Loss of phosphoethanolamine (PEA) from the lipid A of gonococcal strain FA19 results in increased sensitivity to killing by the classical pathway of complement. Here we demonstrate that loss of PEA from lipid A diminishes binding of the complement regulatory protein C4b binding protein (C4BP) to the FA19 porin B (PorB), providing a molecular basis to explain the susceptibility of an lptA null strain of FA19 to killing by normal human serum (NHS). Loss of PEA from lipid A in three additional gonococcal strains that expressed diverse PorB molecules also resulted in decreased C4BP binding, increased deposition of C4b, and increased susceptibility to killing by NHS. Complementation of lptA null strains with lptA restored C4BP binding, decreased C4b deposition, and increased resistance to killing by NHS. These effects of lipid A PEA on C4BP binding to gonococcal PorB and serum resistance were simulated when gonococcal PorB was expressed in a meningococcal background. Loss of PEA from lipid A also affected binding of the alternative pathway regulator factor H (fH) to PorB of some strains. For instance, PorB molecules of lptA null mutants of strains 252 and 1291 bound less fH than those of their parent strains when lipooligosaccharide (LOS) was sialylated, whereas PorB molecules of lptA null mutants of strains FA1090 and 273 retained the ability to bind fH when LOS was sialylated. These data indicate that replacement of lipid A with PEA alters binding of C4BP and fH to PorB and contributes to the ability of gonococci to resist complement-mediated killing.

Neisseria gonorrhoeae causes 106 million of the estimated 498 million new cases of curable sexually transmitted infections that occur globally every year (1). Antimicrobial therapy is a key element of effective management of gonorrhoea, but in the future, drug resistance may compromise the ability to adequately limit infection. N. gonorrhoeae strains that are resistant to multiple classes of antimicrobial drugs, including β-lactams, tetracyclines, and fluoroquinolones, have been well described (2, 3). The recent isolation of ceftriaxone-resistant strains of N. gonorrhoeae in Japan and Europe (4–6) threatens the use of this antimicrobial agent, which has been recommended as first-line treatment by the U.S. Centers for Disease Control and Prevention since 1993 (7). High infection rates coupled with the increasing presence of multidrug-resistant strains could transform this once treatable infection into a global health problem of epic proportions.

To cause infection, gonococci must evade host immune defenses and avoid complement-mediated killing. Activation of the complement system directly facilitates bacterial killing by promoting formation of the membrane attack complex and by enhancing opsonophagocytosis. Antibody (Ab)-dependent classical pathway activation is essential to initiate direct complement-dependent killing of gonococci (8, 9). To thwart the immune response in humans, gonococci highjack host complement-inhibitory proteins that normally function to downregulate complement activation; gonococci use these proteins to inactivate complement on their surfaces. The host uses fluid-phase complement regulators to keep complement activation to a minimum under normal physiological conditions, thereby avoiding inadvertent damage to self-tissues. Factor H (fH) and C4b binding protein (C4BP) are two key fluid-phase regulators that control the alternative and classical pathways of complement, respectively. These proteins function to accelerate the decay of C3 convertases in their respective pathways and act in conjunction with factor I to convert either hemolytically active C3b to the inactive iC3b fragment (fH; alternative pathway) or active C4b to inactive C4d (C4BP; classical pathway).

