Neisserial Lipooligosaccharide Is a Target for Complement Component C4b
INNER CORE PHOSPHOETHANOLAMINE RESIDUES DEFINE C4b LINKAGE SPECIFICITY*

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We identified Neisseria meningitidis lipooligosaccharide (LOS) as an acceptor for complement component C4b (C4b). Phosphoethanolamine (PEA) residues on the second heptose (HepII) residue in the LOS core structure formed amide linkages with C4b. PEA at the 6-position of HepII (6-PEA) was more efficient than 3-PEA in binding C4b. Strains bearing 6-PEA bound more C4b than strains with 3-PEA and were more susceptible to complement-mediated killing in serum bactericidal assays. Deleting 3-PEA from a strain that expressed both 3- and 6-PEA simultaneously on HepII did not decrease C4b binding. Glycose chain extension of the first heptose residue (HepI) influenced the nature of the C4b-LOS linkage. Predominantly ester C4b-LOS bonds were seen when lacto-N-neotetraose formed the terminus of the glycose chain extension of HepI with 3-PEA on HepII in the LOS core. Related LOS species with more truncated chain extensions from HepI bound C4b via amide linkages to 3-PEA on HepII. However, 6-PEA in the LOS core bound C4b even when the glycose chain from HepI bore lacto-N-neotetraose at the terminus. The C4b isoform exclusively formed amide linkages, whereas C4b bound meningococci preferentially via ester linkages. These data may serve to explain the preponderance of 3-PEA-bearing meningococci among clinical isolates, because 6-PEA enhances C4b binding that may facilitate clearance of 6-PEA-bearing strains resulting from enhanced serum killing by the classical pathway of complement.

Complement forms an important arm of the innate immune system that combats neisserial infections. Deficiencies of terminal complement components (C5–C9) predispose individuals to recurrent neisserial infections (1, 2). The classical pathway of complement is essential to mediate in vitro killing by serum (3). Activation of the classical pathway is initiated by binding of the C1 complex to antibodies bound to an antigen on the bacterial surface. Activated C1s in the complex first cleaves C4 to C4b, which binds covalently to the bacterial surface, and then cleaves C2 that binds to C4b, leading to the formation of the C4b2a complex, which is the C3-convertase of the classical pathway. Binding of a C3 molecule to or close to the classical pathway C3-convertase imparts C5-convertase activity to the enzyme complex (reviewed in Ref. 4).

Activation of C4 results from the separation of 77 amino acids (C4a) from the N-terminal end of the C4 α-chain by C1s, resulting in the formation of the metastable C4b molecule. This results in the activation of the internal thioester bond of C4b (5), so that the carbonyl group linked to sulfur (the native thioester) becomes more electrophilic (i.e., an electron acceptor) and reacts readily with nucleophilic groups (i.e., electron-donating groups) such as -OH to form an ester linkage or with -NH₂ to form an amide linkage (6). Alternatively, the carbonyl group can react with water and become hydrolyzed.

There are two isoforms of C4 present in normal human serum, called C4A and C4B (7). A histidine residue in the α-chain at position 1106 imparts to C4B the ability to form ester linkages. The presence of an aspartic acid residue at position 1106 results in C4A functionality and preferential amide bond formation with target -NH₂ groups (8). C4A plays an important role in the clearance of immune complexes from serum, and deficiency of this molecule predisposes individuals to autoimmune disorders (9–11). C4B deficiency was reported to be associated with an increased incidence of bacteremia and meningitis caused by encapsulated organisms (12, 13), but other reports have not supported this finding (14, 15).

Whereas bacterial targets for C3b have been characterized (16–21), no detailed studies on C4b binding to microbial surfaces have been carried out. The classical pathway is important to initiate complement activation on neisseriae (3). In this study, we have identified neisserial LOS1 as an acceptor for

