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Involvement of Phospholipid End Groups of Group C *Neisseria meningitidis* and *Haemophilus influenzae* Type b Polysaccharides in Association with Isolated Outer Membranes and in Immunoassays

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There are several bacterial polysaccharides (PSs) which contain a terminal lipid moiety. It has been postulated that these terminal lipid moieties anchor the PSs to the outer membrane of the bacteria. Our studies have shown that incubation of native PS from group C *Neisseria meningitidis* or *Haemophilus influenzae* type b with isolated outer membrane vesicles results in association of a portion of the PS with the vesicles. Removal of the terminal lipid from the PS by treatment with phospholipase A_2 or phospholipase D eliminates this association. In other studies, it was shown that delipidated PSs are not suitable as solid-phase antigens in a currently used enzyme-linked immunosorbent assay (ELISA). Measurement of antibody units in the reference sera by using delipidated PSs as antigens in an ELISA yielded negligible absorbance compared with native PSs when methylated human serum albumin was used to coat the PSs to the plate. Nevertheless, phospholipase A_2 and phospholipase D treatment did not noticeably affect antigenic epitopes, since soluble group C PS without the terminal lipid bound antibody as effectively as the native PS did, as measured by a competitive inhibition assay. Both hydrophobic and electrostatic interactions are important for the binding of group C *N. meningitidis* PS to the ELISA plate, while charge interactions seem to be sufficient for binding the more negatively charged *H. influenzae* type b PS.

Capsular polysaccharides (PSs) play an important role in the interaction of many bacteria with their host environment and in their ability to cause disease. The mechanisms by which these PSs influence virulence, however, are not clear (5, 17, 18). The polysialic acid capsules from *Neisseria meningitidis* apparently inhibit activation of the alternate pathway of the complement system, enabling the bacteria to evade lysis (11). Studies on the chemical composition and structure of several bacterial PSs have shown that they contain small lipoidal groups covalently linked to the reducing end of the PS (9, 13, 20). The lipid is a glycosidically linked glycerol phosphate moiety which in some cases can be liberated from the PS by treatment with mild acid or alkali. It is likely that the terminal phospholipid moiety is responsible for anchoring the PS to the outer membrane (9). The presence of this group also imparts a micellar behavior to the PS which potentiates aggregation of the PS (9). Furthermore, it is known that the high-molecular-weight group B meningococcal PS complexes with outer membranes as well as bovine serum albumin as a result of the presence of the covalently linked fatty acids, although the nature of the association is not well understood (23).

In this report, we demonstrate that the terminal lipid of *N. meningitidis* group C (NMGC) PS and *Haemophilus influenzae* type b (Hib) PS is essential for association of the PS with isolated outer membrane vesicles released naturally into the medium. We also show that removal of the fatty acid in the *sn*-2 position, but not the residual fatty acid in the *sn*-1 position, by using phospholipase A_2 (PLA_2) is sufficient to prevent this association. The same fatty acid is also essential for efficient attachment of NMGC PS and Hib PS to the microtiter plate in enzyme-linked immunosorbent assays (ELISAs), commonly used for measurement of antibodies against these antigens. By using two different agents to bind the PS to the plate, we demonstrate that both hydrophobic and charge interactions are needed for binding of NMGC PS to the microtiter plate, while binding of Hib PS is more charge related. PLA_2 or phospholipase D (PLD) treatment of NMGC PS and Hib PS does not detectably alter either the antigenic epitopes or antibody binding to the PS.

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MATERIALS AND METHODS

Cultures and sera. NMGC strains C11 and MC19 were from our collection. The meningococcal group B and group C carrier sera used in this study were provided by James Thomas, Los Angeles County Public Health Department. Additional serum samples were from the laboratory's collection.

Purification and characterization of PS. NMGC PS containing *O*-acetyl group (OAc^+) from strain C11 and lacking *O*-acetyl group (OAc^-) from strain MC19 were prepared by the procedure of Gotschlich (8) in modified Frantz medium (4). Native and PLA_2-treated or PLD-treated PSs from Hib were from Merck Research Laboratories (14, 16). Purity of the PS was ascertained by sialic acid (21) and ribose, respectively (12), protein (15), and nucleic acid estimations. The presence and absence of *O*-acetyl groups was determined by ^3H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy performed by William Egan, Center for Biologics Evaluation and Research. NMR spectroscopy was also performed on native, PLA_2-treated, and PLD-treated NMGC and Hib PSs.

