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*J Immunol* 2008; 180:3426-3435; doi: 10.4049/jimmunol.180.5.3426

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Human Factor H Interacts Selectively with Neisseria gonorrhoeae and Results in Species-Specific Complement Evasion

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Complement forms a key arm of innate immune defenses against gonococcal infection. Sialylation of gonococcal lipo-oligosaccharide, or expression of porin 1A (Por1A) protein, enables Neisseria gonorrhoeae to bind the alternative pathway complement inhibitor, factor H (fH), and evade killing by human complement. Using recombinant fH fragment-murine Fc fusion proteins, we localized two N. gonorrhoeae Por1A-binding regions in fH: one in complement control protein domain 6, the other in complement control proteins 18–20. The latter is similar to that reported previously for sialylated Por1B gonococci. Upon incubation with human serum, Por1A and sialylated Por1B strains bound full-length human fH (HuFfH) and fH-related protein 1. In addition, Por1A strains bound fH-like protein 1 weakly. Only HuFfH, but not fH from other primates, bound directly to gonococci. Consistent with direct HuFfH binding, unsialylated Por1A gonococci resisted killing only by human complement, but not complement from other primates, rodents or lagomorphs; adding HuFfH to these heterologous sera restored serum resistance. Lipo-oligosaccharide sialylation of N. gonorrhoeae resulted in classical pathway regulation as evidenced by decreased C4 binding in human, chimpanzee, and rhesus serum but was accompanied by serum resistance only in human and chimpanzee serum. Direct-binding specificity of HuFfH only to gonococci that prevents serum killing is restricted to humans and may in part explain species-specific restriction of natural gonococcal infection. Our findings may help to improve animal models for gonorrhea while also having implications in the choice of complement sources to evaluate neisserial vaccine candidates.

Received for publication August 21, 2007. Accepted for publication January 2, 2008.

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1 This work was supported by National Institutes of Health Grants AI32725 (to P.A.R.) and AI054544 (to S.R.).
2 Address correspondence and reprint requests to Dr. Jutamas Ngampasutadol, Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts, Lazarre Research Building, Room 370T, 364 Plantation Street, Worcester, MA 01605. E-mail address: jutamas.shaghmessa@umassmed.edu
3 Abbreviations used in this paper: NHS, normal human serum; C4BP, C4b-binding protein; LNT, lacto-N-neotetraose; LOS, lipo-oligosaccharide; CMP-NANA, 5′-cytidinemonophospho-N-acetylmuraminic acid; CCP, complement control protein domain; fH, factor H; HuFfH, human fH; NChS, normal chimpanzee serum; FHL-1, fH-like protein 1; FHR-1, fH-related protein; NRI/S, normal rhesus serum; ChFfH, chimpanzee fH; Por, porin.

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killing by using its Por molecules to bind HufH (6). Por molecules are 34–37 kDa proteins comprising eight transmembrane loops, which exist as trimers in their native configurations, and function as selective anion channels (34). Por is an allelic protein consisting of two main isoforms in *N. gonorrhoeae*, Por1A, and Por1B (35). Por1A gonococci frequently cause disseminated disease; Por1B strains usually cause local urogenital disease and pelvic inflammatory disease in women (35, 36). We have shown that Por1A-bearing gonococcal strains bind more HuH than their Por1B (un-sialylated) counterparts (6).

A limitation in understanding the pathogenesis of gonorrhea (e.g., the growth of gonococci in vivo and the immune response to infection) has been the lack of animal models that simulate clinical syndromes caused by *N. gonorrhoeae* infections in humans. The animal model currently used to study gonorrhea is the 17-β-estradiol-treated mouse (37). Attempts to infect or naturally colonize the porin protein specificity of gonococcal serum resistance.

**Table I. Primers used for fH/Fc fusion protein constructions**

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<th>Primers</th>
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**fH/murine Fc fusion proteins**

We generated eight fH/murine Fc fusion proteins that contained contiguous fH CCP domains (CCPs 1–5, 1–6, 1–7, 5–8, 6–10, 11–15, 16–20) fused in-frame at their C-terminal ends to the N terminus of the fH fragment of murine IgG2a (fH/Fc fusion proteins). This allowed us to use the Fc region as a detection site (tag) for symmetric detection of each fusion protein (before flow cytometry) using anti-mouse IgG. Collectively, the fH fragments spanned the entire length of the fH molecule. Using PCR (primers listed in Table I) with pBluescript containing HuH cDNA (gift of Dr. M. K. Pangburn, University of Texas Health Science Center, Tyler, TX) as a template, we cloned each fH fragment into AscI-NcoI sites of the eukaryotic expression vector pcDNA3 (Invitrogen Life Technologies) already containing the Fc fragment of mouse IgG2a (44). All resultant sequences were confirmed by automated DNA sequencing.

