α chain β -Glc and that this structure represents a fourth α chain antigen. In the original description of the mutants, 1291d migrated more slowly than 1291e and therefore received the “d” designation (23).

A Δ *pgm* mutant that does not acquire exogenous glucose 1-phosphate, such as 1291d, has the same phenotype as a Δ lgtF mutant; their LOS consists of a complex basal glucose and the lipid moiety. The 1291d basal glucose is composed of a phosphoethanolaminyl diheptoside, two dOClA residues, and the γ chain GlcNAc. This complex glycolipid probably forms multiple antigens that are not conformed by the α chain, so the number of antigens conformed by 1291wt LOS is almost certainly greater than our estimate. Roughly half of the nLc₄ IgG recognized one or more basal antigens, as shown by binding to 1291d. We did not seek to understand the antigens presented by Δ *pgm*/ Δ lgtF mutants.

Partially hydrolyzed LOS have a single lipid phosphoform (44), so the finding of multiple phosphoforms also was unexpected. MS of intact LOS of the group C strain, 89I, did find multiple lipid phosphoforms (42, 43), but this might have been an exception. What effects, if any, the different lipids might have on glucose antigen formation (48, 49) were not explored.

Also surprising was that the nLc₄ terminal Gal appeared to protect L3,7 but not L2,4 strains from bactericidal activity. The Δ lgtB mutant, which lacks this terminal Gal, was unable to survive in the serum used as a complement source. The nLc₄-nLc₃IgG that required the terminal Gal for binding was not able to overcome its protective effect for L3,7 strains. This IgG did not bind the 7946 Δ lgtB mutant that has the nLc₃ α chain and the same lipids as the parent strain. Thus, we can conclude that it was specific for the nLc₄ terminal Gal and that its specificity was not influenced by basal adornments or its lipid. The sensitivity of L2,4 strains to bacteriolysis by nLc₄-nLc₃ IgG could explain why they cause endemic disease less frequently (10–13).

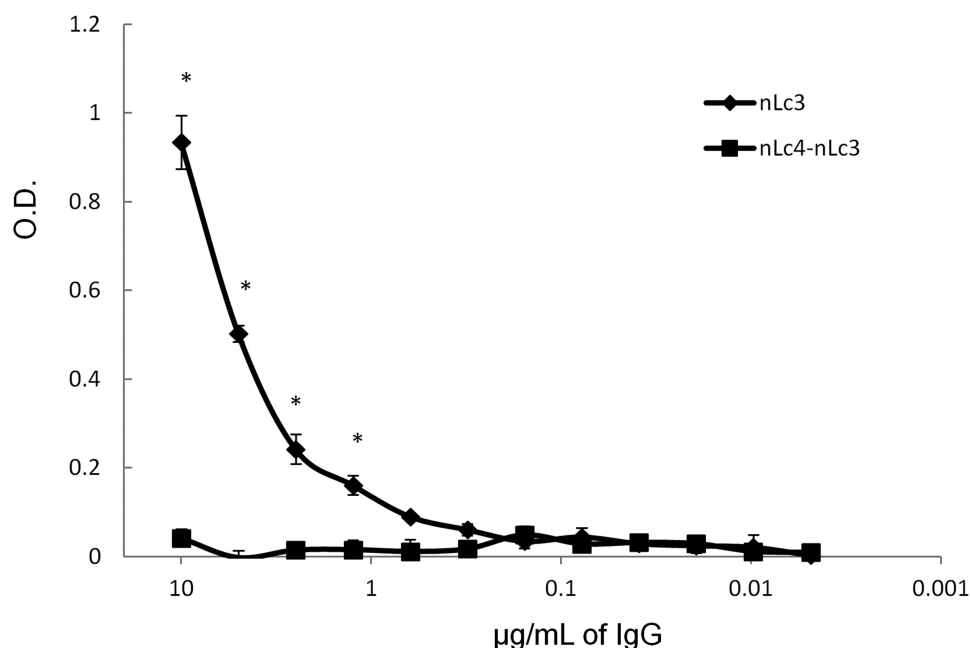


FIGURE 8. Binding of nLc₄-nLc₃ (■—■) and nLc₃ (◆—◆) IgG to 7946ΔlgtB. Error bars, S.D. values; *, $p < 0.05$ (t test; one tail) for the binding of nLc₃ IgG as compared with nLc₄-nLc₃ IgG.

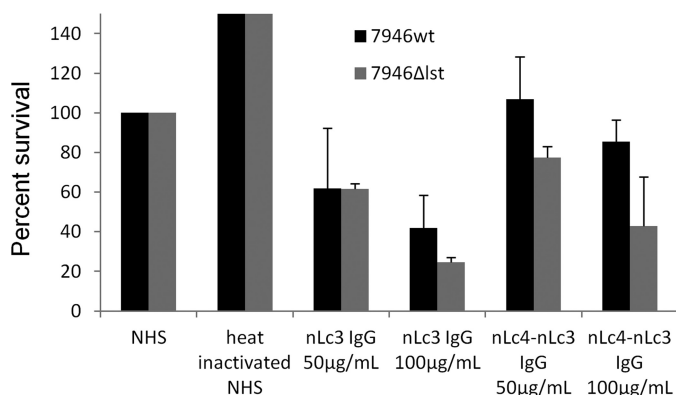


FIGURE 9. Bactericidal activity for 7946wt and its Δlgt mutant of human nLc₄-nLc₃ and nLc₃ IgG. y axis, percentage of cfu present at time 0 that survived after 60 min in the presence of 50 or 100 μg/ml of each IgG added to a human serum (NHS) used as the complement source. Data are normalized to survival in NHS, assigned a value of 100%. Error bars, S.D. values. The NHS added 0.9 μg of nLc₃ IgG and a negligible amount of nLc₄-nLc₃ IgG to each reaction. nLc₄-nLc₃ IgG reduced survival of 7946Δlgt ($p = 0.11$ for the difference in survival between 7946wt and 7946Δlgt in 50 μg/ml nLc₄-nLc₃ IgG, and $p = 0.07$ for the difference in 100 μg/ml nLc₄-nLc₃ IgG).

