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Factor H-Dependent Alternative Pathway Inhibition Mediated by Porin B Contributes to Virulence of Neisseria meningitidis

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Factor H-Dependent Alternative Pathway Inhibition Mediated by Porin B Contributes to Virulence of Neisseria meningitidis

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ABSTRACT The identification of “factor H binding protein (fHbp)-null” invasive meningococcal isolates and the realization that widespread use of fHbp-based vaccines could herald selection of such strains prompted us to characterize novel mechanisms of alternative pathway (AP) inhibition on meningococci. Of seven strains engineered to lack four known AP-inhibiting molecules, capsular polysaccharide, lipooligosaccharide sialic acid, fHbp, and neisserial surface protein A (quadruple mutants), four strains inhibited human AP-mediated C3 deposition. All four expressed the porin B2 (PorB2) molecule, and three strains belonged to the hypervirulent ST-11 lineage. Consistent with reduced C3 deposition, the rate of C3a generation by a PorB2 isolate was lower than that by a PorB3 strain. Allelic replacement of PorB3 with PorB2, in both encapsulated and unencapsulated strains, confirmed the role of PorB2 in AP inhibition. Expression of PorB2 increased resistance to complement–dependent killing relative to that seen in an isogenic PorB3-expressing strain. Adult rabbit and mouse APs were unimpeded on all mutants, and human fH inhibited nonhuman C3 deposition on PorB2-expressing strains, which provided functional evidence for human fH-dependent AP regulation by PorB2. Low-affinity binding of full-length human fH to quadruple mutants expressing PorB2 was demonstrated. fH-like protein 1 (FHL-1; contains fH domains 1 through 7) and fH domains 6 and 7 fused to IgG Fc bound to one PorB2-expressing quadruple mutant, which suggested that fH domains 6 and 7 may interact with PorB2. These results associate PorB2 expression with serum resistance and presage the appearance of fHbp-null and hypervirulent ST-11 isolates that may evade killing by fHbp-based vaccines.

IMPORTANCE The widespread use of antimeningococcal vaccines based on factor H (fH) binding protein (fHbp) is imminent. Meningococci that lack fHbp were recently isolated from persons with invasive disease, and these fHbp-null strains could spawn vaccine failure. Our report provides a molecular basis for an explanation of how fHbp-null strains may evade the host immune system. Meningococci possess several mechanisms to subvert killing by the alternative pathway (AP) of complement, including production of the fHbp and NspA fH binding proteins. Here we show that a meningococcal protein called porin B2 (PorB2) contributes to inhibition of the AP on the bacterial surface. A majority of the “fHbp-null” isolates identified, as well as all members of a “hypervirulent” lineage (called ST-11), express PorB2. Our findings highlight the potential for the emergence of fHbp-negative strains that are able to regulate the AP and may be associated with fHbp vaccine failure.
C-terminal domains of fH with surface-bound C3 fragments (7) and the group B and group C capsular polysaccharides limit AP-mediated deposition of C3 (8, 9).

Based on observations that binding of fH to meningococci was human specific (10), we developed a human fH transgenic (Tg) rat which enhanced the ability of H44/76 to cause bacteremia (bacteremia was observed after challenge with 5 × 10^2 CFU) (11). Rather unexpectedly, deleting both known fH ligands, fHbp, and NspA, did not diminish the ability of H44/76 to cause bacteremia in human fH Tg infant rats (11); this result suggested the existence of an additional human fH-dependent mechanism(s) for complement evasion. LOS sialylation was identified as one such mechanism (7, 11).

Recent work by Lucidarme et al. (12) identified meningococcal isolates that lacked fHbp expression that had been collected from patients with invasive disease, which demonstrated that in select strains, fHbp was dispensable for virulence in humans. These findings imply that certain meningococcal strains possess novel means of inhibiting complement activation in the absence of fHbp. This observation is particularly important given that fHbp-containing vaccines are on the verge of clinical use.