Some serum-resistant gonococcal strains (certain Por1B strains with sialylated LNT LOS and certain Por1A strains), however, do not bind C4BP but bind HuH. We localized the regions in HuH that bind to these Por1A-bearing gonococci and show that the ability of gonococci to bind this complement inhibitor protein directly is restricted to humans and also contributes to the species specificity of gonococcal serum resistance.

**Materials and Methods**

**Bacterial strains**

*N. gonorrhoeae* serotype Por1A strain 252 (6) and serotype Por1B strain, 252PorMS11 were constructed by replacing the Por1A molecule of strain 252 with the Por1B molecule of a fH nonbinding strain MS11 (6) using plasmid pUNCH61 as described previously (41). Transformants were selected on gonococcal agar containing chloramphenicol at a concentration of 25 µg/ml in growth medium to a final concentration of 2 µg/ml CMP-NANA.

**Sera and complement reagents**

Nonimmune NHS was pooled from 10 healthy volunteers with no history of gonococcal infection. Chimpazze, baboon, and rhesus sera were purchased from Bioreclamation. Rabbit and rat sera were purchased from Cedarlane Laboratories and Complement Technology, respectively. The ability of sera to support hemolysis of Ab-sensitized sheep erythrocytes was confirmed using the total complement hemolytic plate assay (The Binding Site). HuH was purchased from Complement Technology. Compartment activity in sera was abrogated by heating at 56°C for 30 min.

**Results**

**Abs and immunochemicals**

Affinity-isolated goat polyclonal Ab against HuH was made by Bethyl Laboratories using purified HuH (Complement Technology) as an immunogen (45). Goat anti-HuH was used in Western blotting experiments to detect free fH previously bound to gonococci during incubations and liberated during electrophoresis (see below). mAb 90X that is specific for mouse IgG (Sigma-Aldrich) was used to detect fH-related protein 1 (FHR-1). Liberated fH, FHL-1, and FHR-1 were detected using alkaline phosphatase (Sigma-Aldrich) at a concentration of 10 µg/ml in carbonate buffer (pH 9.4). Following blocking of nonspecific binding sites with PBS-0.05% Tween 20, serial dilutions of each concentrated supernatant were added to the wells and incubated for 1 h at 37°C. Bound chimeric proteins were detected using protein A conjugated with alkaline phosphatase (Sigma-Aldrich) at a 1/1000 dilution in PBS-0.05% Tween 20. A standard curve was generated using one of the fH/Fc fragments, CCP 18–20Fc, that had been purified over a protein A column and whose concentration was determined both by absorbance at 280 nm and by using the BCA protein assay kit (Pierce).

**Flow cytometry**

Bacteria were grown overnight on chocolate agar plates, washed with HBSS++, and bacterial concentrations were adjusted to 3 × 10⁶ cells/ml. Bacteria (10⁶ organisms) were incubated with concentrated supernatant containing 0.5 µg of recombinant fH/Fc protein in a final reaction volume of 100 µl for 30 min at 37°C. After washing, FITC-labeled goat anti-mouse

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<td>20 NotF</td>
<td>5'-GGGCUCGCTTTTTCACAAGATTGATGACATC-3'</td>
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All forward primers incorporate AscI sites (underlined). All reverse primers incorporate NcoI sites (underlined).

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IgG (Sigma-Aldrich) at a dilution of 1/100 in 1% BSA/HBSS$^+$ was added. Bacteria-bound fH/Fc fusion proteins were detected by flow cytometry (LSRII flow cytometer; BD Biosciences) using FITC-conjugated anti-mouse IgG (Sigma-Aldrich). mAb 90X was also used to detect binding of purified HuH to bacteria; anti-mouse IgG FITC was used as the disclosing fluorophore, as described above. Total C3 (C3b plus iC3b) and C4 binding to bacteria was detected using anti-C3c FITC and anti-C4 FITC as described previously (7). Analysis of binding of fH/Fc fusion proteins, fH and C3, was performed using FloJo data analysis software (www.TreeStar.com).