LOS synthesis by meningococci follows that by gonococci (45), except meningococci do not have an active LgtD. L3 and L7 strains substitute C₃ of Hep2 with PEA (16, 18), catalyzed by Lpt3 (34), whereas L2, L4, and L6 strains substitute C₆ or C₇ of Hep2 with PEA (16, 17, 20), catalyzed by Lpt6 (35). L2 strains substitute C₃ of Hep2 with α-glucose (α-Glc) (17), catalyzed by LgtG (33). An additional β-GlcNac basal substitution has been found on L1 strains (12), and L3 and L4 strains have been reported to have glycine at C₇ of Hep2 (21). We did not explore how these various substitutions affect sensitivity to the bactericidal activity of the two IgG preparations.

40% of 8024 and 7993 bacteria survived 100 μg/ml concentrations of nLc₄-nLc₃ IgG. Both strains make the L3,7 as well as the L2 glycoforms. The genetic basis of the synthesis of the two

glycoforms is such that they are mutually exclusive, and the bacteria making each represents a distinct subpopulation of the global strain population, as has been shown to be the case for both *N. gonorrhoeae* (24, 50) and *N. meningitidis* (51) glycotypes within a strain population. We conclude that the surviving bacteria were the intrinsically resistant L3,7 subpopulations of each strain.

Resistance of L3,7 bacteria to killing by nLc₄-nLc₃ IgG could not be explained by poor binding because it bound to these strains almost as well as did the nLc₃ IgG. Nor could it be explained by any intrinsic defect in its ability to activate complement, because it was bactericidal for L2,4 strains. Surprisingly, LOS sialylation did not affect the binding of either IgG, because binding to the heavily sialylated 8024 bacteria was the same as that to the meagerly sialylated 7946 bacteria.

Bacteria may survive in serum because they intrinsically resist killing by the antibodies present or because the concentration of potentially bactericidal antibodies is inadequate. The ratio of IgG to bacteria in the assay is skewed in favor of survival; 100–300 cfu of bacteria are exposed to μg of IgG in a fixed volume of serum that is not renewed by circulation. IgG specific for the group C meningococcal capsule is bactericidal at concentrations of <2 μg/ml, whereas survival of 7946 was not significantly reduced by exposure to 100 μg/ml of nLc₄-nLc₃ IgG. We conclude that the resistance of 7946 to killing initiated by the nLc₄-nLc₃ IgG was intrinsic and not due to inadequate concentrations of IgG. At high concentrations of IgG, some cross-linking of the bacteria occurs, which can spuriously diminish the observed cfu. In contrast with nLc₄-nLc₃ IgG, IgG specific for one or more of the internal nLc₃ antigens efficiently killed both L3,7 and L2,4 strains, and intrinsic resistance to killing by nLc₃ IgG was not observed.

Why IgG binding to the nLc₄ α chain of the L3,7 and L2,4 glycoforms should result in such different outcomes is not

clear. IgG propagates complement activation through the classical pathway C3 convertase, C4b2a. The Hep2 PEA substitutions provide primary NH₂ groups with which C4b can form amide linkages. The exocyclic PEA of the L2,4 glycoform binds C4b more efficiently than the cyclic PEA of the L3,7 glycoform (52) and thus should maximize the bactericidal effectiveness of bound IgG, but this does not explain the equal sensitivity of L2,4 and L3,7 strains to the bactericidal activity of the nLc₃ IgG.

The exquisite sensitivity to usually fatal meningococcal disease of men with X-linked properdin deficiency and therefore absent alternative complement pathway (ACP) activity (53) shows the importance of the ACP in protection against meningococcal dissemination. When the ratio of IgG to inoculum is low, the density of C3b deposited on the bacterial surface through IgG activation of the classical pathway is not adequate to sustain bacterial lysis without ACP augmentation (54). LOS sialylation interferes with sustained ACP activation (47), and the 7946Δ*lst* mutant, unlike its parent, was modestly sensitive to killing initiated by nLc₄-nLc₃ IgG. But the two L2 strains have more heavily sialylated nLc₄ α chains than 7946, and they were killed by nLc₄-nLc₃ IgG. Furthermore, loss of LOS sialylation had little effect on killing by nLc₃ IgG, so LOS sialylation also does not fully explain the difference in sensitivity to killing of L3,7 and L2,4 bacteria by nLc₄-nLc₃ IgG.

In summary, IgG specific for one or more of the internal nLc₃ antigens was bactericidal for both L2,4 and L3,7 strains; their bactericidal effect was dependent on the concentration of IgG and was not affected by LOS sialylation. In contrast, IgG specific for the nLc₄ terminal Gal was unable to kill bacteria that made the L3,7 glycoform but was bactericidal for those that made the L2,4 glycoform. Resistance of L3,7 strains to this IgG was mediated, in part, by LOS sialylation, whereas sialylation of L2,4 strains did not prevent their killing by the antibody. These data support the conclusion that induction of nLc₃ IgG by *N. lactamica* colonization or vaccination during infancy would protect children from disseminated meningococcal disease.

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