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† The abbreviations used are: LOS, lipooligosaccharide; MOPS, 3-morpholinosopropanesulfonic acid; PEA, phosphoethanolamine; Ab, antibody; mAb, monoclonal antibody; NHS, normal human serum; HBSS, Hank's balanced salt solution; OS, oligosaccharide; MS, mass spectrometry; ES, electrospray; CE, capillary electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
C4b. We have characterized (in detail) the role of the position of phosphoethanolamine (PEA) on the distal heptose (Hepl) chain, and the influence of glycosyl extensions of Hepl chain in determining the nature of the C4b-LOS linkage. We speculated that use of PEA to initiate complement activation may enable the host to circumvent molecular mimicry of host structures by neisserial LOS hexose substitutions (22) and restrict activation of the cascade to the bacterial surface. These findings may serve to explain how LOS phase variation affects complement activation on the bacterial surface. To our knowledge, this represents the first reported study detailing C4b interactions with a microbe.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—**Strains of *N. meningitidis* and their relevant characteristics are listed in Table I. LOS structures and resultant phenotypic features of the mutants are listed in Table II. Every strain or its mutant derivative possessed a single dominant LOS species as determined by silver-stained SDS-PAGE (data not shown), thereby minimizing confounding data by simultaneously expressed species as determined by silver-stained SDS-PAGE (data not shown).

### Table I

| Strain (Ref.) | Serologic classification | LOS phenotype |
|---------------|--------------------------|---------------|
| Y2220 (this study) | Gal-C tolerant (ST-2120) | sialylated LNT⁺ off Hepl; PEA at 3- and 6-positions of Hepl |
| MC58 (45) | Gal-C tolerant (ST-12) | L3 LOS (sialylated LNT off Hepl; 3-PEA on Hepl) |
| 25E (117) | Gal-C tolerant (ST-74) | L2 LOS (sialylated LNT off Hepl; 3-PEA at 3-position and PEA at 6-position of Hepl) |
| 891 (57) | Gal-C tolerant (ST-12) | L4 LOS (sialylated LNT off Hepl; 3-PEA at 3-position and PEA at 6-position of Hepl) |
| 2120 (27) | Gal-C tolerant (ST-12) | L3 LOS (sialylated LNT off Hepl; 3-PEA on Hepl) |

* LNT, lacto-N-neotetraose (Gal→GlcNAc→Gal→Glc→Hepl).

All LOS mutants with a single Glc residue on the Hepl chain (Glc→Hepl) phenotypically similar to galE as described previously (27).

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**ATT TGT CG-3′.** This ampiclon was cloned into the Smal/HindIII site of pK18up (33). A PsIl site was introduced into this plasmid at the beginning of the lgtF coding sequence using primers LGTF-PSTIF (5′-CAA CTG CAG ACA ATA TTT CAA CAA GTT CAA CAA-3′) and LGTF-PSTIR (5′-TGT CTG CTG TTG TCG TAC TGA TAA TGG TAC-3′). This yielded an intermediate plasmid, pNPA2–1psIL. We amplified a region of the a-interposon that confers resistance to spectominycin (34) using primers Omega-F (5′-CCA CTG CAG CAA TTC TCC TGC TCG CGC AGG-3′) and Omega-R (5′-CCA CTG CAG CAG CTT AGT AAA CCC CTC GCT-3′) (PstI sites underlined). Amplification with these primers removed the transcriptional stop signals contained within this interposon, such that the insert would not disrupt the expression of the downstream rfaK gene.

Purification of LOS—LOS was purified from bacterial suspensions by hot water-extraction method, as described previously (43). Deacetylated LOS was prepared as described previously (44) in ~50% yield from the LOS. Core oligosaccharides were prepared accord-
FIG. 1. A low M, molecule is the major acceptor for C4b on the nonsialylated group Y meningococcal mutants: Evidence for amide linkage of C4b to this target. siaD, siaD lst, and siaD lgtA mutant derivatives of strain Y2220 were incubated with 10% NHS for 30 min at 37 °C. Bacteria were washed, lysed, and subjected to Western blotting. C4b bound to bacterial targets detected using a polyclonal anti-C4 Ab. Methylamine (1 M, pH 11) treatment releases only C4b that forms ester linkages with its targets; amide-linked C4b remains unaffected. The position of the free (not bound to a target) α'-chain of C4b (87 kDa) and free α-chain of C4 (95 kDa) are indicated in the lanes marked Pure C4b and NHS, respectively. As a negative control, bacteria were incubated with NHS containing 10 mM EDTA, which blocks all pathways of complement activation.

| Gene inactivated                      | Phenotype                              |
|--------------------------------------|----------------------------------------|
| siaD (polysialytransferase)           | Unencapsulated                         |
| lst (LOS sialytransferase)            | Lacto-N-neotetraose LOS not sialylated  |
| lgtA (LOS glycosyltransferase A)      | Gal → Glc → Hepl                      |
| lgtE (LOS glycosyltransferase E)      | Glc → Hepl                             |
| gaiE (UDP-glucose 4-epimerase)        | Glc → Hepl                             |
| lgtF (LOS glycosyltransferase F)      | HepI unsubstituted                     |
| lpt-3 (LPS phosphoethanolamine transferase-3) | No 3-PEA on HepII                        |