Western blotting

Western blotting was performed as described previously (39). To detect direct bacterial binding of HuH, FHL-1, and FHR-1 and fH from other primates, $10^8$ N. gonorrhoeae, were suspended in HBSS$^+$ and incubated for 30 min at 37°C with 10% (v/v) heat-inactivated NHS or heat-inactivated serum from chimpanzee, baboon, and rhesus in a final reaction volume of 100 μl. Bacteria were washed three times in HBSS$^+$, followed by digestion of bacterial pellets with 4% lithium dodecyl sulfate sample buffer (NuPAGE LDS sample buffer; Invitrogen Life Technologies). In experiments that used organisms incubated with heat-inactivated NHS, liberated HuH, FHL-1, and FHR-1 were resolved on 4–12% Bis-Tris gels using Tris-acetate running buffer and, after transfer, polyclonal anti-HufH and alkaline phosphatase-conjugated secondary Ab were used to detect bound HuH, as described earlier. In experiments designed to compare direct fH binding to gonococci in each of the primate sera, liberated fH was resolved on NuPAGE 3–8% Tris-acetate gels using MES running buffer (Invitrogen Life Technologies), then transferred to a polyvinylidene difluoride membrane (Millipore). Detection was performed using polyclonal anti-HufH at a dilution of 1/100 in 1% BSA/HBSS$^+$, followed by washing and incubation in Tris-acetate buffer with 0.1% BSA. The appropriate secondary Ab was then added, and complexes were visualized using a chemiluminescent detection reagent. Controls consisted of organisms incubated with NHS or the appropriate secondary Ab alone. Total C3 (C3b plus iC3b) and C4 binding to bacteria was detected using anti-C3c FITC and anti-C4 FITC as described previously (7). Analysis of binding of fH/Fc fusion proteins, fH and C3, was performed using FloJo data analysis software (www.TreeStar.com).

Serum bactericidal testing

Susceptibility of gonococci to complement-mediated killing by serum from different species was determined using serum bactericidal assays as described previously (43). Briefly, $10^8$ CFU of bacteria grown to mid-log phase were suspended in HBSS$^+$. Serum was added to a final concentration of 3.3 or 10% (concentration specified for each experiment); the final volume of all bactericidal reaction mixtures was maintained at 150 μl. Twenty-five-microliter aliquots of each reaction mixture were plated onto chocolate agar plates at time 0 and 30 min and incubated at 37°C for 24 h in 5% CO₂. Percent survival was expressed as the number of colonies surviving at 30 min compared with baseline counts at 0 min. In experiments to determine whether the addition of HuH could rescue gonococci from killing by heterologous serum complement, we first added HuH (amount specified for each experiment) to bacterial suspensions, followed by serum of the indicated species to a final concentration of 10% (v/v).

Results

Defining regions in fH that contain Por1A-binding sites

To determine the region of HuH that bound to gonococcal Por1A strains, we constructed fusion proteins genetically that consisted of contiguous HuH CCPs fused at their C-terminal ends to the N terminus of the Fc portion of mouse IgG2a (fH/Fc). The fH fragments spanned the entire length of the HuH molecule. The concentration of fH/Fc fusion proteins in concentrated tissue culture supernatants was measured by ELISA. To validate the use of the fH/Fc fusion proteins to define the CCPs in fH that bind gonococci, we first tested binding of the fusion proteins to Por1B strain F62 whose LOS was sialylated by growth in CMP-NANA-containing medium. We have shown previously that only those recombinant HuH molecules (expressed in a baculovirus system) that contained CCPs 16–20 bound to sialylated Por1B gonococci (11). Consistent with our prior observations and as seen in Fig. 1A, CCP16–20/Fc bound to sialylated F62. We further narrowed the binding site for sialylated gonococci to CCPs 18–20 because a fusion protein that contained only CCPs 18–20 also bound to sialylated F62. All three C-terminal domains (18, 19, and 20) were required for fH binding because deletion of any of these domains singly from CCP 16–20/Fc decreased binding to sialylated gonococci; deleting either CCP 16 or CCP 17 did not affect binding (data not shown). None of the fH/Fc fusion molecules tested showed binding to unsialylated F62 (negative control), which served to confirm specificity of binding of the chimeric proteins. These results were confirmed with another Por1B strain called MS11 that shows augmented fH binding upon sialylation of its LNT LOS (45) (data not shown).
We examined binding of the fH/Fc fusion proteins to Por1A strain 252 (Fig. 1A, right graph). This strain did not bind CCP 1–5/Fc or CCP 11–15/Fc, which suggested that these fH domains did not participate in binding to gonococci. We noted binding of CCP 1–6/Fc, suggesting that in the face of no binding by CCP 1–5/Fc, CCP 6 alone contained a binding site for Por1A. Another CCP-containing fusion protein, CCP 6–10/Fc, also bound to strain 252, which was consistent with CCP 6 containing a Por1A-binding site.

