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Meningococcal Group A Lipooligosaccharides (LOS): Preliminary Structural Studies and Characterization of Serotype-Associated and Conserved LOS Epitopes†

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Structural studies indicate that the neisserial lipooligosaccharides (LOS) are composed of an oligosaccharide (OS) portion with a phosphorylated diheptose (Hep) core attached to the toxic lipid A moiety. A conserved meningococcal LOS epitope, defined by monoclonal antibody (MAb) D6A, is expressed on group A and many group B and C meningococci of different LOS serotypes (J. J. Kim, R. E. Mandrell, H. Zhen, M. A. Apicella, J. T. Poolman, and J. M. Griffiss, Infect. Immun. 56:2631–2638, 1988). This MAb-defined D6A epitope is immunogenic in humans (M. M. Estabrook, R. E. Mandrell, M. A. Apicella, and J. M. Griffiss, Infect. Immun. 58:2204–2213, 1990; M. M. Estabrook, C. J. Baker, and J. M. Griffiss, J. Infect. Dis. 197:966–970, 1993). In this study, we characterize this important MAb-defined LOS epitope. Serotype L10 and L11 group A meningococcal LOS were chemically modified and used to investigate what portion of the LOS molecule is important for expression of the conserved (D6A) epitope and serotype-associated LOS epitopes by use of immunoblotting techniques and selected MAbs as probes. Preliminary structural characterization of the LOS was also accomplished by electrospray ionization-mass spectrometry. Our results indicate the following. (i) Antibodies that recognize the serotype-associated or conserved LOS epitopes recognize the OS portion of the LOS. (ii) The phosphorylated diheptose core region of the OS is essential for expression of the conserved D6A epitope. (iii) The lipid portion of the molecule is important for optimum expression of the LOS epitopes. (iv) The proposed compositions of the O-deacylated LOS are consistent with the presence of a phosphorylated diheptose core and are as follows: for O-deacylated L10 LOS, 3Hex (hexose), 1HexNAc (*N*-acetylhexosamine), 2KDO (2-keto-3-deoxy-D-*manno*-octulosonic acid), 2Hep (heptose), 1PEA or 2PEA (phosphoethanolamine), and O-deacylated lipid A; and for O-deacylated L11 LOS, 2Hex, 1HexNAc, 2KDO, 2Hep, 2PEA, and O-deacylated lipid A. Because the phosphorylated diheptose core region of the LOS is essential for the formation of a conserved LOS epitope (D6A) that is immunogenic in humans, care should be taken to maintain stereochemical requirements for the expression of this conserved epitope in the design of effective, nontoxic LOS vaccines.

Despite the development of meningococcal capsular vaccines, *Neisseria meningitidis* continues to be a major cause of bacterial meningitis worldwide (26). The meningococcal capsular vaccines that have been developed are either nonimmunogenic (capsular serogroup B) or require repeated immunizations in young children for an adequate and long-lasting antibody response (26, 50). Several studies support the potential use of lipooligasaccharides (LOS) in a new meningococcal vaccine. Bactericidal LOS antibodies are produced in young children recovering from meningococcal disease and are also present in normal human sera (12, 47). In addition, monoclonal antibodies (MAbs) directed at LOS are protective in animal models of meningococcal disease (32). Thus, further studies are needed to characterize the LOS of the meningococcus.

Verheul et al. recently provided an excellent review of the immunochemistry of meningococcal LOS, their role as a virulence factor, and the use of LOS in a potential vaccine (39). The LOS contain lipid A and an oligosaccharide (OS) moiety. On the basis of available structural data, a generalized structure of neisserial LOS can be deduced, as shown schematically in Fig. 1 (3, 4, 9, 17, 25, 39, 43). The OS can be further divided into two regions: a basal or core region containing heptose and 2-keto-3-deoxy-D-*manno*-octulosonic acid (KDO) and an outer OS portion. The heptose (Hep) residues may be phosphorylated with phosphoethanolamine (PEA), and the Hep_{II} residue is further substituted with glucosamine (GlcNAc) and in some cases glucose (Glc). The outer OS contains a variable number of sugar residues.

Meningococcal LOS can be separated into different LOS serotypes (L1 to L12) by use of polyclonal antibodies or MAbs (1, 22, 30, 48, 49). Often, the LOS from a single strain reacts serologically with several LOS antisera, e.g., L3 with L7 or L9 or L2 with L4 (48, 49). Common immunodominant structural determinants may explain the serologic cross-reactivity between meningococcal LOS of certain serotypes (39, 48, 49). The meningococcal LOS of serotypes L2, L3, L5, L7, and L9 and most gonococcal LOS contain an outer OS branch with a terminal lacto-*N*-neotetraose structure: Galβ1→4Glc NAcβ1→3Galβ1→4Glcβ1 (3, 9, 17, 23, 25, 39, 42). Serogroup B and C meningococci can endogenously sialylate LOS containing the terminal lacto-*N*-neotetraose structure (24, 43). Differences in the position and extent of phosphorylation,

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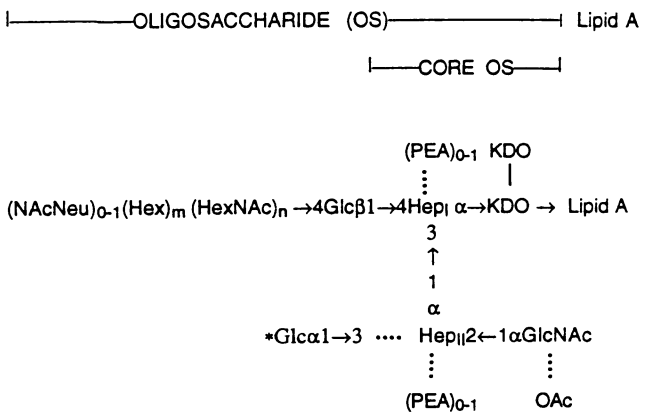

FIG. 1. Generalized structure of neisserial LOS. The OS can be further divided into two regions: a basal or core region containing heptose and KDO and an outer OS portion. The heptose residues may be phosphorylated with PEA, and the Hep_{II} residue is further substituted with GlcNAc and in some cases Glc. The GlcNAc residue may be partially O acetylated (OAc). Attached to the heptose core region is a longer OS branch that contains a variable number of hexose [$(Hex)_m$] *N*-acetylhexosamine [$(HexNAc)_n$], and sialic acid (NAcNeu) residues, depending on the LOS serotype (see the text). For all neisserial LOS structures studied to date, the outer OS contains a →4Glc which is β-linked to C-4 of $heptose_I$ (Hep_I). *, L2 and L5 LOS also contain a Glc α-linked to C-3 of Hep_{II}.

other substitutions of the heptose core, and the presence of other minor LOS components may form the structural basis of certain LOS serotypes (16, 39). The LOS prepared from a single strain may be composed of several components, and immunochemical and structural studies indicate that the higher-molecular-weight LOS components made by an individual strain represent the sequential addition of hexoses or hexosamines to a common basal or core region (Fig. 1) (16, 18, 19, 25, 42).