RESULTS

A Low Molecular Mass Molecule Is the Main Target for C4b: Evidence for Amide Linkages between C4b and the Target—In preliminary experiments, we observed that C4b targets on wild-type serogroup B strains MC58 and H44/76 (capsulated; LOS sialylated) (46, 47) and their siaD lst mutants (unencapsulated; LOS not sialylated) that were incubated with NHS were qualitatively identical in Western blot assays (data not shown); capsular polysaccharide only decreased the amount of C4b binding (48). We proceeded to use the unencapsulated derivatives (siaD, siaD lst, and siaD lgtA deletion mutants) of group Y strain 2220. These mutants bound the highest amounts of C4b in flow cytometry assays (~3-fold more fluorescence intensity than the corresponding derivatives of serogroup B meningococcal strain, MC58; data not shown). The siaD, siaD lst, and siaD lgtA mutants of strain Y2220 were incubated with 10% NHS, and after extensive washing to remove unbound C4/C4b, bacterial lysates were treated with 1 M methylamine, pH 11, to dissociate ester-linked, but not amide-linked, C4b from its target(s) prior to electrophoresis, Western blotting, and probing with anti-human C4 Ab. Organisms that were treated with NHS and then lysed in sample buffer served as controls with all their covalent linkages to C4b intact. Fig. 1 shows that samples treated with buffer alone, the major C4b target adduct has a molecular mass that lies between 87 kDa (the mass of the α'-chain of C4b, as seen in the lane marked pure C4b) and 95 kDa (molecular mass of the intact C4 α-chain, as seen in the NHS lanes).

The adducts formed with siaD lgtA migrated faster than the complexes seen with siaD or siaD lst (Fig. 1). This pattern suggested that LOS was the possible acceptor molecule, because the three isogenic mutants differed only in their LOS molecule, with the siaD lgtA mutant possessing the lowest and the siaD mutant possessing the highest M, LOS.

As expected, methylamine treatment resulted in the appearance of the released C4b α'-chain (87 kDa) previously ester-linked to organisms (Fig. 1). The C4b low M, acceptors on the siaD lgtA strains were least affected by methylamine treatment, and most of the C4b bound to this strain was via amide linkages, as evidenced by minimal “released” C4b.
H9251 H11032 -chain (87 kDa) (Fig. 1). The faint adducts seen at H11011 135 and H11011 140 kDa also resisted methylamine treatment, suggesting that these acceptors too formed amide linkages with C4b (Fig. 1).

LOS Is the low Mr Acceptor Molecule for C4b as Well as C3b—The results above suggested that LOS was the likely acceptor for C4b. To confirm this, we used anti-LOS mAb L8 (specific for the LOS of the siaD lgtA mutant) to co-localize LOS with C4b-related (co-migrating) bands on Western blots. Fig. 2 shows the presence of L8-reactive bands migrating at the same velocity as C4-reactive bands. Organisms alone and organisms incubated with EDTA-treated serum (which blocks complement activation) showed no bands at that site. Methylamine treatment did not diminish the intensity of the L8-reactive band that co-migrated with the C4-reactive bands. A fainter L8- and C4-reactive band was seen at H11011 74 kDa. This could represent LOS bound to a partially processed form of C4b, called C4b’ (49). C4b’ (also termed “nicked” C4b) is generated by (incomplete) cleavage of the α-chain of C4b ~17 kDa from the C-terminal end by factor I and C4b-binding protein and retains the ability to form C3-convertases (50). The “α-chain” of C4b’ that attaches to the target comprises the N-terminal α3 (~25 kDa) and the central C4d (~45 kDa) fragments of the α-chain of the C4b molecule. Attempts were made to co-localize the LOS of the siaD lst strain with the C4-reactive bands using anti-LOS mAb 3F11 (specific for the unsialylated lacto-N-Neotetraose structure) (51) but were not successful, probably because C4b sterically hinders or obscures the 3F11 epitope. L8 reactivity was also seen corresponding to the higher molecular mass C4-reactive band at H11011 170 kDa. This probably represented convertases assembled on LOS (covalent C3b-C4b heterodimers coupled to LOS).