Separately, we also noted binding of CCP 16–20/Fc and CCP 18–20/Fc to strain 252, which suggested a second site in fH that bound to Por1A gonococci. Another HufH-binding Por1A-bearing strain called 15253 (6) also yielded similar results (data not shown). These results suggested that HufH contained two separate regions that bound to Por1A-bearing gonococcal strains, one that resided in CCP 6 and the second in a region spanned by CCPs 18–20.

The higher fluorescence of binding seen with CCP 6–10/Fc compared with CCP 1–6/Fc suggested the possibility that CCP 7 may have also contributed to the interaction between fH and Por1A. To address this, we examined binding of two additional CCP 7-containing fusion proteins (CCP 1–7/Fc and CCP 5–8/Fc) to strain 252 (Fig. 1B). Protein concentration of each recombinant fusion protein was normalized by ELISA. We noted that CCP 1–6/Fc, CCP 1–7/Fc, and CCP 5–8/Fc all bound to CCP 252 with similar fluorescence intensity, suggesting that the presence of CCP 7 did not contribute to the fH-Por1A interaction. The ~2-fold higher fluorescence seen with CCP 6–10/Fc (where CCP 6 is N-terminal) may reflect less steric hindrance for the Por1A-fH interaction rather than a direct contribution of CCP 7.

**Por1A is the molecule that binds to both regions (CCP 6 and CCPs 16–20) in fH**

We have previously shown that gonococcal Por1A directly binds HufH. However, the finding of two distinct regions in HufH that bind to Por1A strain 252 raised the possibility that this strain may possess a second molecule that binds to HufH. Alternatively, Por1A may itself interact with the two sites in HufH, perhaps at two different sites on Por1A. To address these possibilities, we replaced the Por1A molecule of strain 252 with the Por1B molecule of strain MS11. The latter strain does not bind HufH in the unsialylated state (6). The resulting mutant, called 252 PorMS11, did not bind HufH (Fig. 2), indicating that Por1A was the sole HufH-binding molecule and interacted with two distinct regions (CCP 6 and CCPs 18–20) of HufH.

**Binding of FHL-1 and FHR-1 to gonococci**

The ability of Por1A-bearing strain 252 to bind to fH via CCP 6 and CCPs 18–20, and sialylated Por1B gonococci to bind fH via CCPs 18–20 gave rise to the possibility that gonococci might bind to other members of serum proteins related to fH. These include an alternatively spliced variant of fH, called FHL-1 or members of the FHR family, that are encoded by distinct genes. A schematic depiction of the relationships between HufH, FHL-1, and FHR-1 is shown in Fig. 3A. The seven N-terminal CCPs of fH are also present in FHL-1. FHL-1 is spliced to a separate exon, which encodes four unique C-terminal amino acids added to FHL-1 (indicated by the black crescent). FHR-1 is the product of a unique gene, and the three C-terminal CCPs bear amino acid homologies to fH CCPs 18–20 as indicated (48). The binding site for mAb 90X and the Quidel anti-fH mAb is shown. A, Por1A from strain 252 is detected using mAb 90X and the Quidel mAb is shown. B, Strain 252 binds FHL-1 and FHR-1 when incubated with human serum. Strain 252 was incubated with HI-NHS, washed, and pellets were lysed with nonreducing denaturing sample buffer. Liberated fH, FHL-1, and FHR-1 were detected by Western blotting using polyclonal anti-HufH, Quidel anti-fH mAb (specific for CCPs 18–20), and mAb 90X (specific for HufH CCP 1). The locations of the full-length HufH (150 kDa), FHL-1 (43 kDa), and the two glycoforms of FHR-1 are indicated. MW, molecular mass in kilodaltons.

**FIGURE 2.** Por1A on strain 252 is the sole HufH-binding molecule. HufH binding to strain 252 (positive control), MS11 (negative control), and F62 PorMS11 was evaluated by flow cytometry. Bacteria (3 × 10⁷ organisms) were incubated with 10% (v/v) heat-inactivated NHS, and bound HufH was detected using mAb 90X. A representative isotype control with strain 252 where HufH was omitted from the reaction mixture is shown by the broken line. The y-axis represents fluorescence on a log₁₀ scale and the x-axis represents the number of events.