Gonococcal strains vary in the ability to resist killing by normal human serum (NHS); resistance to killing by NHS can be classified as either unstable or stable. Unstable serum resistance is dependent upon growth of the organism in the presence of CMP-N-acetylneuraminic acid (CMP-NANA), simulating what occurs in vivo (10–13). In this case, sialic acid links to terminal lacto-neotetraose (LNT) on gonococcal lipooligosaccharide (LOS) and facilitates fH binding to the gonococcal porin B (PorB) molecule (14). Stable serum resistance is independent of growth conditions, and gonococcal strains that exhibit stable serum resistance either bind fH in the absence of LOS sialylation or bind C4b directly to their PorB molecules (15–17). Previously, we characterized the interactions between C4BP and gonococcal PorB (15) and demonstrated that entirely truncating HepI substitutions on gonococ-
The lipid A structure of neisserial LOS consists of a phosphorylated diglucosamine; phosphoethanolamine (PEA) moieties may be used as substitutions at both the 1 and 4 positions of lipid A (19). Modification of lipid A with phosphoryl substituents generally results in pleiotropic biological effects. Phosphoryl substitution on neisserial lipid A confers activation of NF-κB and dramatically decreased resistance of strain FA19 to killing by NHS (22), a phenotype that was not shared by N. meningitidis lacking lipid A PEA (21). In this study, we sought to identify the underlying mechanisms responsible for serum sensitivity of gonococcal strains lacking lipid A PEA.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions. N. gonorrhoeae strains FA19 (24), 252 (17), 273 (17), FA1090 (25), and 1291 (26) and N. meningitidis strains H44/76 (27) and Y2220 (28) have been described previously. The construction and characterization of the following mutant strains have been described elsewhere, as indicated: FA19 lptA spc (22), FA19 lptA spc lptA′ (22), H44/76 lptA spc (29), H44/76 cap fHbp (H44/76 siaD cat fHbp erm) (29), Y2220 cap lptA spc (Y2220 siaD cat lptA lptA′) (30), Y2220 cap lptA spc (Y2220 siaD cat lptA lptA′) (30). Relevant genotypic and phenotypic information is listed in Table 1.

Neisserial strains were routinely cultured on chocolate agar plates supplemented with IsoVitaleX equivalent at 37°C in an atmosphere of 5% (vol/vol) carbon dioxide. To sialylate the LOS, bacteria were cultured onto chocolate agar plates supplemented with 100 mM CMP-NANA (0.15 mM). Isopropyl-β-d-thiogalactopyranoside (IPTG; 1 mM) was used to induce expression of lptA from the IPTG-inducible promoter in strain FA19 lptA spc lptA′. GC plates supplemented with IsoVitaleX equivalent were used for antibiotic selection. Antibiotics were used when indicated, at the following concentrations: kanamycin, 100 μg/ml; chloramphenicol, 7 μg/ml; erythromycin, 5 μg/ml; spectinomycin, 50 μg/ml; and polymyxin B, 100 μg/ml or 2 μg/ml.

Sera. NHS collected from healthy human volunteers without a history of neisserial disease and who had not received any meningococcal vaccine were used for antibiotic treatment, pooled, aliquoted, and were not under antibiotic treatment were pooled, aliquoted, and were used for antibiotic selection. Antibiotics were used when indicated, at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 125 μg/ml; chloramphenicol, 50 μg/ml; erythromycin, 400 μg/ml; and spectinomycin, 100 μg/ml.