We also observed mAb L8 reactivity in the lane containing opsonized organisms at H11011 70 kDa (marked by an asterisk in Fig. 2), the intensity of which was markedly reduced by methylamine treatment. The molecular mass of this band was consistent with an adduct formed between the α1-chain of iC3b (~68 kDa) and the 3.6-kDa L8 LOS molecule. This was verified in a
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Average mass units were used for calculation of molecular weight based on proposed composition as follows: hexose (Hex), 162.15; heptose (Hep), 192.17; N-acetylhexosamine (HexNAc), 203.19; 3-deoxyxyno-s-2-ketoculosonic acid (Kdo), 220.18; phosphoethanolamine (PEA), 123.05; sialic acid (Sial), 291.18.

| Strain     | Observed ions | Molecular mass | Relative intensity | Lipid A-OH | Core OS | Proposed composition |
|------------|---------------|----------------|-------------------|------------|---------|----------------------|
|            | (M-3H)        | (M-2H)         |                   |            |         |                      |
| N. meningitidis Y2220 | 964.9         | 2898.0         | 0.15              | 934        | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, 2Kdo, Lipid A-OH |
| siaD       | 971.3         | 2897.1         | 0.25              | 952        | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, 2Kdo, Lipid A-OH |
| O-deac     | 998.0         | 2996.7         | 0.15              | 1032       | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, Lipid A-OH |
| 1062.4     | 3190.5        | 3190.8         | 0.45              | 934        | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, 2Kdo, Lipid A-OH |
| 1068.6     | 3208.8        | 3208.8         | 1.00              | 952        | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, 2Kdo, Lipid A-OH |
| 1095.0     | 3288.0        | 3288.7         | 0.50              | 1032       | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, Lipid A-OH |
| 1109.2     | 3330.9        | 3331.8         | 0.40              | 1075       | 1965   | 3Hex, 2HexNAc, 2PEA, 2Hep, 2Kdo, Lipid A-OH |

Core OS     | 870.9         | 1743.1         | 0.35              | 1743       | 1965   | 3Hex, 2HexNAc, 2PEtn, 2Hep, aKdo |
|            | 879.6         | 1761.3         | 1.00              | 1761       | 1965   | 3Hex, 2HexNAc, 2PEtn, 2Hep, Kdo |

a Relative to most intense peak in transformed mass spectrum.

b As determined by MS-MS analyses, following introduction of separated LOS-OH glycoforms into the mass spectrometer by CE.

c As deduced from lipid A-OH size determination.

d aKdo, anhydro-Kdo derivative.

A separate experiment showing that this adduct reacted with anti-C3b mAb G-3E (52) (data not shown).

PEA on LOS Is the Primary Amine That Participates in Forming Amide Linkages with C4b—The only commonly encountered primary amine on LOS that can participate in forming an amide linkage with C4b is PEA. To test the hypothesis that PEA was the acceptor site on LOS for C4b, we analyzed C4b binding to strains that had either a 3-PEA or a 6-PEA substitution (see below). Low M<sub>c</sub> target-C4b adducts were seen in the lanes containing MC58 siaD galE and siaD galE lpt-3 mutants of MC58 (the latter lacks 3-PEA from its LOS) (35). Strains were incubated with 30% NHS and treated with methylamine or buffer, and Western blotting was performed as described above. 30% serum concentration was used to enhance C4b deposition, thereby ensuring that C4b-LOS adducts on the PEA-less strains, if any, were visualized, and also because 3-PEA on HepII binds C4b less efficiently than 6-PEA (see below). Low M<sub>c</sub> target-C4b adducts were seen in the lanes containing MC58 siaD galE and siaD galE lpt-3, when samples were treated with buffer alone (Fig. 3). The C4b-LOS adducts on MC58 siaD galE resisted methylamine treatment. In contrast, methylamine treatment of the siaD galE lpt-3 mutant resulted in the appearance of free (or released) C4b α'-chain, suggesting that C4b bound to this strain predominantly via ester linkages. Collectively, these data suggest that C4b forms amide linkages with PEA in this case 3-PEA) on HepII of LOS.