The LOS serotypes found within serogroup A meningococci (the major capsular serogroup causing epidemics of meningococcal disease) are L8 to L12 (30, 48, 49), and most case isolates are serotypes L9 to L11. The L9 LOS are serologically cross-reactive with the L3 and L7 LOS, and immunochemical studies indicate that the L9 LOS contains the lacto-*N*-neotetraose structure (22, 24, 48, 49). The serotype L10 and L11 LOS are uniquely associated with serogroup A meningococci, and no structural information is available on these LOS (49).

Previously, we analyzed the LOS of group A meningococci (L8 to L11) by polyacrylamide gel electrophoresis (PAGE) and immunoblotting techniques with LOS-specific MAbs (22). The LOS of group A meningococci are composed of one (L10 and L11) or two (L9) major LOS components (22). Serotype (L8 to L11)-specific MAbs were also identified (22). Additionally, a conserved LOS epitope defined by MAb D6A was described. MAb D6A bound LOS from representative LOS serotypes common to group A meningococci (L8 to L11; L12 was not tested). MAb D6A also bound LOS from group B and C meningococci of serotypes L1, L3,7, and L8 as well as some LOS from other *Neisseria* species (6, 21, 22) (see also Materials and Methods). In these experiments, MAb D6A bound major or minor LOS components that were primarily of low molecular masses (usually less than ca. 4.2 kDa).

The LOS serotypes which bind MAb D6A are the major LOS serotypes of strains causing meningococcal disease (39). Additionally, this MAb-defined D6A epitope is immunogenic in humans (5, 6), and MAb D6A is bactericidal. Thus, our goal is to further characterize the structural basis of this conserved (D6A) LOS epitope.

We report here the results of our structural studies of group A meningococcal LOS serotypes L10 and L11 with electrospray ionization-mass spectrometry (ESI-MS) and our investigations of the effect of chemical modifications of group A LOS on the expression of serotype-associated and conserved meningococcal LOS epitopes.

MATERIALS AND METHODS

Bacterial strains. *N. meningitidis* 7880 and 7889, used in this study, are case isolates and are the prototype group A strains for LOS serotypes L10 (7880) and L11 (7889) (49).

Preparation of LOS. Bacteria were grown on supplemented GC agar medium (strains 7880 and 7889) in a CO_2 candle extinction jar, harvested, dehydrated with acetone, and stored at 4°C until ready for use; some preparations of strain 7880 were grown in modified Frantz liquid medium (22).

Acetone-powdered organisms were rehydrated, and LOS were extracted by a modification of the hot phenol-water method of Westphal and Jann (34, 40).

MAbs. MAbs MCA14.2 and 4C4 were used as serotype-associated probes for L10 LOS (MCA14.2) and L11 LOS (4C4) (22). MAb D6A recognizes a conserved LOS epitope. Preparation and characterization of the murine immunoglobulin G MAbs D6A, MCA14.2, and 4C4 were previously described (22). The MAbs were kindly provided by Jan T. Poolman, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands (MAb D6A), Rene Sugasawara, IGSN Inc., Rockville, Md. (MAb MCA14.2), and Michael A. Apicella, University of Iowa, Iowa City (MAb 4C4).

Further characteristics of MAb D6A are as follows. MAb D6A binds LOS of 28 of 28 group A strains (L8 to L11; L12 was not tested). MAb D6A also binds LOS of many group B and C meningococci and other *Neisseria* species (including those of LOS serotypes L1, L3,7, and L8; LOS that is predominantly serotype L7 was not tested) (6, 20–22, 48). MAb D6A does not bind or is only weakly reactive with L2, L4, L5, and L6 LOS (20). A few group A strains that do not bind MAb D6A have been described (2). MAb D6A is bactericidal, i.e., it can lyse meningococci of different LOS serotypes (including L10, L11, L1, and L3,7 strains) (5, 20, 31).

Preparation of OS and HF-treated OS from LOS. The OS of the LOS was released by mild acid hydrolysis (1% acetic acid, 100°C, 2 h) as previously described (46). The OS mixture was lyophilized, redissolved in 100 mM ammonium acetate (5 to 10 mg/ml), and separated by chromatography on a Bio-Gel P-4 column (<400 mesh; 2.6 by 180 cm) equilibrated with 100 mM ammonium acetate. The eluate was monitored by use of a refractometer (R-400; Waters, Millford, Mass.). Two components were obtained: OS-1, $K_{av} = 0.32$; and OS-2, $K_{av} = 0.40$. For the determination of K_{av}, dextran T-40 (Pharmacia) and NaCl were used as standards for V_o and V_t determinations, respectively. Selected fractions were pooled, lyophilized, desalted over a Bio-Gel P-2 column, and relyophilized.

For composition analysis, the OS fractions were dephosphorylated with hydrogen fluoride (HF, 48% [aqueous]; 15 μg of LOS per μl of HF at 4°C for 24 h) (44).

Chemical modification of LOS. To investigate what portion of the LOS molecule is important for expression of the conserved (D6A) epitope and serotype-associated LOS epitopes, we examined LOS epitope expression after dephosphorylation,

removal of the lipid A moiety, or partial deacylation by the following respective chemical modifications of LOS.