**TABLE 1 Bacterial strains and their relevant characteristics**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. gonorrhoeae strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA19</td>
<td>PorB.1A (serotype PIA-1), L8 LOS, binds C4BP, binds fH</td>
<td>24</td>
</tr>
<tr>
<td>FA19 lptA spc</td>
<td>PorB.1A, L8 LOS; lptA spc</td>
<td>22</td>
</tr>
<tr>
<td>FA19 lptA spc lptA′</td>
<td>PorB.1A, L8 LOS; lptA spc lptA′ (FA19 lptA spc complemented with lptA driven from an IPTG-inducible promoter)</td>
<td>22</td>
</tr>
<tr>
<td>273</td>
<td>PorB.1A (serotype PIA-6), LNT (3F11) LOS, binds C4BP, binds fH</td>
<td>17</td>
</tr>
<tr>
<td>273 lptA spc</td>
<td>PorB.1A, LNT (3F11) LOS; lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td>252</td>
<td>PorB.1A (serotype PIA-4), LNT (3F11) LOS, does not bind C4BP, binds fH</td>
<td>17</td>
</tr>
<tr>
<td>252 lptA spc</td>
<td>PorB.1A, LNT (3F11) LOS; lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td>FA1090</td>
<td>PorB.1B (serotype PIB-3*), LNT (3F11) LOS, binds C4BP, binds fH</td>
<td>25</td>
</tr>
<tr>
<td>FA1090 lptA spc</td>
<td>PorB.1B, LNT (3F11) LOS; lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td>1291</td>
<td>PorB.1B (serotype PIB-3*), LNT (3F11) LOS, binds C4BP, binds fH</td>
<td>26</td>
</tr>
<tr>
<td>1291 lptA spc</td>
<td>PorB.1B, LNT (3F11) LOS; lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td>N. meningitidis strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H44/76 cap−</td>
<td>Unencapsulated; siaD cat</td>
<td>29</td>
</tr>
<tr>
<td>H44/76 cap fHbp</td>
<td>Unencapsulated; siaD cat fHbp ermC</td>
<td>29</td>
</tr>
<tr>
<td>H44/76 cap lptA spc</td>
<td>Unencapsulated; siaD cat lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td>H44/76 cap fHbp lptA spc</td>
<td>Unencapsulated; carries insertional inactivation of LOS sialyltransferase; siaD cat lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td>Y2220 cap lst</td>
<td>Unencapsulated, carries insertional inactivation of LOS sialyltransferase; siaD cat lptA lptA′</td>
<td>This work</td>
</tr>
<tr>
<td>Y2220 cap lst lptA spc</td>
<td>Unencapsulated, carries insertional inactivation of LOS sialyltransferase, has Y2220 cap lptA′</td>
<td>This work</td>
</tr>
<tr>
<td>Y2220 cap lst PorFA19</td>
<td>Unencapsulated, carries insertional inactivation of LOS sialyltransferase, has Y2220 PorB2 replaced with FA19 PorB1A; binds C4BP and fH; siaD cat lptA lptA′ PorB1A lptA spc</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The FA1090 and 1291 PorB molecules are 97% identical, with 11 amino acid differences in 348 amino acids.
Lipid A PEA alters complement inhibitor binding. In vivo, complement inhibition is essential in controlling intravascular pathogen spread, whereas in the extracellular space it is less important (34). Therefore, pathogen lipopolysaccharide (LPS) often contains inhibitors of complement components or receptors that bind those inhibitors (35). In this study, we characterized a new complement inhibitor, the LNT epitope, which is found on many LPSs from N. gonorrhoeae strains, and we compared it with the well-characterized IgM-binding LPS (IbpA) from Neisseria meningitidis strains (36). We showed that the LNT epitope is not a binding target for IgM but blocks the IgG- and IgA-binding LPS (lptA) (37). We also demonstrated that the LNT epitope is a major determinant of complement evasion (38).

In a series of experiments, we investigated the properties of the LNT epitope and its role in complement evasion. First, we examined whether the LNT epitope is present in all N. gonorrhoeae strains. We used a monoclonal antibody (3F11) that recognizes the LNT epitope and a line-blot assay to determine the presence of the LNT epitope in all strains of N. gonorrhoeae (39). We found that all strains contained the LNT epitope, with the exception of the lptA mutant strain FA19 (36).

Next, we investigated the role of the LNT epitope in complement evasion. We used a complement assay in which bacteria are incubated with complement components and then washed to remove free components. We then added antibody to the complement components and examined the amount of antibody bound to the bacteria. We found that the LNT epitope blocked the binding of antibody to the bacteria (38).

Finally, we investigated the role of the LNT epitope in complement evasion in vivo. We used a guinea pig model of N. gonorrhoeae infection and found that the LNT epitope was present in the bacteria that were recovered from the guinea pigs (39). This suggests that the LNT epitope plays a role in complement evasion in vivo.

In summary, we have shown that the LNT epitope is present in all N. gonorrhoeae strains and that it blocks the binding of complement components. We have also shown that the LNT epitope is present in the bacteria that are recovered from guinea pigs. These results suggest that the LNT epitope plays a role in complement evasion in vivo.

REFERENCES

the serum-resistant wild-type FA19 strain than on FA19 \textit{lptA::spc}. This result is consistent with the serum-sensitive phenotype of FA19 \textit{lptA::spc}. Complementation of FA19 \textit{lptA::spc} with wild-type \textit{lptA} restores serum resistance (22). Consistent with this result, complementation also resulted in a decrease in C4b deposition (Fig. 1, FA19 \textit{lptA::spc} + IPTG). None of the anti-C4-reactive bands was detected in control samples lacking NHS (data not shown). These data indicate that in the absence of lipid A PEA, gonococci regulate the classical pathway less effectively, as reflected by the higher levels of C4b detected.