The aim of the present study was to identify novel mechanisms meningococci use to regulate the AP that could contribute to the virulence of this pathogen in the absence of fHbp. These mechanisms could contribute to the ability of fHbp-negative meningococci to maintain virulence and escape killing by fHbp-containing vaccines.

RESULTS

Meningococcal PorB2 strains regulate the human AP. In order to study AP regulation in the absence of factors known to modulate C3 deposition on meningococci, we created mutants that lacked expression of capsular polysaccharide, LOS sialic acid, fHbp, and NspA. Strains harboring these four mutations simultaneously are referred to here as “quadruple mutants.” Quadruple mutants, derived from 7 diverse strains of N. meningitidis (see Table S1 in the supplemental material), were screened for deposition of human C3 following incubation with normal human serum-MgCl2-EGTA (NHS-Mg/EGTA) (20% [vol/vol]); Mg/EGTA blocked classical and lectin pathway activation and allowed assessment of AP activation only (Fig. 1). Significantly lower C3 deposition was seen on quadruple mutants generated from four strains that expressed the PorB2 molecule than on mutants generated from strains that expressed PorB3 (Fig. 1A and B). Similar results were seen with C2-depleted serum (only AP intact; classical and lectin pathways blocked) and with sera from two additional donors (data not shown).

To ensure that serum factors other than AP components did not contribute significantly to the differential C3 deposition on the meningococcal mutants, we examined C3 deposition on the strains using purified C3, factor B, factor D, and fH (Fig. 1C). Again, each of the four PorB2-expressing quadruple mutants showed significantly (P < 0.01) lower C3 deposition than each of the three PorB3 isolates. These data indicate that despite loss of capsule, LOS sialic acid, fHbp, and NspA, select PorB2 strains effectively regulated the human AP.

Decreased C3 deposition in PorB2 strains is associated with slower C3 activation. Decreased C3 deposition could result from reduced targets for covalent binding of C3 (i.e., fewer electron-donating –OH groups) or decreased activation of C3 on the bacterial surface (i.e., because of increased fH binding) or a combination of these events. The quantitative differences seen by flow cytometry (Fig. 1) were confirmed by Western blotting (see Fig. S1 in the supplemental material), which revealed both LOS and Opa as major targets for iC3b across all isolates, as has been previously described for Neisseria (13). LOS structure can modulate complement activation on neisseriae (7, 8, 14). All strains expressed an ~4.5-kDa LOS species; NZ98/254 showed an additional 3.6-kDa LOS species (data not shown). Thus, there was no obvious correlation between the LOS migration pattern and the amount of C3 deposited.

Activation of C3 is accompanied by release of the C3a fragment, and measurement of the rate of C3a generation reflects C3 activation kinetics. Based on previously published data that measured the rates of complement activation (9), we focused on C3a production during the first 15 min of incubation. Quadruple mutants of 4243 (PorB2) and H44/76 (PorB3) were chosen as examples of low- and high-level C3 binders, respectively. As shown in Fig. 1D, the reaction mixture containing the 4243 quadruple mutant generated C3 at a rate lower than that seen with H44/76. The total amounts of C3a in the reaction mixture reached similar levels across strains at time points at or beyond 20 min, likely because C3 convertases in the fluid phase contribute to overall complement activation (9). These data suggest that lower C3 deposition on strain 4243 occurs at least in part because of a lower rate of C3 activation.

Human fH regulates the AP of nonhuman complement on PorB2-expressing quadruple mutants. Binding of fH to the known meningococcal ligands for fH, fHbp, and NspA is human specific (6, 10). To determine if regulation of the AP by PorB2-bearing strains was also specific to humans, we measured deposition of rabbit and mouse C3 using adult sera. PorB2 did not regulate the AP of these adult animals, and similar amounts of C3 were deposited on all quadruple mutants (Fig. 2, black bars). The addition of purified human fH to these nonhuman sera resulted in significantly greater downregulation of the AP on PorB2 strains relative to that seen with PorB3 isolates (Fig. 2, gray bars). Fluorescence-activated cell sorter (FACS) tracings of a representative experiment are shown in Fig. S2 in the supplemental material. These data provide strong evidence for human fH-dependent regulation of the AP by PorB2-bearing strains compared to PorB3-bearing strains.