(i) HF treatment. LOS was reacted with a 48% HF solution (10 mg of LOS per ml) in the dark at 4°C for 48 h (46). After evaporation of HF, the residues were resuspended in H_2O and lyophilized. Treatment of LOS with aqueous HF results in the removal of phosphate groups.

(ii) NaOH treatment. LOS was treated with 50 mM NaOH (12 mg of LOS per ml) at 80°C for 20 min; this treatment was followed by dialysis and lyophilization. This method has been used to detoxify LOS for vaccine studies. The NaOH treatment results in the removal of some fatty acids (O and N linked) from the lipid A moiety and *O*-acetyl groups (4, 46).

(iii) Periodate oxidation. LOS was suspended in a solution of 50 mM $NaIO_4$ in 100 mM phosphate buffer (pH 7.0) (12 mg of LOS per ml) and incubated at 4°C for 24 h; this step was followed by dialysis and lyophilization (46). Periodate treatment oxidizes vicinal hydroxyl groups on the OS portion of the LOS.

ESI-MS analysis. LOS were O deacylated by the procedure of Helander et al. (15) as described by Phillips et al. (29). In brief, LOS were mixed with anhydrous hydrazine (1 to 10 mg of LOS per ml) and incubated at 37°C for 20 min. Samples were cooled, precipitated with chilled acetone, pelleted, resuspended in H_2O, and lyophilized.

Samples of O-deacylated LOS were analyzed by ESI-MS with a VG Bio-Q mass spectrometer with an electrospray ion source; scans were taken in the negative-ion mode with H_2O-acetonitrile (75%:25% [vol/vol]) containing 1% acetic acid as a solvent (10).

A general approach to the analysis of electrospray data is as follows (7). During the ionization process, a molecular species of mass M may produce a series of ion species, $(M - nH)^{n-}$, of mass M − nH, that vary in their degree of deprotonation. ESI-MS of the molecular species (negative-ion mode) will yield a series of peaks with mass/charge (*m/z*) ratios of $(M - nH)^{n-}/n$. After identification of an ion series pair of charge n_1 and $n_2 = n_1 + 1$, with corresponding *m/z* of $(M - n_1H)^{n_1-}/n_1$ and $(M - n_2H)^{n_2-}/n_2$, respectively, mass M and charge state n_1 can be determined.

ESI-MS was recently used to analyze LOS of several species of gram-negative bacteria, including *Neisseria* species (10). For O-deacylated LOS, the deprotonated species formed are preferentially in charge state $z = 3$ or, to a lesser extent, charge state $z = 2$; additional *m/z* peaks which correspond to the loss of a water molecule during ion formation are often observed (10).

A corresponding molecular weight for each major *m/z* peak can be calculated on the basis of the assigned charge state (z = 2 or 3) as previously described (10). In brief, neisserial LOS are composed of hexoses, *N*-acetylhexosamines (HexNAc), heptose, KDO, sialic acid, PEA, and *O*-acetyl groups linked via a KDO residue to a lipid A moiety (13, 24). A computer program is used (W. Hines, University of California, San Francisco) to generate all possible compositions for an O-deacylated LOS of a specified molecular weight and containing these possible moieties. The OS recovered after acid hydrolysis of neisserial LOS contains two heptose residues and only one KDO residue (4, 9, 17, 18, 25, 42). However, on the basis of mass spectrometric studies of O-deacylated LOS, the intact LOS contains two KDO residues (10). Thus, we considered compositions that yielded a calculated mass that was approximately equal to the observed mass and that also contained a minimum of two heptose residues, two KDO residues, and an O-deacylated lipid A moiety.

For calculations of the predicted mass, the following interval average mass units were used: H, 1.00794; H_2O, 18.015; hexose, 162.142; heptose, 192.169; HexNAc, 203.195; KDO, 220.179; sialic acid, 291.258; PEA, 123.048; and O-deacylated lipid A, 953.009. The mass of O-deacylated lipid A is based on the mass of the diphosphorylated diacyl lipid A moiety from an *N. gonorrhoeae* strain, as determined by Takayama et al. (35).

Carbohydrate composition analysis. For composition analysis of neutral sugars, 20 μg (ca. 10 nmol) of dephosphorylated OS was dissolved in 200 μl of H_2O, treated with 200 μl of 4 M trifluoroacetic acid (TFA), and heated for 4.25 h at 100°C (14). For quantification of amino sugars, samples were prepared as described above except that concentrated HCl was substituted for 4 M TFA (14). The hydrolysate was evaporated to dryness in a Speed-Vac concentrator, and monosaccharide separation and quantification were carried out by high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection as previously described (14, 28, 29). To elute the monosaccharide components, the gradient was slightly modified as follows: (i) 20 mM NaOH for 22 min, (ii) linear to 50 mM NaOH in 10 min, (iii) linear to 100 mM NaOH and 100 mM sodium acetate in 3 min, and (iv) linear to 160 mM sodium acetate in 15 min (with NaOH kept constant at 100 mM). A standard monosaccharide mixture containing fucose, $GalNH_2$, $GlcNH_2$, galactose (Gal), and Glc (Dionex, Sunnyvale, Calif.) was used for quantification. Authentic monosaccharide liberated from the OS of *Salmonella typhimurium* Ra was used as a standard for the identification of L-*glycero*-D-*manno*-heptose (28).

PAGE and immunoblot analysis. LOS and LOS derivatives (0.06 to 0.12 μg) were separated by PAGE with a Bio-Rad PROTEAN 2 Cell as previously described (22). One gel was silver stained by the method of Tsai and Frasch, and a duplicate gel was electroblotted to nitrocellulose (22, 37). The electroblotted LOS were analyzed for their reactivity with the above-mentioned MAbs as previously described (22), except that alkaline phosphatase-labelled goat anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, Mo.) and substrate (naphthol-X-phosphate and Texas red; Sigma) were used to detect bound mouse MAbs.

The silver staining of the LOS was overdeveloped to better visualize the modified LOS. Immunoblots were allowed to develop maximally in enzyme substrate and could be compared quantitatively for differences in MAb binding.