**Lipid A PEA expression is required for maximal C4BP binding to \textit{N. gonorrhoeae}**. Decreased C4b detected on wild-type FA19 may have resulted from fewer C4b molecules having been deposited initially on the organism, for example, because of less IgG and/or IgM binding. We did not detect differences in IgG or IgM binding to these strains by FACS (data not shown), which suggested that processing of C4b was impaired on the mutant strain rather than diminished deposition of C4b on wild-type bacteria at the outset.

\textit{N. gonorrhoeae} strain FA19 was isolated from the cervix of a patient with disseminated gonorrhea and is stably serum resistant (24, 40). PorB on FA19 binds the complement regulatory protein C4BP (16), and bound C4BP serves as a cofactor in the factor I-mediated cleavage of C4b to its inactive C4c and C4d fragments. A large fraction of deposited C4b is converted to C4d on strains that bind C4BP. Preventing C4BP from binding to PorB on FA19 (using the antigen binding fragment [Fab] derived from anti-C4BP MAb 104) results in killing by NHS (16). C4BP binding to FA19, FA19 \textit{lptA::spc}, and FA19 \textit{lptA::spc lptA } was detected by Western blotting following incubation with 50% NHS (100 \mu g/ml C4BP in the reaction mixture) (Fig. 2A). C4BP binding was greatly decreased in strains lacking PEA on lipid A compared to that of wild-type FA19. Complementation of the \textit{lptA::spc} mutant strain with wild-type \textit{lptA}, which restores PEA expression on lipid A (22), resulted in a recovery of C4BP binding. Diminished binding of C4BP to strain FA19 \textit{lptA::spc} was confirmed using FACS to detect C4BP bound to the surfaces of gonococci after incubation with either 20% or 2% NHS (Fig. 2B and 3B, respectively). Taken together, these data indicated that the PEA moiety on lipid A was important for maximal binding of C4BP to FA19 PorB; strains that lacked PEA bound less C4BP and were killed by NHS (were serum sensitive).

**C4BP binding to PorB.1A and PorB.1B strains and influence of lipid A PEA**. We next sought to investigate the role of lipid A PEA in C4BP binding to other PorB.1A and PorB.1B strains (16). We constructed \textit{lptA} mutations in the following strains that bind C4BP, measured the mutant strains’ ability to bind C4BP, and compared complement killing with that of the parent strains.

**FIG 1** C4 processing on \textit{N. gonorrhoeae} strain FA19 that lacks lipid A PEA. C4 deposition on gonococcal strains incubated in 50% NHS (vol/vol) was determined by Western blotting. C4b binding to bacterial targets was detected with a polyclonal anti-C4 Ab. As evidenced by increased binding of C4b to LOS, Opa, and Por, more C4b remained unprocessed on strains that lacked lipid A PEA (FA19 \textit{lptA::spc} and FA19 \textit{lptA::spc lptA } [no IPTG]), with LOS-, Opa-, and PorB-C4b complexes migrating to 90 kDa, 100 kDa, and 130 kDa, respectively, as characterized previously (33). Higher-molecular-mass (>200 kDa) complexes are indicated by a bracket and represent C4b-containing heterodimers (C5 convertases) linked to bacterial targets (33). The positions of the 95-kDa C4b α and 75-kDa C4b chains are indicated to the left. The portion of the membrane containing proteins of 10 kDa was excised prior to blocking and stained with Coomassie blue. The Coomassie blue-stained load control is shown at the bottom of the figure.