Preparation of outer membrane vesicles. The outer membrane vesicles from a nonencapsulated variant of group B

meningococcal strain M986-NCV are naturally released into the culture broth during growth and were isolated from the medium after 72 h of growth as described by Frasch and Peppler (5). Protein in the outer membrane preparations was measured by the method of Lowry et al. (15).

PLA_2 treatment. The *sn*-2 fatty acid from the terminal lipids of NMGC and Hib PSs were removed by treatment with PLA_2 (from bee venom; Sigma Chemical Co., St. Louis, Mo.). Five-milligram samples were incubated with 13 μl of PLA_2 (5 to 15 U/mg of protein) for 6 h at 37°C in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.2]) buffer containing 0.22 M NaCl, 0.02 M $CaCl_2$, and 1 mM EDTA as described by Gotschlich et al. (9). After PLA_2 treatment, the PS was phenol extracted to remove the enzyme and then ethanol precipitated and lyophilized.

PLD treatment. NMGC and Hib PSs were treated with PLD (from *Streptomyces chromofuscus*; Sigma) to remove both of the terminal fatty acids. The PS was dissolved in 10 mM Tris (pH 7.4) containing 5 mM $CaCl_2$ and 0.3% Triton X-100, and then 0.3% (wt/wt) PLD was added. An equal volume of methyl *tert*-butyl ether-ethanol (9:1 [vol/vol]) was then introduced, and the reaction was carried out at 35°C for approximately 2.5 h as described by Lee et al. (14). The PS-containing aqueous phase was extracted by phenol, and the PS was ethanol precipitated.

The fatty acid composition. The fatty acid composition and content of the NMGC and Hib PSs before and after PLA_2 and PLD treatment were measured by capillary gas chromatography (GC). The samples were saponified, and then the fatty acids were derivatized to their methyl esters and subsequently extracted into hexane-diethyl ether prior to injection into GC. Each sample is run with an internal standard. The capillary GC assay employs a splitless mode of injection which provides a limit of detection of 0.001% (wt/wt) for the individual fatty acid methyl esters (16a).

Column chromatography. Native and PLA_2-treated NMGC PSs were each chromatographed on Sepharose CL-4B (Pharmacia Fine Chemicals). Five milligrams of PS dissolved in 0.2 M ammonium acetate (pH 7.0) was loaded per run. The CL-4B column (1.5 by 90 cm) was equilibrated and eluted with 0.2 M ammonium acetate and 0.02% sodium azide. Fractions of 2 ml were collected, and sialic acid determinations were performed on each fraction, using the recorcinol reagent as described by Svennerholm (21).

Association of NMGC and Hib PSs with outer membrane vesicles. Various amounts of OAc^+ and OAc^- NMGC and Hib PSs were incubated overnight at 4°C with 2 mg each of outer membrane vesicles in phosphate-buffered saline (PBS) to determine the saturation level of lipid-containing PS binding. Similarly, PLA_2- and PLD-treated PSs were also incubated with isolated membranes. Maximum PS association with the outer membrane vesicles was achieved by incubation of equal amounts of PS and isolated membranes, although there were small increases with higher amounts of PSs. Subsequent association studies were carried out below that level. An outer membrane protein control without PS was also incubated with PBS. Each sample was layered on 10 ml of potassium tartrate solution (density, 1.15 g/ml) and centrifuged at 150,000 × *g* for 4 h. The supernatant containing the unassociated PS was carefully removed. The pellets were resuspended in 0.5 ml of water and analyzed for sialic acid (21) or ribose (12) to determine the amount of PS associated with the membrane.