Analysis of LOS of Strain Y2220: Enhanced C4b Binding via Amide Linkages Is Due to Two PEA Residues on the HepII Chain—The prominence of C4b that was amide-linked to the LOS of the siaD lst mutant of Y2220 (Fig. 1) compared with the predominantly ester-linked C4b to MC58 siaD lst mutant (see below; Fig. 6) raised the possibility that either strain possessed an additional PEA residue or the position of the PEA differed from that of MC58 PEA. We therefore analyzed the LOS of strain Y2220 siaD, paying particular attention to the number and position of the PEA residues. O-Deacylated LOS was prepared by hydrazinolysis, and initial analyses were carried out by ES and CE-MS (Table III). Determination of the size of the lipid A moiety by MS-MS enabled the size of the core OS to be deduced and suggested a composition consistent with the presence of two PEA residues. Core oligosaccharide was also isolated and examined by ES-MS, confirming the studies on the O-deacylated material (Table III). Further MS-MS experiments on the OS located both PEA moieties to the HepII residue following identification of characteristic ions m/z 996<sup>−</sup> and m/z 642<sup>−</sup> (Fig. 4). These studies were corroborated and extended by NMR analyses that confirmed, by comparison with previous data (44), that the HepII residue was substituted at both the 3- and 6-positions simultaneously by PEA molecules (data not shown).

6-PEA Is the Preferred Acceptor for C4b—The finding of PEA residues simultaneously at the 3- and 6-positions of HepII prompted us to investigate the contributions of each of these PEA residues in C4 binding. We compared binding of C4b to Y2220 siaD, Y2220 siaD lst, and Y2220 siaD lst galE (PEA at the 3- and 6-positions of HepII) with their corresponding isogenic lpt-3 mutant derivatives (6-PEA only) by flow cytometry and found that deletion of 3-PEA did not decrease C4b binding (Fig. 5A) (IgG and IgM binding to the pairs was the same (data not shown)). This suggested that 6-PEA was the more efficient C4b acceptor or more readily accessible to C4b.

We next asked whether the nature of the linkage between C4b and LOS was altered by deleting 3-PEA. Western blot analysis of C4b-LOS bonds revealed no differences in the apparent amount or the nature of C4b-LOS linkages between the siaD and siaD lst mutants and their respective lpt-3 derivatives (Fig. 5B). We compared C4b binding to strains that had either a 3-PEA...
alone (MC58 and a serogroup C strain 2120), a 6-PEA alone (35E and 89I), or a 3-PEA and 6-PEA simultaneously (serogroup Y strain 2220) (Fig. 6) to evaluate C4b binding to HepII-linked PEAs across strains. IgG and IgM binding were also quantified to ensure that differences in C4b binding were not explained solely by differences in Ig binding. We used siaD mutants, because the presence of capsular polysaccharide decreases C4 binding to meningococci (48). Furthermore, we introduced an lgtE mutation, because HepI chain substitutions have an impact on binding of C4b to LOS, and optimal binding of C4b to PEA occurs in lgtE and lgtF mutants (Glc_3 HepI and HepI unsubstituted, respectively) (see below). Strains with a 6-PEA bound the highest amounts of C4b (Fig. 6). There was no direct correlation between the amounts of IgG or IgM and C4b binding between the strains.

The phenotype of the strains paralleled the amounts of C4 binding; strains with a 6-PEA were killed 100% in 2.5% NHS, whereas the MC58 and 2120 derivatives survived 100% in 2.5% NHS (Table IV). Collectively, these data suggest that 6-PEA-bearing strains are more sensitive to the bactericidal action of NHS.

Influence of the HepI LOS Chain on C4b Binding—The HepI chain of LOS is phase variable, largely in large part because of slipped-strand mispairing within the homopolymeric tract of G residues in the lgtA locus (53). We investigated the influence of alterations in HepI chain length on binding of C4b to LOS. Strains MC58 (3-PEA), 89I (6-PEA), and Y2220 (3-PEA and 6-PEA) were rendered unencapsulated by constructing siaD mutants, and these were further altered genetically to progressively shorten the length of the glycose substitution of HepI to yield the lgtA (Gal → GlcNAC → Gal → Glc → HepI), lgtE (Glc → HepI), and lgtF (HepI unsubstituted) mutants. Fig. 7 shows that almost all C4b binding to the LOS of the siaD lst mutant of MC58 occurred via ester linkages, as evidenced by the complete release of the α'-chain of C4b bound to LOS upon methylamine treatment. A decrease in HepI length was clearly associated with a shift toward amide linkage formation between LOS and C4b in strain MC58. On 6-PEA-bearing strains 89I and Y2220, amide linkages were evident even in the presence of the lacto-N-neotetraose LOS species (i.e. in the lst mutants).