**FIG 2** Effect of lipid A PEA substitution upon C4BP binding to PorB.1A of strain FA19. (A) C4BP binding was assessed by Western blotting. Bacteria were treated with 50% NHS, washed, and lysed, and proteins were separated in a 4 to 12% SDS-PAGE denaturing gel. The gel was cut horizontally and probed with sheep anti-human C4BP Ab (top) and anti-PorB.1A MAb 1EA (bottom). Bound primary Abs were detected using alkaline phosphatase-conjugated anti-sheep and anti-mouse IgGs, respectively. The location of the 70-kDa freed C4BPα chain is indicated with an arrow. C4BP was not detected in control reaction mixtures in which NHS was omitted (− NHS). (B) C4BP binding to FA19 and FA19 \textit{lptA::spc} was assessed by FACS analysis. Bacteria were incubated in 20% NHS (−40 μg/ml C4BP) for 30 min at 37°C, and bound C4BP was detected using sheep anti-human C4BP Ab followed by anti-sheep IgG–FITC. Median fluorescence values for a typical experiment are shown to the right. Data from one experiment of at least three independent experiments are shown.
Wild-type gonococcal strains 273 (PorB.1A), FA1090 (PorB.1B), and 1291 (PorB.1B) each bound more C4BP than the corresponding lptA mutant (Fig. 3A and B); strains 273 and FA1090 were fully resistant to killing by 10% NHS (Fig. 3C) and bound more C4BP than strain 1291 (Fig. 3A), which was less resistant (Fig. 3B). These data indicated that PEA substitution of lipid A, required for maximal binding of C4BP to PorB, was generalizable to diverse gonococcal PorB molecules that bound C4BP.

Lipid A PEA contributes to serum resistance by modulating fH binding. Some gonococcal strains possess PorB molecules that do not bind C4BP, yet these strains are serum resistant (14, 16). We explored the role of lipid A PEA in serum resistance of one such strain, strain 252 (PorB.1A), by constructing an lptA::spc mutation and comparing the mutant’s ability to resist killing by NHS with that of the wild type. Interestingly, 252 lptA::spc was killed more readily than the parent strain by NHS, with 0% survival compared to 92% survival in 10% NHS (Fig. 4A). Lack of C4BP binding to wild-type 252 was confirmed as reported previously (16). As expected, 252 lptA::spc did not bind C4BP, either by Western blot assay (data not shown) or by FACS (Fig. 4B). This indicates that a separate mechanism exists to mediate lipid A PEA-enhanced serum resistance of strain 252.

While the PorB of strain 252 (PorB.1A) does not bind C4BP, it does bind the soluble alternative pathway regulatory protein fH in the absence of LOS sialylation (14, 17). We compared fH binding to strains 252 and 252 lptA::spc by FACS. As expected, wild-type 252 bound fH (14, 41), but the lptA::spc mutant of 252 did not (Fig. 4C). Loss of the ability to bind fH in the 252 lptA::spc mutant likely accounts for its sensitivity to killing by NHS.

The observation that lipid A PEA modulates fH binding to strain 252 raised the possibility that lipid A PEA may play a broader role in binding of complement-inhibitory proteins to the surfaces of gonococci. Sialylation of LNT-expressing LOS that occurs in vivo also enhances fH binding to gonococci to promote resistance to killing by NHS (14), and we sought to examine the role of lipid A PEA upon binding of fH to sialylated gonococci that otherwise bind fH minimally in the unsialylated state. All strains were confirmed to express the LNT epitope in the absence of CMP-NANA by their ability to react with MAb 3F11. Sialylation was confirmed by a loss of 3F11 reactivity on Western blots (data not shown). As shown in Fig. 5A, all wild-type strains bound fH following growth in CMP-NANA. With the exception of 252, which binds fH even in the unsialylated state, binding of fH to the other unsialylated gonococcal strains was similar to that of the isotype controls (data not shown). The effect of lipid A PEA on fH binding to sialylated gonococci was strain specific. fH binding to sialylated FA1090 and 273 was not (or minimally) reduced by loss of lipid A PEA, while fH binding to sialylated strain 1291 was abrogated in the lptA::spc mutant. Loss of lipid A PEA also reduced
 binding of fH to sialylated 252, although to a lesser extent than in sialylated strain 1291 lptA::spc.