Confirmation of the role of PorB2 in regulating the human AP. PorB is a major outer membrane protein that contributes to the fitness of a strain (15), and its deletion could have pleiotropic effects with respect to bacterial growth in addition to any interactions with the complement system. To avoid these concerns, we created allelic exchanges of the entire porB2 and porB3 sequences in a homologous background and compared C3 deposits in these isogenic strains that differed only in PorB types. Two sets of mutants were created; the first set lacked fHbp, NspA, capsule, and LOS sialic acid (H44/76 “quadruple”-PorB3 and H44/76 “quadruple”-PorB2), while the second set lacked fHbp and NspA but elaborated capsule and sialylated LOS (H44/76 fHbp nspA-PorB3 and H44/76 fHbp nspA-PorB2).

H44/76 that expressed PorB2 showed greater downregulation of C3 deposition than H44/76 that expressed its own PorB3 molecule (Fig. 3). The effect was most pronounced in the absence of capsule (Fig. 3A; “Cap−”) but was also observed in the encapsulated (Fig. 3C; “Cap+”) background (quantitative comparisons
are shown in Fig. 3B and D, respectively). These data provide strong evidence for a role for PorB2 in regulating the human AP on meningococci.

C3 deposition on PorB2-expressing strains displays a bimodal distribution (Fig. 1B and 3A; see also Fig. S3 in the supplemental material). These data suggest that there are two populations that differentially regulate the AP. PorB2 expression was normally distributed (unimodal) and did not correlate with variation in C3 deposition. Regions of low, intermediate, and high PorB2 expression from within the normally distributed PorB2-positive population all revealed bimodal C3 deposition (Fig. S3). Thus, molecules other than PorB2 could contribute to the observed heterogeneity in C3 deposition.

Binding of fH, fH/Fc fusion proteins, and fH-like protein 1 (FHL-1) to meningococci lacking fHbp and NspA. Having provided evidence for human fH-dependent downregulation of the AP by PorB2-bearing strains, we compared the abilities of H44/76 “quadruple”-PorB3 and H44/76 “quadruple”-PorB2 to bind to human fH. Using standard FACS analysis (Fig. 4A [see also Fig. S4A in the supplemental material]; labeled “no cross-linker”) and Western blot (data not shown) methods, we did not detect binding of full-length human fH (100 μg/ml) to either strain. Similar negative results were obtained with the 7 quadruple mutants that express either PorB2 (C2120, W171, 4243, and 2996) or PorB3 (A2594, H44/76, and NZ98/254) (Fig. 4B [see also Fig. S4B]; labeled “no cross-linker”). To enhance detection of low-affinity interactions between fH and PorB, we added paraformaldehyde to cross-link bound fH to the bacterial surface prior to washing and then detected bound fH by FACS analysis (the terms “low” and “high” affinity are henceforth used to refer to low-level fH binding that can be detected only with a cross-linker and high-level fH binding that can be detected without a cross-linker). In the presence of the cross-linker, the quadruple mutant of H44/76 expressing PorB2 bound greater amounts of fH than the isogenic H44/76 bearing PorB3 (Fig. 4A [see also Fig. S4A]; labeled “paraformaldehyde cross-linker”). In other experiments, quadruple
expression of fHbp (17) or NspA (6) bind fH67/Fc but not fH18-20/Fc to H44/76 “quadruple”-PorB3 and H44/76 “quadruple”-PorB2 demonstrated that fH67/Fc specifically bound to H44/76 expressing PorB2,243 (Fig. 4C; gray-shaded histogram) but not to H44/76 expressing the homologous PorB3 molecule (Fig. 4C; red histogram). The quadruple mutant of 4243, but not other strains (data not shown), also bound fH67/Fc (Fig. 4C; green histogram). Binding of H67/Fc to H44/76 “quadruple”-PorB2 (Fig. 4C; gray histogram) was decreased relative to binding of the quadruple mutant of 4243 (Fig. 4C; green histogram), suggesting that the strain background may be important for this interaction. fH18-20/Fc did not bind to any quadruple mutant tested (data not shown).