**FIGURE 3.** Binding of FHL-1 and FHR-1 to N. gonorrhoeae Por1A strain 252. A, Schematic of fH, FHL-1, and FHR-1. FHL-1 is an alternatively spliced variant of fH that contains the seven N-terminal CCPs. FHL-1 is spliced to a separate exon that encodes for the four C-terminal amino acids unique to FHL-1 (indicated by the black crescent). FHR-1 is the product of a unique gene, and the three C-terminal CCPs bear amino acid homologies to fH CCPs 18–20 as indicated (48). The binding site for mAb 90X and the Quidel anti-fH mAb is shown. B, Strain 252 binds fH and FHR-1 when incubated with human serum. Strain 252 was incubated with HI-NHS, washed, and pellets were lysed with nonreducing denaturing sample buffer. Liberated fH, FHL-1, and FHR-1 were detected by Western blotting using polyclonal anti-HufH, Quidel anti-fH mAb (specific for CCPs 18–20), and mAb 90X (specific for HufH CCP 1). The locations of the full-length HufH (150 kDa), FHL-1 (43 kDa), and the two glycoforms of FHR-1 are indicated. MW, molecular mass in kilodaltons.
C-terminal CCP domains of FHR-1 (CCPs 3, 4, and 5) exhibit 100, 100, and 97% amino acid homology with the corresponding CCPs (18, 19, and 20) of full-length HufH, respectively (47, 48). Consequently, we speculated that FHL-1 and FHR-1 were likely to bind to strain 252, and FHR-1 likely bound sialylated F62. Alternative FHRs, named 2–5, bear lower degrees of homology with fH (47) and seemed less likely candidates to bind to gonococci.

To identify the molecules that belong to the fH family of proteins (48) that bind to gonococci, we used anti-fH mAbs with specificities against different regions of fH. mAb 90X recognizes HufH CCP 1 (46) and therefore binds to full-length fH and FHL-1. Another HufH-specific mAb (Quidel) was found to react selectively with fH/Fe fusion proteins containing CCPs 16–20 and CCPs 18–20 (data not shown), thereby localizing specificity of this mAb to an epitope spanned by CCPs 18–20; Quidel mAb was used to detect FHR-1 binding to gonococci. A polyclonal Ab directed broadly across the fH molecule recognized all three fH-related species.

Fig. 3B shows parallel samples run on the same blot that were probed with the three different anti-fH Abs. We first examined the binding of HufH and its related proteins to strain 252 using polyclonal anti-HufH. As seen in Fig. 3B (left blot), strain 252 bound the full-length fH. In addition, two prominent bands were seen between ~35 and ~45 kDa. Two glycoforms of FHR-1 exist in human serum: one migrates at 37 kDa (FHR-1α) and the other at 43 kDa (FHR-1β) (48). We speculated that these two bands may represent the two glycoforms of FHR-1 (49).

The middle section of the Western blot was probed with the Quidel mAb, and the lane marked 2 (bacteria incubated with heat-inactivated serum (HI-NHS)) showed reactivity with fH as well as bands that migrated in parallel with the two prominent bands seen at ~37 and ~43 kDa in the left blot, confirming their identity as FHR-1α and FHR-1β. It is noteworthy that the intensity of these bands disclosed with the Quidel mAb in the lane containing bacteria incubated with serum was greater than in the control lane with serum alone, indicating that the bacteria were concentrating FHR-1 molecules on their surface.

When we used mAb 90X to detect binding of full-length fH and FHL-1 to strain 252 (Fig. 3B, right blot), weak binding of FHL-1 to bacteria was observed relative to fH binding. In contrast to FHR-1 binding, FHL-1 binding by mAb 90X was greater in serum relative to bacteria. In the aggregate, these data indicate that when incubated with human serum Por1A strain 252 binds to full-length fH and FHR-1 (α and β) preferentially, but only weakly to FHL-1. We also detected binding of FHL-1s to sialylated Por1B strain F62 (binds fH via CCPs 18–20). As expected, we did not detect any binding of FHL-1 to this strain (data not shown).