C4 Isoform Determines the Nature of the Linkage between C4b and LOS—Two isoforms of C4, called C4A and C4B, exist in human serum. C4A forms amide linkages with amino groups, whereas C4B preferentially forms ester linkages with hydroxyl groups on surface targets (8, 54). We used serum that contained either C4A alone or C4B alone and examined the nature of the targets formed between C4b and meningococci. Strain Y2220 siaD lst was incubated with either C4A-deficient or C4B-deficient serum, and C4b-target linkages were examined by Western blotting (Fig. 8). NHS (10%), which contained both C4 isoforms, served as a positive control. There was no
evidence of ester linkages formed between C4A and meningo-
cocci, as evidenced by the lack of "released" C4b/H9251/H11032-chain upon methylamine treatment of bacteria incubated with serum containing C4A alone (Fig. 8, far right lane). C4B-specific serum formed predominantly ester linkages, although some residual C4b-LOS adducts were seen despite methylamine treatment.

These data provide further evidence for amide linkages between C4b and LOS and also illustrate the acceptor specificity of C4 isoforms on neisserial surfaces.

**DISCUSSION**

The importance of the antibody-dependent classical pathway in defense against microbes was described almost a century ago (reviewed in Ref. 55). The chemistry of the complement pathway has subsequently been elucidated in great detail. However, to our knowledge, targets for C4b on microbial surfaces and the nature of the linkages formed between C4b and bacteria have never been reported.

Efficient serum killing of neisseriae requires initiation of complement activation by the classical pathway (3). In this study, we have identified LOS as a major acceptor for C4b. The novel finding is the importance of PEA residues as targets for C4b. Participation of PEA in complement activation on the bacterial surface may have pathophysiologic implications. The LOS of neisseriae mimics host structures (22). For example, the lacto-N-neotetraose LOS (Gal3GlcNAc3Gal3Glc3HepI) resembles glycosphingolipids. The use of bacterial PEA by the host to bind C4b and initiate activation of the complement cascade may be a means to circumvent host mimicry. This would allow complement to be activated selectively on the bacterial surface, thereby minimizing damage to host structures. Phosphatidylethanolamine, which is similar to PEA because it is also a strong nucleophile, is not normally exposed on host cells, but in certain pathological conditions such as sickle cell anemia, it becomes exposed on erythrocyte membranes and has been shown to activate the alternative pathway of complement (56).

The presence of ester linkages between C4b and the LOS of Y2220 siaD lst, but not the more truncated LOS of Y2220 siaD lgtA (Fig. 1; methylamine-treated samples) raises the possibility that C4b may bind to -OH residue(s) of the terminal lactosamine from the oligosaccharide extension at HepI. The presence of complexes containing a C4b molecule (either dimers of C4b or C4b/C3b heterodimers) were also demonstrated on LOS,
as evidenced by mAb L8 reactivity at ~170 kDa, that colocalized with C4b (Fig. 2). The exact nature and constituents of these high molecular mass complexes remain to be determined. We also observed a mAb L8-reactive band at ~70 kDa. The reactivity of this band with an anti-iC3b mAb (data not shown) indicates that LOS also binds to C3b (which is further processed to iC3b).

The preponderance of amide linkages between C4b and LOS of Y2220 (Fig. 1; methylamine-treated samples) derivatives prompted a detailed structural analysis of PEA residues in the core OS. We observed that strain Y2220 bore 2 PEA residues simultaneously at the 3- and 6-positions of HepII, which is not typical of any of the described meningococcal LOS immunotypes (L1–L12).

Further experiments showed that it was the presence of the 3-PEA alone, rather than the two PEAAs on HepII, that was responsible for the preponderance of amide linkages between C4b and Y2220 siaD lst. The dominant role of the 3-PEA in forming amide linkages in the presence of a lacto-N-neotetraose HepI chain is clearly borne out in the comparison between Y2220 siaD lst (3- and 6-PEA) and Y2220 siaD lst lpt-3 (only 6-PEA); no differences in the extent of amide linkages were seen between these strains (Fig. 5B). Additional evidence for the selective C4b binding ability of 6-PEA across strains is based on the observation that strains with a 6-PEA on HepII (35E, 89I, and Y2220) bound more C4b than strains with only a 3-PEA on HepII (MC58 and 2120), an effect that was mostly independent of the amount of IgG or IgM binding (Fig. 6). Collectively, the above data provide strong circumstantial evidence of the role of 6-PEA in binding C4b. The functional counterpart of enhanced C4b binding by the 6-PEA-bearing strains (35E, 89I, and Y2220) was illustrated by the enhanced susceptibility of these strains to complement-mediated killing (Table IV).