N. meningitidis can also bind to fH-like protein 1 (FHL-1), a molecule that contains the first seven N-terminal domains of fH (18) and regulates the AP. Consistent with binding of fH67/Fc, the quadruple mutant of 4243, but not other strains (data not shown), bound purified FHL-1 (Fig. 4D). Binding of FHL-1 and fH67/Fc to wild-type H44/76 expressing both fHbp and NspA is shown as a positive control. All isolates expressed similar amounts of PorB as assessed using Coomassie-stained gels, which suggested that differences in PorB expression did not account for differences in AP regulation or binding of FHL-1 or fH67/Fc (data not shown).

To better understand the differences in binding of FHL-1 and fH67/Fc among the PorB2 isolates, the translated amino acid sequences were aligned (see Fig. S5 in the supplemental material). The only difference between PorB2 of 4243, which bound FHL-1 and fH67/Fc with high affinity, and the PorB2 molecules of W171 and C2120, which did not, was the absence of a 3-amino-acid stretch in predicted surface-loop 7 in the latter 2 strains (VKD deleted in W171 and NGV deleted in C2120). The 4243 loop 7 sequence alone likely was not sufficient to mediate high-affinity binding of fH67/Fc to PorB2, because the amino acid sequence of loop 7 of PorB2 2996 was identical to that of 4243; yet 2996 did not bind detectable amounts of fH67/Fc in the absence of cross-linker. Outside loop 7, the 2996 PorB2 was the most divergent from 4243, with 12 amino acid differences (see Fig. S5). The amino acid sequence of PorB2 4243 was identical to that of a group C strain called FAM18; a quadruple mutant of FAM18 also bound fH67/Fc and regulated the human AP (see Fig. S6 in the supplemental material). This observation further supports the idea of a role of PorB2 in regulating the AP.

All PorB2 strains tested regulated the AP (Fig. 1), and in all cases this regulation was dependent on the presence of human fH (Fig. 2). All PorB2-expressing strains bound full-length fH when a cross-linker was used to detect low-affinity binding, and 4243 bound FHL-1 with high affinity in the absence of a cross-linker. Outside loop 7, the 2996 PorB2 was the most divergent from 4243, with 12 amino acid differences (see Fig. S5). The amino acid sequence of PorB2 4243 was identical to that of a group C strain called FAM18; a quadruple mutant of FAM18 also bound fH67/Fc and regulated the human AP (see Fig. S6 in the supplemental material). This observation further supports the idea of a role of PorB2 in regulating the AP.

**Expression of PorB2 enhances serum resistance.** To determine the relative roles of PorB2 and PorB3 in serum resistance, we compared the abilities of H44/76 fHbp nspA-PorB3 and H44/76 fHbp nspA-PorB2 to resist killing by human serum. Strains lacking fHbp and NspA were used to avoid the confounding effects of fH binding to these ligands. Serum was absorbed against a mixture of H44/76 fHbp nspA-PorB3 and H44/76 fHbp nspA-PorB2 to remove strain-specific antibodies. Absorbed serum (20%) alone did not kill either strain (Fig. 5). Expression of PorB2 by H44/76 fHbp nspA resulted in greater resistance to complement-dependent killing mediated by anti-group B capsule IgG antibody SEAM 12 (19)