* N. gonorrhoeae strains FA19 (22), 273, FA1090 (Fig. 3C), and 252 (Fig. 4A) fully resisted killing by NHS, while the ability of strain 1291 to resist killing was “intermediate” (Fig. 3C). lptA mutants of all of these unsialylated strains were fully killed by NHS (22) (Fig. 3C and 4A). Upon LOS sialylation, the ability of lptA mutant strains to bind fH was variable. To determine if fH binding correlated with serum resistance of sialylated lptA::spc mutants, we performed serum killing assays on organisms grown in the presence of CMP-NANA (Fig. 5B) to sialylate LOS. The FA19 wild type did not express LNT LOS (data not shown) and was not tested. As predicted, sialylation of LOS did not increase the survival of mutant strains 1291 lptA::spc and 252 lptA::spc, which had already showed impaired fH binding resulting from the lptA mutation (Fig. 5A). Although binding of factor H in both 273 lptA::spc and FA1090 lptA::spc was maintained and not enhanced upon LOS sialylation (Fig. 5A), sialylation of LOS did increase survival in strain 273 lptA::spc but not strain FA1090. Sialylated FA1090 lptA::spc remained sensitive to killing by NHS, despite fH binding. Overall, these data indicate that the role of lipid A PEA in modulating binding of fH and resistance to killing by NHS of sialylated gonococci is strain specific.

Confirmation of the role of lipid A PEA in modulating serum resistance in a heterologous background. Meningococci do not bind significant amounts of C4BP in the presence of isotonic buffers (42), and unlike those of gonococci, meningococcal porin molecules have not been reported to contribute to binding of fH to intact bacteria as detected by FACS assays (29, 37, 43). Meningococci bind to fH via two surface proteins, fH binding protein (fHbp) and neisserial surface protein A (NspA) (29, 37). Loss of lipid A PEA in *N. meningitidis* resulted in increased sensitivity to polymyxin B but did not increase sensitivity to NHS (21). The effects of losing lipid A PEA were further examined in *N. meningitidis*, first in an unencapsulated (cap−) *N. meningitidis* strain and then in a cap− *N. meningitidis* strain genetically modified to express *N. gonorrhoeae* PorB.
Lipid A PEA Alters Complement Inhibitor Binding

DISCUSSION

Neisserial LOS exhibits a wide range of structural variation, and the composition of the LOS outer core influences binding of complement-regulatory proteins, such as fH and C4BP, to gonococcal porin. Here we report that variations in PEA content of the lipid A moiety of gonococcal LOS also influence binding of the complement inhibitors C4BP and fH to gonococcal PorB. To our knowledge, this is the first report of modulation of complement regulator binding to microbes by lipid A PEA substitutions. A summary of how neisserial surface structures modulate complement activation and inhibition is provided in Table S1 in the supplemental material.

Loss of PEA residues from the lipid A of gonococcal strain FA19 results in increased killing of the organism by the classical pathway of NHS. We provided a molecular basis for the extreme susceptibility of FA19 lptA::spc to killing by NHS by demonstrating that a loss of PEA from lipid A diminishes binding of C4BP to gonococcal PorB. Additional gonococcal strains that bind C4BP were tested, and in all cases, the loss of PEA from the lipid A resulted in a dramatic decrease in C4BP binding compared to the wild-type parent. Loss of C4BP binding, as previously described (16), was associated with an increase in deposition of C4b and susceptibility to killing by NHS in the absence of LOS sialylation. Despite larger amounts of C4b deposited on their surfaces (Fig. 1), gonococcal mutant strains lacking lipid A PEA bound less C4BP (Fig. 2B), which suggested that C4BP bound to bacteria did not occur through C4b deposited on the bacterial surface. Binding of purified C4BP parallels binding of C4BP in the context of NHS and is inversely proportional to C4b deposited on the bacteria (16), lending strong support to the fact that C4BP bound to bacteria in the context of NHS does not occur through C4b. Complementation studies further demonstrated that restoration of PEA to the lipid A of the FA19 mutant restored C4BP binding, decreased C4b deposition, and increased resistance to killing by NHS.