**Binding of nonhuman fH to N. gonorrhoeae**

Natural infection with *N. gonorrhoeae* is restricted to humans. We have shown previously that gonococci bind human C4BP (and in some instances, chimpanzee C4BP) selectively, which may at least in part explain species specificity of gonococcal infections (39). We speculated that binding of fH to gonococci was also likely to be species specific.

To test this hypothesis, we examined direct binding of fH from different primate species to *N. gonorrhoeae* by Western blotting. Because we had available to us only polyclonal anti-HufH as a means to measure direct fH binding to bacteria and this reagent does not cross-react with fH from nonprimates such as rabbit or rat, we were unable to directly assess nonprimate fH binding to gonococci. *N. gonorrhoeae* strains 252, sialylated F62 (and unsialylated F62 serving as negative control) were incubated with 10% heat-inactivated (to destroy heat labile complement components that permit activation of complement on the bacterial surface) human and primate sera at 37°C for 30 min which permits assessment of direct binding of (heat stable) fH to bacteria (11, 50). Unheated serum was also used for incubation (see below) to assess the functional role of fH that binds to C3b bound to bacteria in complement-active serum. Western blots were probed with polyclonal goat anti-HufH Abs after first demonstrating the ability of these Abs to react with fH from all the primate sera tested; although fH from all primates were detected, binding to baboon and rhesus fH was weaker (Fig. 4, *upper blot*). We found that HufH bound well to both strains that resisted killing by human serum (Fig. 4, *middle two blots*). Weak binding of chimpanzee fH (ChfH) was seen with 252 (Fig. 4, *second blot from top*); sialylation of strain 252 did not result in an increase in ChfH binding (data not shown). Neither strain bound fH when incubated with heat-inactivated baboon or rhesus sera. The negative control strain F62 showed minimal binding to HufH, and as expected did not bind fH from any animal species (Fig. 4, *lower blot*).

**Correlation between fH binding and serum resistance of *N. gonorrhoeae***

We examined whether direct binding of added HufH to *N. gonorrhoeae* resulted in resistance to killing by heterologous serum complement from several animal species. We used gonococcal strains that do not bind to C4BP to avoid confounding that would result from the binding to both of these complement inhibitors. We also examined whether sialylation of the LNT LOS structure enabled bacteria to resist killing by heterologous animal complement with and without added HufH.

As expected, serum bactericidal assays showed that both sialylated F62 and 252 (unsialylated) strains resisted killing (>50% survival) by 10% human serum (Fig. 5A, ). Por1A strain 252 was killed by all heterologous animal (chimpanzee, baboon, rhesus, and nonprimate (rabbit and rat)) sera tested. Sialylated F62 and also sialylated 252 survived in human and chimpanzee serum, but were killed by all other animal sera. The nonsialylated (control) strain F62 was killed >99% (0–1% survival) by serum from every species tested including human (data not shown).
The increase in HufH binding that occurs with sialylation of gonococcal LNT LOS is dependent on the inherent specificity of gonococcal Por (45). Having shown, however, that sialylation of Por1B strain F62 conferred resistance to chimpanzee serum in the absence of direct binding of ChfH to gonococci (Fig. 4), we also tested whether sialylation of Por1A strain 252 would enable bacteria to escape killing by chimpanzee complement. Sialylation of Por1A strain 252 also conferred full resistance to killing by 10% NChS (data not shown).

The experiments above examined the ability of gonococci to resist killing by 10% complement. Although low complement concentrations are relevant at mucosal surfaces, bacteria encounter higher complement concentrations in the bloodstream (as would occur during the course of disseminated gonococcal infection) or in the female genital tract during menses. To test this hypothesis, we added HufH at a physiologic concentration of 500 μg/ml to heterologous sera for use in bactericidal assays (Fig. 5A). Adding purified HufH at this physiologic concentration to primate sera restored the serum resistance phenotypes to strains that bound HufH (Por1A strain 252 and sialylated F62). Addition of physiologic concentrations of HufH restored full serum resistance (100% survival) of strain 252 against rabbit complement but resulted in only 7% survival in rat serum. Sialylated F62 showed 20% survival when HufH was added to rabbit serum, while only a 2% increase in survival was evident when HufH was added to rat serum. We speculate that the greater evolutionary distance of rabbits and rats from humans and consequently the greater diversity of the fH sequence (51–53) decreases the efficacy of HufH to regulate complement activation on the gonococcal surface by the sera of these species. To illustrate this, we used a lower concentration of rat serum (3.3%) in the bactericidal assay and added increasing amounts of HufH to the reaction mixture. In this setting, we observed that adding HufH exogenously in a dose-responsive manner rescued both sialylated strain F62 and strain 252 (unsialylated) from killing by rat serum (Fig. 5C). At this lower (3.3%) concentration of rat serum, physiologic amounts of HufH added to the serum (500 μg/ml) resulted in 35% survival of strain 252 and 83% survival of sialylated F62; supraphysiologic amounts of HufH further enhanced survival. The control strain, F62 (weak fH binder), was not rescued by the addition of HufH to serum even at a concentration as high as 2000 μg/ml (four times normal), confirming that restored serum resistance of the other strains was not the result of nonspecific inhibition of complement by supraphysiologic fH levels (i.e., the sum of native fH and added HufH).