Dot blot analysis. Samples of LOS and LOS derivatives were dried over phosphorous pentoxide in vacuo and dissolved (1.0 mg/ml) in 0.2% sodium deoxycholate (Sigma); the OS released by acetic acid hydrolysis was lyophilized and dissolved in water (10 mg/ml). Serial twofold dilutions of LOS and LOS derivatives were dotted onto silica gel-impregnated glass fiber sheets (Gelman Sciences, Ann Arbor, Mich.). Sheets were coated with 0.5% methacrylate and incubated sequentially in 0.5% casein, MAb, and ^{125}I-labelled goat anti-mouse immunoglobulin G (Amersham) (46). Autoradiographs were analyzed with a Zeineh (Sunnyvale, Calif.) video densitometer; densitometer measurements of the modified LOS were obtained relative to the absorbance intensity of the native LOS.

RESULTS

As determined by PAGE analysis, the LOS of meningococcal strains 7880 (serotype L10) and 7889 (serotype L11) consist of major components with estimated molecular masses of 4.1 and 3.6 kDa, respectively, and MAbs that bind either L10 or L11 or both LOS have been identified (Fig. 2) (22). The L10

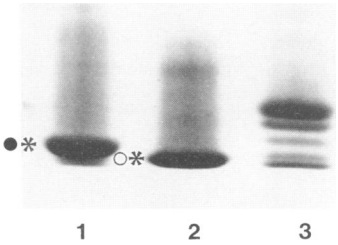

FIG. 2. Silver-stained PAGE of meningococcal LOS and MAb binding patterns for L10 LOS (7880) (lane 1) and L11 LOS (7889) (lane 2). For comparison, the LOS of strain 8002 (group B; LOS serotype not determined) is shown in lane 3. MAb MCA14.2 binds to the major L10 component (●). MAb 4C4 binds to the major L11 component (○). MAb D6A binds to a conserved epitope expressed on both L10 and L11 LOS (*). The silver staining of the L10 LOS is overdeveloped and demonstrates both a major component and a slightly more rapidly migrating minor component.

LOS also contains an additional minor component with a molecular mass of ca. 3.6 kDa.

To gain insight into the structures of the L10 and L11 LOS, we analyzed these samples by ESI-MS (Fig. 3 and 4). LOS were O deacylated to improve solubility for ionization. The major molecular ion species and the proposed compositions of the species are summarized in Table 1.

ESI-MS analysis of O-deacylated L10 LOS indicates that there are two major species, with observed molecular weights of 2,589.9 and 2,711.6. These species correspond to the observed triply charged, $(M - 3H)^{3}$, peaks at *m/z* 863.0 and 903.7 and their doubly charged, $(M - 2H)^{2}$, counterparts at *m/z* 1,292.9 and 1,353.6, respectively. In ESI-MS analyses of O-deacylated LOS, additional *m/z* peaks which correspond to the loss of a water molecule from the parent molecular ion are often observed (10). Thus, the slightly less abundant peaks at *m/z* 856.9 and 897.9 represent species due to the loss of a water molecule from the parent molecular ions, $(M - H_2O - 3H)^{3}$. Similar peaks corresponding to the doubly charged anhydro forms of the LOS are also observed (Fig. 3 and Table 1).

On the basis of the observed molecular weights of the O-deacylated LOS, we have proposed compositions for the major molecular ion species of the O-deacylated L10 LOS (Table 1). The O-deacylated L10 LOS is composed of two major components, which contain an O-deacylated lipid A moiety, 3Hex (hexose), 1HexNAc, 2Hep (heptose), 2KDO, and 1PEA or 2PEA. The heterogeneity in the number of PEAs and the formation of ion species with a loss of an H_2O molecule contribute to the complexity of the electrospray ionization spectra.

ESI-MS analysis of O-deacylated L11 LOS was done in a similar fashion (Fig. 4), and the assignment of the peaks and proposed compositions are summarized in Table 1. There is one major species (observed molecular weight, 2550.1) corresponding to the triply charged peak at *m/z* 850.0 and the doubly charged peak at *m/z* 1,272.6 and their respective anhydro forms. A small peak at *m/z* 809.2 (and 802.7) may correspond to an O-deacylated LOS lower in mass (123 Da) (lacking PEA) (and the corresponding anhydro form).

ESI-MS provides a rapid method of accurately determining the molecular weights of bacterial LOS (10). On the basis of the observed mass of intact gonococcal lipid A, we can calculate expected molecular weights for native L10 and L11 LOS of 3,472.8 and 3,311.3, respectively, with 2PEA (+761.2 Da for O-linked fatty acids) (35). These are in the range of our previous estimates of molecular masses of 4.1 and 3.6 kDa, respectively, based on electrophoretic mobility, with *Salmonella* rough mutants as a standard (22).

In summary, on the basis of ESI-MS results, we propose the following compositions: for L10 LOS (7880), 3Hex, 1HexNAc, 2Hep, 2KDO, 1PEA or 2PEA, and lipid A; and for L11 LOS (7889), 2Hex, 1HexNAc, 2Hep, 2KDO, 2PEA, and lipid A. (Note that the HexNAc of some neisserial LOS may be O acetylated [3, 4, 9, 18, 25]. The acetate groups would be cleaved during preparation of the LOS for ESI-MS analysis.)

To verify the proposed composition of the L10 LOS, we determined the composition of the OS portion of the LOS of