Loss of PEA from lipid A affected binding of fH to some gonococcal PorB molecules but not others; this effect did not correlate with a specific porin type (not specific to either PorB.1A or PorB.1B). fH binding to the PorB molecules of strains 252 and 1291 was impaired by the loss of lipid A PEA, even in the presence of LOS sialylation. However, the PorB molecules of strains FA1090 and 273 retained the ability to bind fH in the absence of lipid A PEA when their LOS was modified with sialic acid. Wild-type gonococcal strains vary widely in the ability to bind C4BP and/or fH. Gonococcal porins range in size from 34 to 37 kDa and possess eight antigenically variable surface loops. While the N-terminal loop of PorB.1A on select strains appears to be necessary for C4BP binding, loops 5 to 7 of PorB.1B on some strains may contain the binding site for C4BP (16). Variations in PorB binding to fH are also common. Most gonococci require LOS sialylation for maximal interaction of fH with PorB; however, the PorB molecules from certain strains, such as 252 and 15253, bind fH well in the absence of LOS sialylation (17, 41). In addition, subtle differences in the PorB amino acid sequence may explain the differential effects of lipid A PEA on fH binding to sialylated gonococci. For example, a linear peptide that represents FA19 loop 5 blocks fH binding to the homologous strain but not to heterologous strains with distinct loop 5 sequences (17). Diversity in PorB sequences, differences in the presentation of PorB on the surface of

FIG 6 Lipid A PEA does not affect fH binding or resistance to killing of N. meningitidis by NHS. fH binding and resistance to killing by NHS were determined for isogenic lptA mutants of unencapsulated (cap−) H44/76. (A) Serum bactericidal assays were performed on H44/76 cap− and its isogenic lptA mutant, using 2.5% NHS (2.5% NHS is capable of killing N. meningitidis [see Fig. 7]). Values represent the mean (± range) survival in NHS, calculated from three or more separately performed experiments. (B) fH binding to isogenic lptA mutants of N. meningitidis. Meningococci were incubated with purified human fH (5 μg/ml), and bound ligand was detected using anti-fH MAb 90X and anti-mouse IgG–FITC. A mutant that also lacked fHbp (H44/76 cap− fHbp lptA::spc) was included as a negative control. A representative control where fH was omitted from the reaction mixture is shown (shaded gray).

Each experiment was repeated at least three times.

(31). Strain H44/76 expresses low levels of NspA; fH binding to this strain is attributed mainly to expression of fHbp (37) (Fig. 6B). We confirmed that lipid A PEA did not play a role in killing of meningococcal strain H44/76 cap− (Fig. 6A) and further showed that loss of PEA (H44/76 cap− lptA::spc) did not influence factor H binding to meningococci (Fig. 6B).

Unencapsulated, LOS sialylation-deficient meningococcal strain Y2220 (Y2220 cap− lst) bound C4BP minimally (Fig. 7A), did not bind fH in amounts that were detectable by FACS (Fig. 7B), and was susceptible to killing by 2.5% NHS (Fig. 7C). We replaced the PorB molecule of N. meningitidis strain Y2220 cap− lst with PorB.1A from gonococcal strain FA19 (Y2220 cap− lst PorFA19) to simulate gonococcal PorB C4BP and fH binding capabilities (Fig. 7A and B) and to increase resistance to killing by NHS (Fig. 7C). Introduction of the lptA mutation into Y2220 cap− lst PorFA19 resulted in a loss of binding to both C4BP and fH (Fig. 7A and B) and in an increased susceptibility to killing by NHS (Fig. 7C). These data further validate the role of lipid A PEA in modulating killing of gonococci by NHS and specifically implicate the added role of lipid A PEA in modulating C4BP and fH binding to gonococcal PorB.
each strain, and heterogeneity of LOS structures likely affect the binding of complement inhibitors to PorB. Considering these observations, it is not surprising that the effects of lipid A PEA on fH varied across gonococcal strains.

The binding of complement regulators to other Gram-negative bacterial pathogens is similarly affected by lipopolysaccharide (LPS) structure. *Yersinia enterocolitica* LPS does not contribute directly to complement resistance but does so indirectly, by modulating binding of both fH and C4BP to the organism (44–46). Two proteins, YadA and Ail, bind fH and C4BP and contribute to resistance to killing by NHS. YadA, a trimeric lollipop-shaped protein, projects 30 nm out from the outer membrane, while Ail, a 17-kDa integral membrane protein, is predicted to contain four short extracellular loops. Binding of both fH and C4BP to Ail is masked by the O antigen and the outer core of LPS. YadA is well exposed on the surface, and LPS does not affect binding of either fH or C4BP to YadA (44, 46). Meningococci express two fH binding proteins that act in similar ways. The 27-kDa fHbp, tethered in the outer membrane by a lipid anchor, binds fH in an LOS-independent manner, while binding of fH to the more membrane-proximal, ~17-kDa NspA protein, an integral membrane protein predicted to contain 4 very short surface-exposed loops, is influenced by the chain length of HepI and the sialylation of LOS (37).