LOS sialylation leads to inhibition of the classical pathway of complement on N. gonorrhoeae

The data in Fig. 5A show that sialylated gonococci resist only NHS and NChS, but not normal sera from lower primates such as rhesus macaques or baboons. We sought to understand the reason for the selective resistance of sialylated strains to human and chimpanzee complement.

We measured C3 binding to strains 252, F62, and their sialylated counterparts (Fig. 6, upper panel). Gonococci were incubated with NHS (kills only unsialylated F62, but not 252 or sialylated F62), NChS (kills unsialylated F62 and 252 but not their sialylated counterparts), and NRhS (kills even sialylated gonococci). Sialylation of F62 (left graph) resulted in decreased C3 fragment binding in NHS and NChS (despite direct binding to HufH only). The highest level of C3 binding was seen with NRhS and the decrease seen with sialylation was modest (consistent with the observation that NRhS killed sialylated F62; Fig. 5A). Strain 252 binds HufH even in the unsialylated state, therefore, sialylation resulted in only
a marginal decrease in human C3 binding. Consistent with its serum-sensitive phenotype in NChS and NRhS (and its inability to bind to ChfH or rhesus fH), 252 bound high levels of chimpanzee and rhesus C3. Akin to the observations with sialylated F62, sialylation of 252 also resulted in a marked decrease in chimpanzee and rhesus C3. Akin to the observations with sialylated F62, and 252 bound high levels of chimpanzee C3, but only a small decrease in rhesus C3 binding.

We hypothesized that the differences in the levels of rhesus and chimpanzee C3 binding to the sialylated strains were likely related to differences in the level of classical pathway activation. The independent classical pathway is essential for efficient complement activation on and killing of gonococci (54). We have shown previously that the mannose-binding lectin pathway does not contribute to killing of even unsialylated gonococci in the context of NHS because the lectin pathway is regulated by C1 inhibitor and α2-macroglobulin (55). We next measured C4 binding to the strains (Fig. 6, lower panel) that were incubated with normal human or primate sera (all of which contained naturally occurring antibacterial Ab). We noted that sialylation of F62 and 252 decreased C4 binding, suggesting that gonococcal LOS sialylation leads to inhibition of the classical pathway of complement. Higher levels of C4 binding to sialylated bacteria were seen with NRhS compared with NChS, which were consistent with the high rhesus C3 binding and resultant serum sensitive phenotype in NRhS.

Discussion

Complement forms a key arm of the innate immune system in combating neisserial infections. Evasion of complement-mediated killing is essential for survival of Neisseria in vivo (6, 7, 11). We have shown previously that N. gonorrhoeae can bind fH and C4BP, which are key soluble-phase inhibitory molecules of the alternative and classical pathways, respectively, and thus permit resistance of gonococci to complement-mediated killing by NHS (6, 7, 11).

In this study, we have shown that gonococcal Por1A can interact with two distinct regions in HuF, namely CCP 6 and a region spanning by CCPs 18–20. S. pneumoniae PspC has also been shown to interact with two separate regions of HuF, CCPs 8–11, and 19–20 (56). More recently, Pseudomonas aeruginosa elongation factor Tuf was also shown to bind to two distinct regions (CCPs 6–7 and 19–20) of HuF and to FHR-1 (57). We have also further narrowed the sialylated Por1B gonococcal fH-binding site to a region spanned by CCPs 18–20 (we showed previously that CCPs 16–20 contained the binding site for sialylated Por1B gonococci (11)).