PS ELISA. Methylated human serum albumin (mHSA) was used to facilitate binding of NMGC and Hib PSs to Immulon 1 plates (Dynatech Laboratories, Chantilly, Va.), as both PSs coat poorly on the plate by themselves (1). Native, PLA_2-treated, or PLD-treated NMGC or Hib PS (5 μg/ml) was mixed with 5 μg of mHSA per ml, and 100 μl per well was added to Immulon 1 plates (Dynatech). The plates were coated for 6 h at 28°C and then washed. Appropriate dilutions of reference and test sera were added and incubated overnight at 4°C. The plates were washed and then incubated for 2 h with an appropriate dilution of anti-human immunoglobulin-alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The color reaction with the substrate was carried out as described previously (1). The antibody concentration in the test sera was determined by using a reference curve constructed by linear regression of logit-log transformed data obtained with a human standard serum (6). ELISA was also performed with HSA and poly-L-lysine (PLL) instead of mHSA to coat the PS on the plate. HSA (5 μg/ml) was mixed with the PS and coated. PLL (5 μg/ml) was precoated for 30 min, plates were washed with water, and then the PSs were added to the wells.

TABLE 1. Composition of major fatty acids of NMGC and Hib PSs

PS	Composition (% of dry wt)			
	LPS, 14:0	PS lipid end groups		
		16:1 *cis* 9	16:0	Total
NMGC				
Native	0.008	0.036	0.034	0.078
PLA_2 treated	0.004	0.01	0.021	0.035
PLD treated	<0.001	0.006	0.002	0.009
Hib				
Native	0.092	0.078	0.116	0.286
PLA_2 treated	0.002	<0.001	0.017	0.019
PLD treated	<0.001	<0.001	<0.001	<0.001

Quantitation of native and PLA_2-treated NMGC PSs attached to polystyrene tubes. Polystyrene tubes (10 by 75 mm) were coated overnight with 5 μg of mHSA and NMGC PS (native or PLA_2 treated) per ml and then washed with ELISA wash buffer. The PS attached to the tubes was hydrolyzed with 0.01 M sulfuric acid for 30 min at 80°C. Sialic acid determinations were done by the thiobarbituric acid method (21).

Inhibition ELISA. NMGC PSs (native and PLA_2 treated) were serially diluted starting from 10 μg/ml, and each was incubated for 4 h at room temperature on a rotary shaker with sera containing antibodies to the PS. An antibody concentration giving an absorbance of approximately 1.0 at 50 min in the midpoint of a direct ELISA was selected. At the end of the incubation, 100 μl of the incubation mixture was transferred to a native NMGC PS-coated Immulon 1 plate for measurement of PS bound to the antibody by an ELISA. Similar inhibition experiments were carried out with OAc^- NMGC PS.

RESULTS

To confirm that PLA_2 and PLD treatments removed only the intended fatty acids and that no other structural alterations of the PSs had taken place, determination of fatty acid composition, NMR spectroscopy, and Sepharose CL-4B chromatography of the native and phospholipase-treated PSs were performed before the PSs were used for in vitro association experiments.

Fatty acid content and composition of native and PLA_2- and PLD-treated NMGC and Hib PSs. Table 1 gives the composition of three main fatty acids, namely, myristic acid (14:0, characteristic of lipopolysaccharide [LPS], which is present in low quantities in the native PSs), palmitic acid (16:0), and palmitoleic acid (16:1 *cis* 9), measured as a percentage of dry

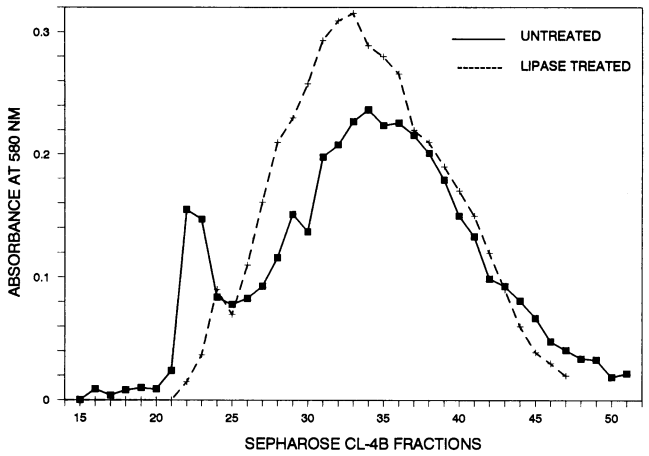

FIG. 1. Elution profile of native and PLA_2-treated NMGC PSs on Sepharose CL-4B.

weight for the various PSs. Even for the native PSs, the fatty acid weight percent is extremely low because of the small size of the lipid compared with the total PS polymer, and only a fraction of the PS contains lipid. The GC results also indicate that the fatty acid compositions of the native NMGC and Hib PSs are qualitatively similar. Reaction with PLA_2 results in significant removal of the fatty acid in the *sn*-2 position (16:1 cis 9), while PLD cleaved both of the fatty acids. Apparently, the fatty acid of LPS can also be removed by enzymatic treatment with PLA_2 or PLD.