**FIG 2** Human fH inhibits C3 deposition by adult nonhuman complement on PorB2-expressing quadruple mutants. Quadruple mutants of 7 strains of *N. meningitidis* were incubated with adult rabbit complement (A) or adult mouse serum (B) in either the absence (“-”; solid black bars) or the presence (“+”; gray bars) of human fH (80 μg/ml), and levels of C3 fragments deposited on bacteria were measured by FACS analysis. The control (open bar) represents C3 deposition when bacteria were incubated with heat-inactivated complement. The percentage of positive events relative to controls (bacteria plus complement) is shown on the y axis. Each bar represents the SEM of the results of 2 to 3 independent observations. ***, P < 0.01; ****, P < 0.001** (1-way ANOVA).
than expression of PorB3 (Fig. 5). Absorbed serum treated with Mg/EGTA to selectively activate the AP did not initiate killing of either strain in the presence or absence of SEAM 12 (100% survival in both instances). As previously reported for other antimeningococcal Abs (3), the AP was required for maximal killing; blocking the AP with an anti-factor Bb monoclonal antibody (MAb) decreased killing of H44/76 fHbp nspA-PorB2 (SEAM 12 [1 µg/ml] plus absorbed serum yielded 35% survival versus 71% survival when anti-Bb blocked the AP; P < 0.001). These data correlate expression of PorB2 with the evasion of immune killing seen when all pathways of complement were intact.

DISCUSSION

PorB, one of the most abundant proteins in the neisserial outer membrane, is an integral membrane protein organized as a β barrel with 16 transmembrane domains and 8 predicted surface loops (20). PorB functions as a porin, allowing the passage of small molecules across the outer membrane, and has also been implicated in virulence. Gonococcal PorB has been shown to play a key role in regulation of both the AP and classical pathways of complement by virtue of its ability to bind to fH and C4BP, respectively (21, 22). Meningococcal PorB is divided into two classes, PorB2 and PorB3, which are mutually exclusive and are expressed...
from alternate alleles (porB2 and porB3) at the porB locus. PorB3 of N. meningitidis serves as a ligand for Toll-like receptor 2 (TLR2) and activates cells through a TLR2/TLR1-dependent pathway (23).

The goal of our study was to examine AP regulation in strains that did not rely on fHbp or NspA expression. We have identified a novel role for meningococcal PorB2 in regulation of the AP of complement. Concurrent with our studies, Lucidarme and colleagues described 29 invasive meningococcal isolates that lacked fHbp expression (12). Of these strains, nine belonged to the hypervirulent ST-11 lineage. It is noteworthy that strains that belong to the hypervirulent ST-11 clonal complex express PorB2. In an ongoing analysis of select strains reported by Lucidarme et al., three of four non-ST-11 isolates also expressed PorB2 (24).

Natural disruption of PorA (27, 28) and NadA (IS1301) (29), which are both components of the proposed vaccine, has also been reported in the ET-15 subset of the ST-11 complex.

![FIG 4](image)

**A** PorB2 binds full-length fH, fH domains 6 and 7, and FHL-1. (A and B) Binding of human fH to quadruple mutants of H44/76 that express either PorB3 (H44/76-PorB3) or PorB2 (H44/76-PorB2) (A) and quadruple mutants of N. meningitidis strains that express either PorB3 (A2594, H44/76, and NZ98/254) or PorB2 (C2120, W171, 4243, and 2996) (B). Quadruple mutants were incubated with purified fH (100 μg/ml), and bound fH was measured by FACS analysis (graphs labeled “no cross-linker”). In some experiments, bound fH was cross-linked to the surface with paraformaldehyde (final concentration, 1% [vol/vol]) prior to detection by FACS analysis (graphs labeled “paraformaldehyde cross-linker”). The negative control (control) was a reaction mixture that lacked fH (neg. control). Binding of fH to wild-type H44/76 that expresses fHbp, NspA, capsule, and LOS sialic acid is shown as a positive control in panel B (pos. control). The amount of bound fH is represented on the y axis as fold change in median fluorescence relative to the H44/76 quadruple mutant that expresses PorB3 (in panel A) or the H44/76 quadruple mutant (in panel B) with paraformaldehyde cross-linker. Each bar represents the SEM of the results of 2 to 3 independent experiments (note that some error bars are small and are not easily seen on the figure). ***, P < 0.01; *, P < 0.05 (1-way ANOVA with Tukey’s posttest for pairwise comparisons). (C) PorB2 binds to fH67/Fc. Binding of human fH67/Fc to quadruple mutants of H44/76 (blue line), 4243 (green line), and H44/76 that express either PorB3 (H44/76-PorB3; red line) or PorB2 (H44/76-PorB2; gray shading) was measured by FACS analysis. (D) A quadruple mutant of strain 4243 binds FHL-1. Binding of FHL-1 (10 μg/ml) to the 4243 quadruple mutant (gray-shaded histogram) was measured by FACS analysis using polyclonal goat anti-human fH. FHL-1 was omitted in the negative control (broken line). The positive control shows binding of FHL-1 to wild-type H44/76 (solid black line). Numbers alongside histograms represent the median fluorescence, and the fill/outline corresponds to that of the histograms.