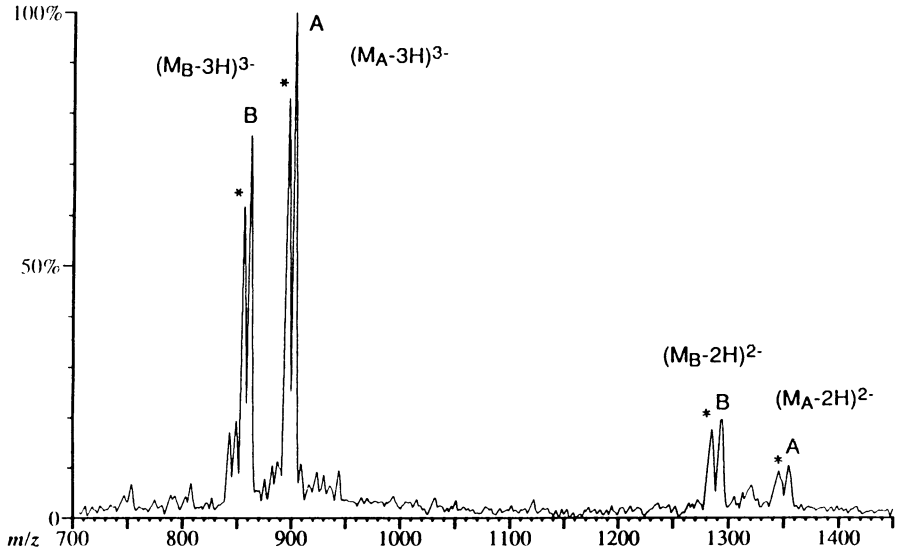

FIG. 3. ESI-MS of O-deacylated L10 LOS (strain 7880). The masses and proposed structures for the different LOS species (A and B) are listed in Table 1. The peaks designated by an asterisk represent adjacent ion species with an additional loss of an H_2O molecule.

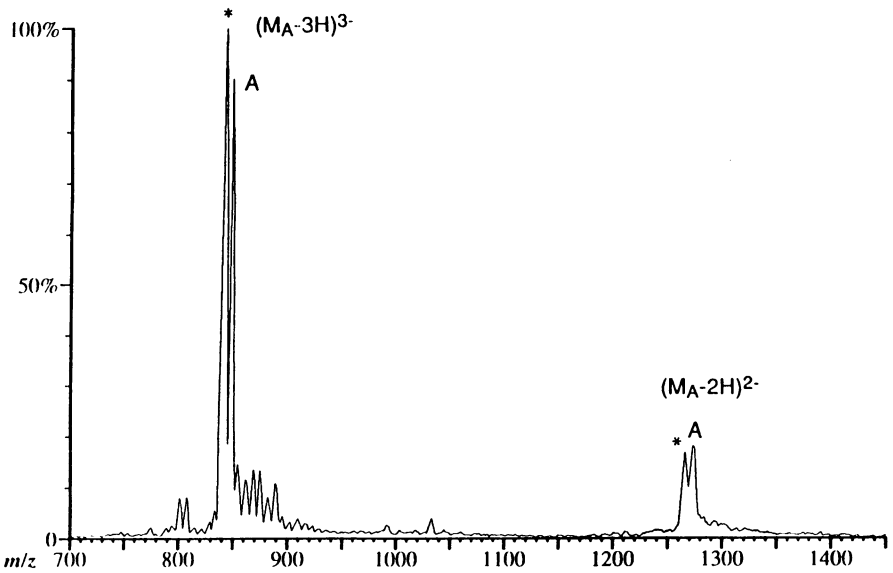

FIG. 4. ESI-MS of O-deacylated L11 LOS (strain 7889). The masses and proposed structures for the LOS species (A) are listed in Table 1. The peaks designated by an asterisk represent adjacent ion species with an additional loss of an H_2O molecule.

strain 7880. The chromatogram of the separation of the OS by size exclusion chromatography is shown in Fig. 5. Fractions were pooled into two components, OS-1 (fractions 81 to 86) and OS-2 (fractions 90 to 96), for further analysis. To determine the monosaccharide composition, dephosphorylated samples of OS-1 and OS-2 were hydrolyzed with TFA (neutral sugars) or HCl (amino sugars) and analyzed by HPAEC. Analysis of hydrolysates of dephosphorylated samples of OS-1 and OS-2 yielded identical compositions; therefore, the OS-1 and OS-2 samples differ in the degree of phosphorylation.

Monosaccharide analysis of 7880 OS yielded a Gal/Glc/$GlcNH_2$ (glucosamine) molar-equivalent ratio of 1.0:2.1:1.0, consistent with the compositions predicted by ESI-MS (Table 1). (Note that the native amino sugars are N acetylated but are deacetylated during hydrolysis.) Heptose was detected but not quantified. There was incomplete hydrolysis of the basal or core region when TFA was used (41), and HCl hydrolysis degraded the neutral sugars (14, 41). The KDO moiety could be readily detected by HPAEC (28, 42). Thus, our composition analysis of 7880 LOS confirmed the presence of 3Hex, 1HexNAc, and 2Hep, and the composition analysis of the dephosphorylated OS fractions indicated that these two fractions differ in the degree of phosphorylation, as was proposed by ESI-MS analysis.

Our ultimate goal is to investigate the use of group A meningococcal LOS in a vaccine against meningococcal disease. Because LOS must be detoxified prior to use in a vaccine, important LOS epitopes must be characterized and maintained during chemical modification of LOS.

We sought to characterize the effect of chemical modification on L10 and L11 LOS epitope expression by using several previously described MAbs as probes. As illustrated in Fig. 2, MAb D6A binds to the LOS of both the L10 and the L11 strains, whereas MAb MCA14.2 binds to only L10 LOS and MAb 4C4 binds to only L11 LOS (22).

Our ESI-MS and composition data indicate that the L10 and

TABLE 1. Proposed compositions and molecular weights of the major O-deacylated LOS of *N. meningitidis* serotypes L10 and L11

Strain (serotype)	Proposed composition[a]	Mol wt		*m/z* (*z*)
		Calculated	Observed[b]	
7880 (L10)	3Hex, 1HexNAc, 1PEA, 2Hep, 2KDO, and O-deacylated lipid A[c]	2,590.4	2,589.9	863.0 (3), 856.9[d]; 1,292.9 (2), 1,284.2[d]
	3Hex, 1HexNAc, 2PEA, 2Hep, 2KDO, and O-deacylated lipid A[c]	2,713.5	2,711.6	903.7 (3), 897.9[d]; 1,353.6 (2), 1,345.0[d]
7889 (L11)	2Hex, 1HexNAc, 2PEA, 2Hep, 2KDO, and O-deacylated lipid A[f]	2,551.3	2,550.1	850.0 (3), 844.0[d]; 1,272.6 (2), 1,265.2[d]

[a] Hex, hexose; Hep, heptose.
[b] Based on the average of the molecular weights obtained from the two charge states of a given species.
[c] Species B in Fig. 3.
[d] For each major species, an additional, lower-mass species, corresponding to a loss of an H_2O molecule, $(M - zH - H_2O)^{z-}$, was also observed. The corresponding ion species differed by ca. 18 Da/*z*.
[e] Species A in Fig. 3.
[f] Species A in Fig. 4.