NMR spectroscopy of native and PLA_2- and PLD-treated PSs. NMR spectroscopy was performed on native and PLA_2- and PLD-treated NMGC and Hib PSs to determine whether there were structural changes other than removal of the terminal lipid groups. No differences in the NMR spectra of native and phospholipase-treated PSs were observed.

Effect of PLA_2 on the molecular size of NMGC PSs. Native and PLA_2-treated NMGC PSs were each fractionated on Sepharose CL-4B, and the sialic acid content of the fractions was determined. Elution profiles are shown in Fig. 1. The void-volume peak present for the native PS was absent after PLA_2 treatment. The void-volume peak represents high-molecular-weight aggregates of the PS formed as a result of the presence of the terminal lipid. Similar results have been observed for the Hib PS (16a).

Association of NMGC and Hib PSs with outer membrane vesicles. We studied the association of native and delipidated PSs with outer membrane vesicles by incubating PLA_2- or PLD-treated PS and untreated PS with outer membrane vesicles. The associated PS was then separated from the free PS by density gradient centrifugation. About 15% of the native NMGC PS and none of the PLA_2- or PLD-treated PS associated with outer membrane vesicles, as measured by sialic acid determination, indicating that the phospholipid end groups are important for in vitro association of the PS with membrane vesicles (Table 2). Similarly, about 15 to 30% of the native Hib PS and none of the phospholipase-treated PS associated with the membrane vesicles. The results also indicate that it is sufficient to remove only the fatty acid in the *sn*-2 position (cleaved by PLA_2) in the terminal lipids of NMGC and Hib PSs to disrupt the association with the membrane. Removal of the entire glycerol phosphate moiety by PLD treatment is not required for this effect.

ELISA using native and PLA_2- or PLAD-treated NMGC and Hib PSs. To investigate whether the presence of the terminal lipid group may also be necessary for effective binding of the PS to the surface of the microtiter plate, an ELISA was performed with NMGC and Hib PSs with and without PLA_2 or PLD treatment. Serial dilutions of *Haemophilus* and meningococcal reference sera were used to measure the specific antibody content. There was very little absorption of the reference sera antibody on the plates coated with PLA_2-treated PS (Fig. 2A).

To examine the type of interactions involved in the binding of the PS to the plate, mHSA, HSA, and PLL were used for coating NMGC and Hib PSs onto Immulon 1 plates. The PSs

TABLE 2. Association of NMGC and Hib PSs with outer membrane vesicles from M986 NCV-1

PS	Ratio, PS/OMV[a]	% Association of PS with OMV/mg of PS[b]
NMGC		
Native	1:1	15
	1:2	18
	1:3	20
PLA_2 or PLD treated	1:1	Nil
Hib		
Native	1:1	22
PLA_2 or PLD treated	1:1	Nil

[a] OMV, outer membrane vesicle.
[b] Values are geometric means of four independent experiments.