Loss of binding of complement regulators to gonococci variably correlated with the ability to resist killing by NHS. Strains such as 1291 lpta::spc and 252 lpta::spc, which did not bind either fH or C4BP, were readily killed by NHS, even when they were sialylated. On the other hand, strains FA1090 lpta::spc and 273 lpta::spc lost the ability to bind C4BP but retained the ability to bind fH when they were sialylated. Binding of fH to sialylated 273 lpta::spc promoted resistance to killing by 10% NHS, while binding of fH to sialylated FA1090 lpta::spc did not. Resistance of gonococci to killing by NHS is a balance between activation and regulation of the complement system. The strain background dictates the ability of a specific strain to activate and also to subvert complement-mediated killing by NHS. Gonococcal strains that resist killing by NHS may bind only C4BP, only fH, or both (14, 17). Strains such as FA1090 may require both C4BP and fH to offset complement activation, while strains like 273 may prevent killing by NHS with either C4BP or fH alone. A possible explanation for the ability of NHS to kill sialylated FA1090 lpta::spc may be the presence of kinetically overwhelming classical pathway activation (the lpta::spc mutant does not bind C4BP) that can over-ride regulation by the alternative pathway (binding of fH). As an example, expression of a terminal GalNac distal to the LNT structure of the HepI chain of LOS is a variable that results in increased killing by NHS; GalNac expression is under the control of the phase-variable gene lgtD (47). Differences in serum sensitivity of sialylated derivatives of FA1090 lpta::spc and 273 lpta::spc may result from subtle alterations in LOS structure (i.e., LOS micro-heterogeneity). This may include, for example, the amount of terminal GalNac (lgtD gene product) expressed, which could account for the serum sensitivity of certain strains, such as FA1090 (16, 47), but not others, such as 273 lpta::spc. Killing of *Neisseria* by complement is driven by a number of factors in addition to binding to C4BP and fH, and understanding the differences between sialylated lpta mutants of FA1090 and 273 will require additional studies.

LOS is an important and wide-reaching determinant of neisserial pathogenesis that plays a primary role in modulating immune defenses. Understanding the impact of structural variations of LOS on innate immune responses increases our understanding of
pathogenesis and the host immune response to Neisseria. Specifically, phosphoryl substitutions of the lipid A component of LOS, including substitution(s) involving PEA, may play a major role in the bioactivity and pathogenesis of LOS (19–22, 48, 49). Jarvis et al. recently reported a positive correlation between the level of phosphorylation of lipid A and induction of the TLR4–MD-2 pathway; greater amounts of phosphoryl substitutions on neisserial lipid A were correlated with a greater ability to induce innate immunity through activation of NF-κB (20). Preliminary studies indicate that the presence of PEA on lipid A increases the in vivo competitive fitness of gonococci in a murine model of lower genital tract infection (R. Packiam and A. Jerse, personal communication). Hence, substitutions of lipid A involving PEA likely contribute to the capacity of gonococci to resist innate host defenses during infection and may help to dictate overall fitness in vivo. Our studies demonstrate that the composition of the lipid A moiety, specifically the presence or absence of PEA, can also modulate binding of the complement regulators C4BP and FH to gonococci. Understanding the impact of LOS variation upon neisserial pathogenesis will increase our understanding of the pathogenesis and immune response to these organisms.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI32725 and AI08408 to P.A.R. and AI054544 to S.R. and by a VA merit award to W.M.S. W.M.S. is the recipient of a Senior Research Career Scientist Award from the Department of Veterans Affairs Medical Research Service and is also supported in part by NIH grant U19 AI031496 (to P.F. Sparling, University of North Carolina-Chapel Hill).

REFERENCES


32. Lewis LA, Gray E, Wang YP, Roe BA, Dyer DW.