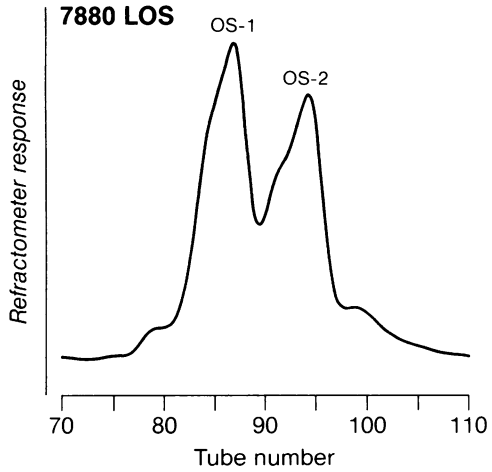

FIG. 5. Chromatogram of the OS of the L10 LOS (strain 7880) separated over a Bio-Gel P-4 column with an elution buffer containing 0.1 M ammonium acetate, showing the refractive index of the eluate. The OS contains two major components: OS-1 (tubes 81 to 86; K_{av}, 0.32) and OS-2 (tubes 90 to 96; K_{av}, 0.40).

L11 OSs are phosphorylated and, as indicated from previous structural studies, it is likely that the diheptose portion of the LOS is the site of phosphorylation (13, 39). Thus, we hypothesized that the phosphorylated diheptose portion of the LOS molecule contributes to the formation of the conserved D6A epitope.

To test this hypothesis and gain insight into other structural requirements for the expression of the D6A LOS epitope and

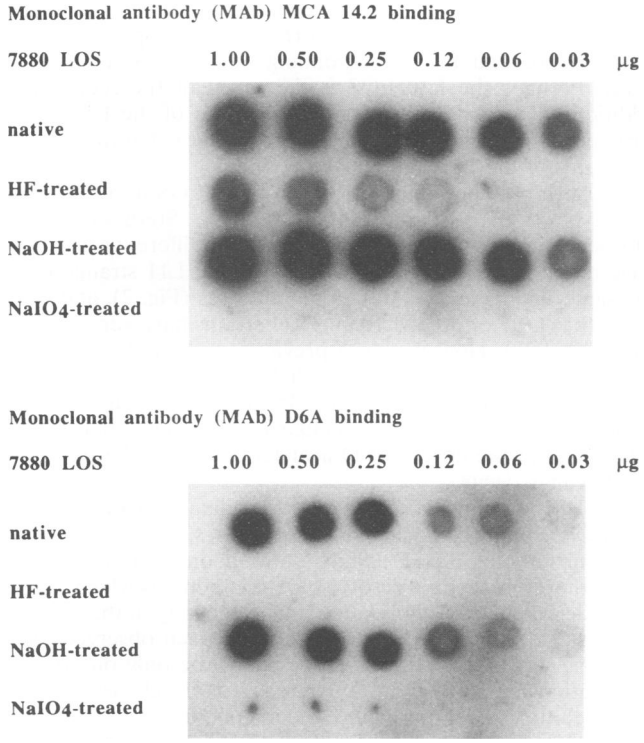

FIG. 6. Dot blot analysis of L10 LOS and LOS derivatives with MAbs MCA14.2 and D6A.

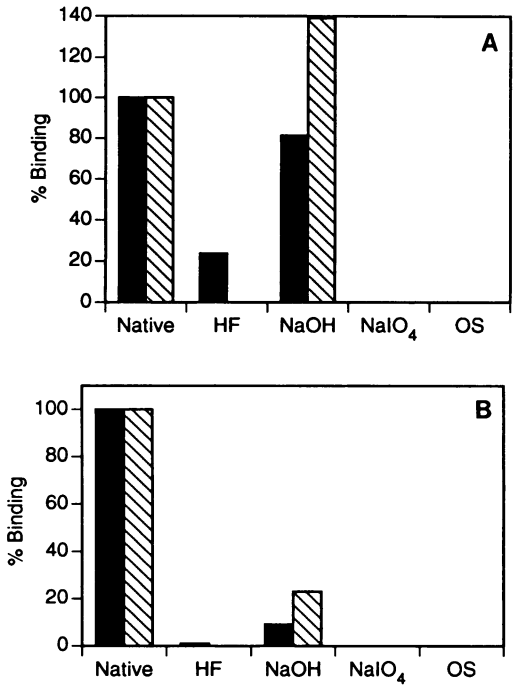

FIG. 7. Percent binding by MAbs of modified L10 (A) and L11 (B) LOS relative to that of the native (unmodified) LOS. Percent binding is defined as the relative densitometry reading [(optical density of treated sample/optical density of native sample) × 100]. Densitometry readings were taken for 1.0 μg of native or treated LOS. For OS, densitometry readings were taken for 10.0-μg samples. Symbols: ■ MAbs MCA14.2 (for L10 LOS) and 4C4 (for L11 LOS); ▨, MAb D6A (for both LOS).

L10 and L11 serotype-associated LOS epitopes, we examined the effect of dephosphorylation, modification of the OS, or modification of the lipid A portion of the LOS molecule on MAb binding by dot blot analysis. A representative dot blot analysis of derivatized 7880 LOS reacted with MAbs MCA14.2 and D6A is shown in Fig. 6, and a summary of the results of an analysis of the dot blots by densitometry is shown in Fig. 7.

PAGE and immunoblot analysis of native and derivatized LOS were also performed and confirmed the results obtained by dot blot analysis. Representative PAGE of native and modified 7880 and 7889 LOS and a corresponding immunoblot analysis with MAb D6A are shown in Fig. 8.

Dot blot, PAGE, and immunoblot studies showed that MAb binding was altered after treatment of the LOS. However, the effect on MAb binding differed, depending on the treatment, the LOS type, and the MAb examined. The results were as follows.