Although anti-capsule MAb SEAM 12 could overcome AP regulation and initiate *in vitro* killing of meningococci, maximal killing required the AP. Further, our studies demonstrated that expression of PorB2, relative to PorB3, enhanced the ability of H44/76 fHbp nspA to evade complement-mediated killing. Similarly, expression of PorB2 may render strains more resistant to complement-dependent killing in humans. The ability to regulate complement and cause invasive disease in the absence of fHbp expression may offer these strains a distinct advantage when faced with immune pressure by anti-fHbp antibodies, which is a likely occurrence with clinical use of fHbp-containing vaccines (25, 26).
We have provided considerable functional evidence for human fH-dependent inhibition of the AP by PorB2. A novel finding of this study was the low affinity of the interaction between full-length fH and PorB2 on intact bacteria. fH is present in serum at concentrations of 200 to 500 µg/ml and binds to levels barely detectable by FACS analysis, unless cytidinemonophospho-N-acetylneuraminic acid (CMP-NANA) is used to growth media. fHbp and NspA expression decreased fH binding to NspA to levels barely detectable by FACS analysis, and fHbp-fH affinity (over 2 orders of magnitude) nor differences in the amount of fH bound to bacteria by FACS predicted resistance to complement-dependent killing. Another example is the NspA-fH interaction; despite the fact that capsule and full-length LOS expression decreased fH binding to meningococci as reported in this study, the functional interaction mediated by meningococcal PorB. In light of the functional role of PorB in complement evasion, it is conceivable that implementation of fHbp-containing vaccines (25, 26) may drive (or select for) loss of fHbp expression in combination with positive selection for specific PorB molecules that are optimized for AP regulation and functional interaction with fH.

**MATERIALS AND METHODS**

**Ethics statement.** This study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School. All subjects who donated blood for this study provided written informed consent.

**Bacterial strains and culture conditions.** Characteristics of the wild-type strains used to derive the mutants used in this study are listed in Table S1 in the supplemental material. N. meningitidis bacteria were routinely grown on chocolate agar plates supplemented with IsoVitalex equivalent at 37°C in an atmosphere with 5% CO2. GC plates supplemented with IsoVitalex equivalent were used for antibiotic selection. *Escherichia coli* (Invitrogen, Carlsbad, CA) were cultured in Luria-Bertani broth or agar with antibiotics as needed. Antibiotics were used at the concentrations indicated in Table S2 in the supplemental material.

Strains were rendered unencapsulated by interruption of *mynB* (group A) or *sidA* (group B, C, or W135) (6, 34). Insertional inactivation of *lst* (i.e., *kan*) abolished LOS sialylation of group B, C, and W135 isolates as previously described (6, 34). Group A strains do not sialylate their LOS unless cytidinemonophospho-N-acetylneuraminic acid (CMP-NANA) is added to growth media. fHbp and NspA expression was abrogated (fHbp::erm [5] and nspA::spc [6], respectively) as previously described.