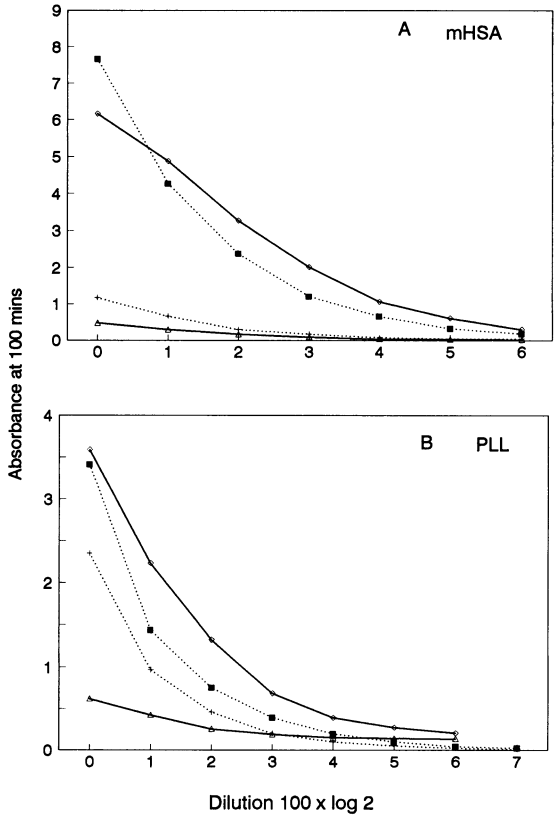

FIG. 2. Measurement of antibodies against native (◇) and PLA_2-treated (△) NMGC PS and against native (■) and PLA_2-treated (+) Hib PS in the meningococcal and Hib reference sera, using mHSA (A) and PLL (B) to coat the PS onto the microtiter plates.

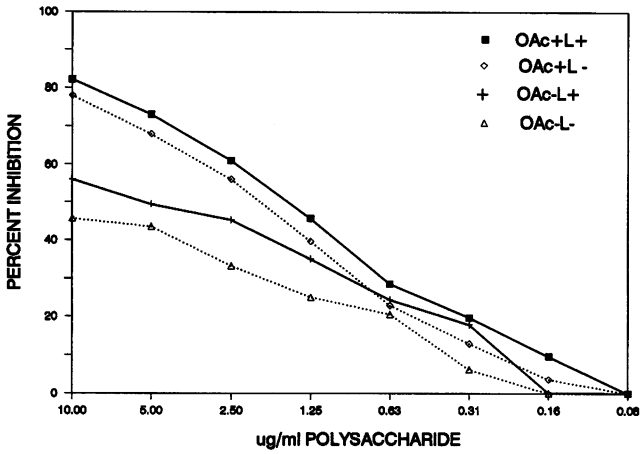

FIG. 3. Competitive inhibition ELISA with native (solid lines) and PLA_2-treated (broken lines) NMGC PSs.

by themselves are negatively charged, although they have some hydrophobic nature due to the contribution of the lipid tail, and have poor binding to the plate. HSA is both hydrophobic and negatively charged, mHSA is more hydrophobic and positively charged, and PLL is a highly positively charged polymer. Native NMGC and Hib PSs exhibited little or no binding to Immulon 1 plates when mixed with HSA but bound very well when mixed with mHSA or when the plates were precoated with PLL (Fig. 2B). PLA_2-treated NMGC and Hib PSs exhibited very little binding to Immulon 1 plates when mixed with mHSA (Fig. 2A). However, PLA_2 or PLD treatment did not significantly reduce the binding of Hib PS to PLL-precoated plates. Binding of PLA_2-treated NMGC PS was reduced compared with binding of native PS to PLL-precoated plates, although it was slightly better than binding to mHSA plates (Fig. 2B).

Sialic acid contents of native and PLA_2-treated NMGC PS-coated polystyrene tubes were measured to determine whether the low absorbance values in ELISA using delipidated PS was due to poor binding of the PS to the tubes. Estimation of sialic acid bound to polystyrene tubes coated with mHSA alone and NMGC PS with and without terminal lipid mixed with mHSA, using the more sensitive thiobarbituric acid method, revealed negligible amount of sialic acid in tubes coated with mHSA alone and in those coated with PLA_2-treated PS compared with PS containing terminal lipid (data not shown).

Inhibition studies with NMGC PS before and after PLA_2 treatment. To further confirm that removal of the terminal lipid did not remove an important epitope, native and PLA_2-treated NMGC PSs were used to competitively inhibit the binding of antibody to native PS-coated Immulon 1 plates. Figure 3 shows the inhibition curves obtained by incubating various concentrations of untreated and PLA_2-treated PSs with the serum. Similar results were obtained with three other sera. The inhibition patterns of both PSs are similar, demonstrating that removal of the terminal lipid group does not apparently alter the antigenic binding sites or specificities of the PS. Similar results were obtained with OAc^- PS with and without the lipid moiety. These results show that important antigenic epitopes of the PS are not altered due to the removal of the terminal lipid group.