After the removal of phosphate groups by aqueous HF treatment, the binding of all MAbs studied was decreased, but MAb D6A binding was most greatly affected by dephosphorylation. After HF treatment, there was a dramatic loss of the ability of 7880 and 7889 LOS to bind MAb D6A. The binding of MAb D6A to 0.03 μg of untreated LOS (the lowest amount tested) was easily visualized. In contrast, 1.0 μg of HF-treated LOS failed to bind MAb D6A (Fig. 6 and 7). PAGE demonstrated that the HF-treated LOS samples were not degraded and migrated as a narrower band with a slightly higher apparent molecular weight. These HF-treated LOS no longer bound MAb D6A (Fig. 8, lanes 3 and 9). The charge of the PEA group and the heterogeneity in the extent of phosphor-

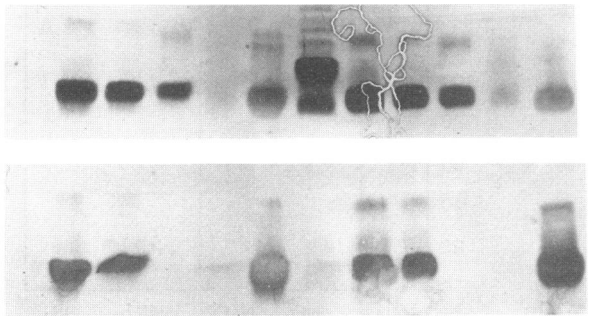

FIG. 8. Silver-stained PAGE and immunoblot analysis of meningococcal LOS and LOS derivatives. (A) Silver-stained electrophoresis gel. (B) Immunoblot with MAb D6A. Lanes 1 to 5 contain L10 LOS and LOS derivatives, and lanes 7 to 11 contain L11 LOS and LOS derivatives. Samples include native LOS (0.125 μg, lanes 1 and 7; 0.063 μg, lanes 2 and 8), HF-treated LOS (0.125 μg, lanes 3 and 9), $NaIO_4$-treated LOS (0.125 μg, lanes 4 and 10); and NaOH-treated LOS (0.125 μg, lanes 5 and 11). The LOS of a meningococcal group Y strain, 8032 (LOS serotype not determined), is shown in lane 6. The very faint silver staining of the periodate-treated samples is at the level of migration of the unmodified LOS and most likely represents minimal incompletely reacted LOS.

ylation (for L10 LOS) may have contributed to the migration of the LOS as a broad band in PAGE.

In comparison, after HF treatment, binding of the L10 serotype MAb (MCA14.2) to L10 (7880) LOS was only decreased to 23%, as compared with MAb binding to the native (i.e., untreated) L10 LOS. Although binding of the L11 serotype MAb (4C4) was still detectable by autoradiography, it was greatly decreased (to <1%) after HF treatment.

NaOH treatment of the LOS had a different effect on LOS epitope expression. Although the LOS was altered in electrophoretic mobility and was not silver stained well after the removal of fatty acids by NaOH treatment, it still bound MAb D6A (Fig. 8, lanes 6 and 11). Using densitometry to quantify MAb binding, we found that NaOH treatment of 7880 LOS did not significantly alter MAb MCA14.2 binding and had a slight enhancing effect on MAb D6A binding. After NaOH treatment of 7889 LOS, the binding of both tested MAbs was somewhat decreased.

Periodate treatment of LOS oxidizes available *cis* or vicinal hydroxyl groups on carbohydrate residues. After periodate oxidation of the LOS, MAb binding could no longer be detected (Fig. 8, lanes 4 and 10). Periodate-treated LOS were also no longer visualized by silver staining because vicinal hydroxyl groups on the carbohydrates are needed for the staining procedure (37).

The reactivities of OS samples and native LOS with the MAbs were compared in dot blot assays. As little as 0.03 μg of untreated LOS was sufficient to bind the MAbs, as shown in Fig. 6. However, after the removal of the lipid A moiety by acetic acid hydrolysis, there was no detectable binding of the MAbs to the OS portion, even when 10.0 μg of OS was reacted in a dot blot on silica gel sheets (data not shown).

The results of our immunochemical studies on the reactivities of the conserved D6A and serotype-specific epitopes after chemical modification of the LOS suggest the following. (i) The MAbs used in this study recognize the OS portion of the LOS. Periodate oxidation of the carbohydrate portion results in a loss of epitope expression. (ii) The phosphorylated diheptose core region of the OS is essential for expression of the MAb-defined D6A epitope on both L10 and L11 LOS. The phosphorylated substituents are also important for optimal expression of the L10 and L11 serotype-associated epitopes that we studied. (iii) The lipid A portion of the molecule is important for optimal expression of these LOS epitopes. After removal of the lipid A moiety by acetic acid hydrolysis, the resultant OS no longer binds the MAbs. Partial deacylation of the lipid A portion of the molecule can affect LOS epitope expression.

DISCUSSION

On the basis of previous immunochemical and structural studies of neisserial LOS (Fig. 1), we hypothesized that there is a conserved core OS region of the LOS of group A and many group B and C meningococci which is recognized by MAb D6A. Our present data support this hypothesis.

Although the LOS of the L10 (7880) and L11 (7889) group A meningococci are serologically distinct from the LOS of other meningococcal serotypes, our immunochemical and structural data for the group A L10 and L11 LOS are consistent with the generalized model of neisserial LOS shown in Fig. 1. The observed molecular weights obtained by ESI-MS yield proposed compositions that are consistent with the presence of 1PEA or 2PEA, 2Hep, 2KDO, and hexose and hexosamine residues. On the basis of previously determined neisserial LOS structures, it is likely that the core region of the L10 and L11 LOS contains a phosphorylated diheptose diKDO moiety.

The compositions of the L10 and L11 LOS differ by the presence of an additional hexose for the larger, L10 LOS, and the PAGE profile of the L10 LOS demonstrates a minor component that migrates at the same position as the L11 LOS (Fig. 2). ESI-MS of the L10 LOS also shows minor species at *m/z* 850.0 and 844.0 that would correspond to a species which lacks hexose and the corresponding anhydro form, respectively. Perhaps the L10 LOS is the result of the sequential addition of hexose to the outer OS portion of the L11 LOS structure. Further studies are in progress to determine the structures of the L10 and L11 LOS.