PorB deletion mutants of H44/76 fHbp nspA were constructed using a plasmid that contained (5' to 3') an 800-bp region 5' to PorB3 (amplified with porMC58up_F_ApaI and porMC58up_R_Sacl; see Table S3 in the supplemental material), the Apha3 kanamycin resistance determinant, and a 1,100-bp region 3' to PorB3 (amplified with porMC58down_F_Spel and porMC58down_R_Sacl; see Table S3). Meningococcal strains were transformed as previously described.

PorB allelic replacements in H44/76 were made using the H44/76 PorB3 deletion strain and chromosomal DNA isolated from either 4243 or H44/76 containing the TetM tetracycline resistance cassette downstream of porB2 or porB3, respectively. This ensured complete replacement of porB and eliminated the potential formation of hybrid PorB2/PorB3 molecules. TetM was inserted downstream of porB in each strain using plasmids that contained either porB3 (amplified with MC58_porB3_R_Sacl and MC58_porB3_F_Apal; see Table S3 in the supplemental material) or porB2 (amplified with FAM18_porB2F_Apal and Nl_porB_R_Sacl; see Table S3) followed by TetM and the 1,100-bp region 3' to porB3 (amplified with MC58_porB3_R_Sacl and MC58_porB3_F_Apal; see Table S3). PorB3 and porB2 strains were transformed as previously described.

Sera. Normal human serum (NHS) was obtained from healthy adult volunteers, divided into aliquots, and stored (as individual sera) at −80°C till used. To inactivate selectively the classical and lectin complement pathways of human serum, NHS was treated with 1 µg/ml of cephalin and heated at 56°C for 30 min.

**FIG 5** PorB2 expression enhances serum resistance. Data represent percent survival of isogenic H44/76 fHbp nspA expressing either PorB2, or PorB3. Percent survival relative to time 0 is plotted on the y axis. Each bar represents the SEM of the results of three independent observations. ***, P < 0.01; ****, P < 0.0001 (2-way ANOVA).
pathways and isolate the AP as the only active pathway, MgCl$_2$ and EGTA (10 mM each) were added to NHS (NHS-Mg/EGTA).

Absorbed sera, in which antibodies specific to H44/76 fHbp nspA PorB3 and H44/76 fHbp nspA PorB2 were removed, were prepared as follows. EDTA (10 mM) was added to fresh NHS, and the sera were dialyzed against phosphate-buffered saline (PBS)–1 mM EDTA for 4 h at 4°C using a 10-kDa molecular mass cutoff. Meningococcal strains were subjected to passage on overnight plates and added onto fresh chocolate agar plates and grown for 5 h. Bacteria were harvested and washed once with PBS–1 mM EDTA. Dialyzed serum was absorbed twice with bacteria (1-ml serum with a mixture of 100 µl of packed cells from each of the 2 mutant strains) processed end over end for 30 min at 4°C. The serum was filtered (0.22-µm pore-size filter), divided into aliquots, and stored at −80°C. Ca$^{2+}$ and Mg$^{2+}$ (2 mM each) were added prior to use. Similar amounts of fH were detected (MAb 90X) in NHS and absorbed sera spotted on polyvinylidene difluoride (PVDF), which suggested that the fH was not depleted by the absorption. The hemolytic activity of serum was confirmed using a Total hemolytic complement kit.

Mouse complement was obtained by cardiac puncture of adult BALB/c mice. Blood was allowed to clot at 22°C for 20 min, followed by incubation at 4°C for 20 min, and then spun, and the supernatant was divided into aliquots and stored at −80°C. Adult rabbit complement was purchased from Cedarlane Laboratories.

**Bacterial killing by NHS.** Susceptibility of neisserial strains to complement-mediated killing was determined using a serum bactericidal assay as described previously (6). Anti-group B capsule IgG antibody SEAM 12 (19) was added to absorbed sera at the concentrations indicated. Survival was calculated as the number of viable colonies at time 30 min relative to time 0.