DISCUSSION

Escherichia coli, *N. meningitidis*, and *H. influenzae* have all been found to contain terminal phospholipid groups at the reducing end of their capsular PSs (9, 13, 20). In all capsular PSs studied, only about 15 to 50% of the molecules in a preparation appear to be phospholipid substituted, although this is difficult to assess quantitatively because of the lability of the glycosyl-phosphate linkage (10). The mechanism of addition of phosphatidic acid and the subcellular location at which it occurs are not clear, but its addition is expected to be involved in anchoring the PS to the outer membrane.

Our studies with NMGC and Hib PSs support the hypothesis that the terminal lipid groups are involved in the association of the PS with the outer membrane. The membranes that we have used are isolated as blebs which are released naturally into the fermentation broth and hence are likely to be representative of the natural process. In vitro incubation of the native NMGC PS with outer membrane preparations resulted in about 15% association of the PS with the membrane, and this was eliminated after treatment of the PS with PLA_2. Similar results were obtained with Hib PS except for between 15 and 30% association for different preparations, indicating that the association is not limited to NMGC PS and is not likely to be receptor mediated (Table 2). In an earlier study, Zollinger et al. showed that the meningococcal group B PS binds to bovine serum albumin, which is known to have binding sites for fatty acids (23).

PLA_2 or PLD treatment did not lead to any other structural or conformational changes in NMGC and Hib PSs, as assessed by NMR studies. The fatty acid composition data clearly indicate which fatty acids have been removed with PLA_2 and PLD treatments. It is interesting that the association of the PS with isolated membranes is eliminated by the removal of palmitoleic acid in the *sn*-2 position and that the removal of the entire phosphatidylglycerol moiety is not required. It is possible that the attachment of the PS to the membrane may be mediated by the fatty acid in position 2.

Our results on measurement of antibodies by ELISA in reference sera against NMGC and Hib PSs, using native and PLA_2- and PLD-treated PSs, show that this treatment reduced the binding, as measured by optical absorbance, by over 90% in comparison with native PS (Fig. 2A). Therefore, the terminal lipid moiety appears to be important for attachment of the PS to the ELISA plate. This conclusion is also supported by the finding that there was little sialic acid present on the polystyrene tubes coated with PLA_2-treated PS compared with native NMGC PS.

Antigenic epitopes on the NMGC PS are not altered by the PLA_2 treatment, as shown by the competitive inhibition ELISA using various human sera mixed with PLA_2-treated and native PSs (Fig. 3). Hence, the apparent failure of NMGC and Hib PSs without the terminal lipid to act as antigens in the PS ELISA was due to their inability to bind to the microtiter plates. The attachment of the PS to the microtiter plate probably involves several interactions. Serum albumin is known to bind both lipid and detergent molecules by hydrophobic interactions. However, when HSA was mixed with NMGC PS and coated, there was no absorption, indicating that hydrophobic interactions alone are not primarily responsible for the binding the PS to the plate. When HSA is methylated at the carboxyl groups, the negative charge of the carboxyl group is neutralized and replaced by hydrophobic methyl groups, resulting in both a more positively charged and more hydrophobic mHSA (19). This finding suggests that both charge and hydrophobicity play an important role in the

attachment of the NMGC PS to the plate and perhaps to the membrane. PLL, which is highly positively charged, does not facilitate binding of the PLA_2-treated NMGC PS. The Hib PS (polyribitol phosphate) is more negatively charged than NMGC PS, and when PLL is used for precoating, the charge interaction seems to be sufficient for binding of the PS to the plate even following PLA_2 or PLD treatment (Fig. 2B).

These studies provide a possible model system with which to determine whether a given capsular PS possesses terminal phospholipid groups and a method to estimate the relative proportion of molecules possessing such moieties. These results also suggest that the extent of binding of both native and delipidated PSs may differ with different PSs, depending on their composition, charge, and hydrophobicity. Therefore, these factors should be taken into consideration in the development of an ELISA for anti-PS antibodies.

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