We have provided data on L10 and L11 LOS derived from prototype strains 7880 and 7889, respectively. Studies of a few LOS structures to date indicate that LOS of different serotypes have unique structures (39). Other L10 and L11 strains have the same PAGE and MAb binding patterns (Fig. 2), and it is likely that LOS of other L10 and L11 strains have very similar structures (22). However, in a previous study, LOS obtained from two L11 meningococci had slightly different Bio-Gel P-4 column chromatographic profiles (45). It is likely that the LOS of both L11 strains have very similar OS structures, but minor differences in the degree of O acetylation and/or the position of PEA may occur.

Our ESI-MS and P-4 chromatography data indicate that the L10 LOS actually contains two major species, which differ by a PEA substituent. PAGE (which showed one major component), therefore, cannot resolve LOS components which differ in the degree of phosphorylation. Heterogeneity in the degree of phosphorylation of neisserial LOS has been observed, but for most neisserial LOS, there appears to be only one phosphorylation state (4, 9–11, 25). There is also a slight asymmetry of the elution profile of each OS component, suggesting that there may be some additional microheterogeneity in the mixture. This may be due, in part, to variability in O acetylation (4). Of note is the fact that any *O*-acetyl groups are cleaved in

the preparation of the LOS compounds for analysis by ESI-MS; therefore, the presence and extent of O acetylation cannot be determined by this method.

Our monosaccharide composition analysis and ESI-MS data indicate that the L10 LOS is composed of 1Gal, 2Glc, 1GlcNAc, 2Hep, 2KDO, 1PEA or 2PEA, and a lipid A moiety. These findings are consistent with preliminary data indicating that the L10 OS contains a Glcβ1→4Glcβ moiety (16). On the basis of immunologic data, others have suggested that the L10 LOS may contain a sialylated lacto-*N*-neotetraose structure (36). However, we believe that this structure is unlikely for several reasons. Frosch et al. have shown that, in contrast to group B and C meningococci, group A meningococci lack the genes necessary for the synthesis of sialic acid (8). We also have no such evidence to support the presence of a sialylated lacto-*N*-neotetraose structure. Our present composition analysis and ESI-MS data on L10 LOS are not consistent with the presence of a Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1 structure with or without sialic acid; in addition, we previously found no evidence that the L10 LOS contains the lacto-*N*-neotetraose structure by using well-characterized MAbs (22, 24).

Our immunochemical studies on the reactivity of the MAb D6A and the serotype-specific MAbs after chemical modification of the LOS indicate that although the MAb-defined epitopes in this study are directed at the OS portion of the LOS molecule, the presence of at least a portion of the lipid A moiety is important for optimal expression of these LOS epitopes. It is interesting that after NaOH treatment, there is a mild increase in the binding of MAb D6A to the deacylated L10 LOS but a decrease in the binding to the deacylated L11 LOS. One explanation may be that, after deacylation, the phosphorylated diheptose regions of L10 and L11 LOS adopt slightly different conformations, which lead to differences in affinity for MAb D6A.

Our results show that the PEA substituents are essential for the binding of MAb D6A. Thus, on the basis of a correlation with known neisserial structures (Fig. 1), MAb D6A may bind directly to a portion of the phosphorylated diheptose core region that is common to most neisserial LOS. Alternatively, the conformation of smaller LOS molecules (e.g., L10 and L11 LOS) which express the D6A epitope may be strongly influenced by the presence of the PEA groups. Further studies will be necessary to define the minimal LOS structure which expresses the D6A epitope and to determine the position and extent of phosphorylation of meningococcal LOS.

In comparison, the binding of the L10 MAb MCA14.2 is only mildly affected by HF treatment of the L10 LOS, and that of the L11 MAb 4C4 is more substantially affected. For larger LOS molecules, dephosphorylation does not affect the binding of MAbs that recognize the outer OS portion of the LOS molecule (46).

Therefore, on the basis of the generalized model of neisserial LOS (Fig. 1), the L10 serotype-associated MAb is likely to be directed at the unique OS sequence of the outer portion of the L10 OS, and dephosphorylation of the core portion of the L10 LOS does not have a substantial influence on L10 MAb binding. In contrast, the L11 LOS is a smaller molecule, and the L11 epitope is determined, in part, by the PEA groups.

In support of our hypothesis that the phosphorylated diheptose region is essential for the formation of the D6A epitope, we previously showed that LOS which express the D6A epitope are usually those components with molecular masses of ≤4.1 kDa, as estimated by PAGE (6, 22). Additionally, a 4.5-kDa LOS component that contains the lacto-*N*-neotetraose structure does not bind MAb D6A (22, 23), but a smaller, 3.6-kDa LOS component with only Galβ1→4Glc linked to a phosphorylated heptose core does bind MAb D6A (20). Thus, in larger LOS molecules, the addition of glycose moieties may obscure this epitope. Neisseriae can undergo phase variation of their LOS in vivo and in vitro to produce smaller or larger LOS molecules (31, 33); additional studies will be necessary to determine LOS epitope expression in vivo.

Native LOS is toxic, and several strategies have been proposed for the preparation of an LOS vaccine. These include detoxification of the lipid A moiety by NaOH treatment of the LOS, liposomal preparations, or conjugation of the OS or core portion of the LOS to a protein carrier (27, 38, 39).

Meningococcal LOS components that bind MAb D6A, such as 7880 and 7889 LOS, are possible candidates for a broadly reactive meningococcal LOS vaccine. Detoxification of LOS by NaOH treatment has little effect on MAb D6A binding to L10 (7880) LOS but decreases MAb D6A binding to L11 (7889) LOS; therefore, LOS of strain 7880 would be preferred for detoxification by NaOH treatment to preserve the conserved D6A epitope. LOS vaccines made with OS-protein conjugates would also show minimal toxicity; however, important substituents, e.g., PEA, can be modified during OS preparation and subsequent conjugation (39).

Our structural and immunochemical analyses suggest that the phosphorylated diheptose core region creates a conserved LOS epitope (defined by MAb D6A). Because this conserved LOS epitope is bactericidal and immunogenic in humans, care should be taken to maintain stereochemical requirements for the expression of this conserved epitope in the design of effective, nontoxic LOS vaccines.

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