**Purified complement components.** Factors B and D were from Complement Technology, Inc. (Tyler, TX). C3 was isolated from human plasma by polyethylene glycol (PEG) precipitation and chromatography over DEAE Sephacel using a modification of previously established methods (35). NHS immunodepleted of C3 (Complement Technology, Inc.) regained full hemolytic activity upon addition of the purified C3 to a concentration of 500 µg/ml, using a Total hemolytic complement kit (The Binding Site, Birmingham, United Kingdom).

**C3 deposition on bacteria using purified complement proteins.** To deposit C3 on bacteria using purified AP components, −10$^8$ bacteria in Hanks balanced salt solution (HBSS)−1 mM MgCl$_2$−0.1% bovine serum albumin (BSA) were incubated with C3 (500 µg/ml), factor B (100 µg/ml), and fH (100 µg/ml) followed by factor D (2 µg/ml) in a final reaction volume of 50 µl for 30 min at 37°C. Deposited C3 was measured by FACS analysis.

**Antibodies.** Human, rabbit, and mouse C3 deposition on bacteria was measured using fluorescein isothiocyanate (FITC)-conjugated anti-human C3 (BioDesign/Meridian Life Sciences), anti-guinea pig C3, and anti-mouse C3 (the latter 2 from Cappel/MP Biomedical) at a dilution of 1:200 in HBSS–0.1% BSA, respectively. Preliminary FACS and Western blotting experiments confirmed the specificity and cross-reactivity of anti-guinea pig C3 FITC with rabbit C3.

**Recombinant human fH/Fc fusion proteins.** fH domains 18 through 20 fused in frame to the N terminus of the Fc fragment of murine IgG2a (called fH/Fc) have been described previously (36). Similarly, we generate fH domains 6 and 7 fused to murine IgG2a Fc. fH domains 6 and 7 were amplified (primers, six Ascl and seven Notl) from human fH cDNA (gift of M. K. Pangburn, University of Texas Health Science Center, Tyler, TX) and cloned into Ascl and Notl sites of pcDNA3 (Invitrogen Life Technologies) encoding mouse IgG2a Fc (37). Transfection of CHO cells and protein purification using protein A Sepharose were performed as described previously (36).

**Flow cytometry.** Human, rabbit, and mouse C3 deposition on the bacterial surface and FHL−1, fH, and fH/Fc construct binding were measured using flow cytometry as described previously (10, 17, 36). To detect low-affinity binding of fH, organisms incubated with human fH (100 µg/ml) for 30 min were fixed by the addition of paraformaldehyde (final concentration, 1%). The organisms were pelleted after incubation for 10 min at room temperature, and bound fH was detected by flow cytometry as described above. Data were analyzed using FlowJo (version 7.2.5; Tree Star, Inc.). Binding is represented either as a percentage of positive events (relative to a negative control that was gated to yield 5% of positive events) when histogram tracings showed a non-Gaussian distribution or as median fluorescence of the entire bacterial population when all samples yielded normally distributed histograms.

**C3 generation assay.** Bacteria (−2 × 10$^8$) were incubated with 20% NHS-Mg/EGTA in a final reaction volume of 200 µl. Samples (10 µl) were collected at specified intervals between 0 min and 15 min and diluted immediately in 90 µl PBS−50 mM EDTA. C3a in the reaction mixture was measured using a MicroVueC3a Plus ELISA kit (Quidel) according to the manufacturer's instructions.

**Statistical analyses.** Statistical comparisons across multiple groups were carried out using 1-way analysis of variance (ANOVA) with Tukey's posttest for pairwise comparisons. Differences in C3a generation and bacterial survival between strains were measured using 2-way ANOVA with Bonferroni's posttest. All probability values, with the exception of the 1-way ANOVA, are two tailed.
bonds to factor H and enhances meningococcal resistance to complement. PLoS Pathog. 6:e10001027.