Binding of C4b to 3-PEA was influenced by HepI hexose substitutions. In lacto-N-neotetraose-bearing strains with only a 3-PEA on HepII (such as MC58), almost all of the C4b bound to LOS via ester linkages (Fig. 7; no residual LOS-C4b chain adduct when MC58 siaD lst was subjected to methylamine treatment). However, truncation of the oligosaccharide extending from HepI causes a shift in C4b linkages from ester to amide. Nevertheless, the 6-PEA (strains 89I and Y2220) appears to be accessible even in the presence of lacto-N-neo-
HepI LOS substitutions in meningocci are lacto-N-heptose sugar bears a LNT substitution. Extension of the HepI chain prevents the C4b from gaining access to the 3-PEA. In contrast, the 6-PEA can bind C4b even when the HepI sugar bears a LNT substitution.

The preponderance of 3-PEA among clinical isolates may suggest a role for this residue in pathogenesis. This is supported by the observation that MC58 siaD galE lpt-3 (no PEA on HepII) bound more IgM and C4 (−1.5-fold increase in fluorescence in a flow cytometry assay for both components) than MC58 siaD galE (3-PEA on HepII) (data not shown). Consistent with greater C4b binding, siaD galE lpt-3 was more sensitive to the bactericidal action of NHS than siaD galE (~7% survival versus ~73% survival in 5% NHS). A schematic summary of the interactions between C4b and the most common clinically encountered meningococcal LOSs (bearing either lacto-N-neotetraose or lactose substitutions on HepI and either a 3- or a 6-PEA on HepII) is provided in Fig. 9. These findings may serve to explain why over 70% of meningococcal strains isolated from humans possess a 3-PEA on HepII (i.e. the L1, L3, L7, and L8 LOS immunotypes) and the L2, L4, and L6 (all with 6-PEA) are relatively rare.

The lgtG gene product that adds a Glc at the 3-position of HepI is infrequently expressed in N. meningitidis and is seen with the L2 LOS immunotype (e.g. strain 35E in this study). The lgtG gene contains a homopolymeric tract of cytidine (poly(C)) and is subject to phase variability. The LgtG gene product (i.e. when lgtG is in frame) competes with lpt, and a Glc substitution occurs at the 3-position of HepII (35). The presence of a Glc at the 3-position of HepII does not decrease the amount of C4b binding (Fig. 5, strain 35E); nor does it appear to affect the ability of concomitantly present 6-PEA to form amide linkages with C4b (similar LOS-C4b binding patterns were seen with 35E siaD galE (3-Glc and 6-PEA on HepII) and 89I siaD galE (only 6-PEA on HepII; data not shown).

Two isoforms of C4, called C4A and C4B, are present in NHS. C4Ab forms amide linkages, whereas C4Bb preferentially forms ester linkages with its targets (8, 54). Although the functional consequences of C4Ab and C4Bb binding to the meningococcal surface with respect to serum bactericidal activity and binding to CR1 remain to be determined, in our studies we used the isoforms separately to validate the results of the methylamine release assays in predicting amide versus ester linkages between C4b and LOS. As expected, the bond formed between C4Ab and LOS was not disrupted by methylamine. In contrast, C4Bb formed predominantly ester linkages with the bacteria and could be released from its targets by methylamine (Fig. 8).

In conclusion, these data shed light on an important aspect of classical pathway activation on meningocci. Enhanced understanding of how complement is activated on bacteria will shed light on the pathogenesis of meningococcal disease and may contribute to developing effective vaccines that rely on Ab-mediated (complement-dependent) immunity. Studies have shown that qualitative aspects of complement activation (i.e. the site of complement activation) may be an important factor in determining serum bactericidal activity (57). It is possible that the target for C4b is altered by Abs of varying specificity, and this may explain differences in bactericidal action mediated by different Abs. One such example is blocking Ab directed against N. gonorrhoeae (and perhaps against N. meningitidis as well), where C3b deposition sites may be altered, resulting in a situation where no killing occurs despite complement activation.

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