A comparison of the amino acid sequences of HuF (binds Por1A) and ChfH (binds Por1A very weakly) in CCP 6 reveals differences at four amino acid positions and we speculate that one or more of these are important in forming the motif in CCP 6 that binds Por1A. In addition, we believe that of the three C-terminal HuF CCPs (18–20), CCP 20 is most likely to contain the gonococcal-binding site because this domain differs at 11 amino acid positions between the two species; CCP 18 of HuF and ChfH are identical and CCP 19 differs by only two amino acids.

In addition to binding the full-length fH molecule, Por1A gonococci also bind both FHL-1 and FHR-1; the former less compared with fH binding, the latter more. FHR-1 is associated with specific lipoprotein particles that are composed of apolipoprotein A-1 (58). The three C-terminal CCP domains of both human FHR-1 and fH, whose sequences are highly homologous, bind to heparin and C3b. However, the known complement regulatory regions are located at the N terminus within CCPs 1–4 in HuF, which are not present in FHR-1. Although the physiological role of FHR-1 remains uncertain, it also binds to certain complement regulator-acquiring surface proteins on B. burgdorferi (59). It is noteworthy that when Por1A strain 252 was incubated with human serum, the bacteria bound full-length fH preferentially over the alternatively spliced FHL-1 molecule. Our preliminary data (unpublished) show that on a molar basis, ~5-fold more recombinant FHL-1 compared with full-length HuF is required to protect N. gonorrhoeae from killing by heterologous sera. We hypothesize that preferential binding of HuF over FHL-1 enables the bacteria to more efficiently regulate complement.

N. gonorrhoeae is a human-specific pathogen. Animal models that have been used to simulate disease in humans by inoculating gonococci into their genital tracts include the chimpanzee (38, 60, 61), which can sustain gonococcal infection and more recently the 17-β-estradiol-treated mouse (37). Previously, we identified that
human (and in some instances, chimpanzee) C4BP selectively interacts with *N. gonorrhoeae* and results in species-specific infection (39). This mechanism partly explains the restriction of serum resistance of *N. gonorrhoeae* to humans, and in some instances to chimpanzees. However, certain Por1A strains, such as 252, and several Por1B strains (including their sialylated derivatives), such as F62, used in the current studies do not bind C4BP. In this report, we have shown that selective binding of HuH is an additional mechanism of species-specific complement evasion by *N. gonorrhoeae*. We show that, like C4BP, HuH also rescues *N. gonorrhoeae* from killing by heterologous sera (Fig. 5). The ability of HuH to rescue gonococci from killing by heterologous sera was best seen with primate sera, and less so with sera from lower animals. It is likely that HuH is less efficient in regulating complement from lower animals compared with primates, which are evolutionarily less distant from humans. Despite limited homology between human and nonprimate HuH, HuH displays regulatory activity over endogenous C3b from other species. For example, there is only 63% homology at the amino acid level between human and rat HuH (52). Despite the ancient nature of the complement system and its long evolution, conservation of critical structure function relationships persists in certain other important complement components as well. For example, activated forms of the human third (C3b) and fourth (C4b) complement components are cleaved at identical internal sites by enzymatic activity present in the plasma or serum of the ancient barred sand bass, *Paralabrax nebulifer* (62).

*B. burgdorferi* (the causative agent of Lyme disease) exhibits wider host specificity and expresses several fH-binding surface proteins that can be broadly divided into two classes (63): the class I fH-binding proteins comprise members of the outer surface protein E lipoproteins (64), while the class II proteins in- clude the complement regulator-acquiring surface proteins (65, 66). *B. burgdorferi* can express different outer surface protein E-related proteins, each of which exhibits differential affinity for fH from different animal hosts (67), thus permitting differential complement-mediated resistance in each host.

We observed that sialylated gonococci were resistant to NCChS, although they did not bind to ChH directly. Binding of chimpanzee, baboon, and rhesus HuH to both sialylated and unsialylated sialylated gonococci were noted, but only in the presence of an intact complement system, and occurred through C3b bound to the bacterial surface (data not shown). However, the indirectly bound HuH did not confer protection to bacteria as evidenced by the serum-sensitive phenotype of unsialylated F62 to all sera tested, and killing of sialylated bacteria by rhesus and baboon sera. The ability of sialylated gonococci to resist killing by NCChS, but not lower primate (such as rhesus macaque) complement prompted us to compare the binding of primate C3 and C4 to gonococci. LOS sialylation resulted in decreased C3 binding. We observed that the effect was more dramatic in NCChS than in NRHS. It is noteworthy that sialylation also resulted in decreased C4 binding to the bacteria. Differences in C3 binding in NCChS and NRHS were explained by differences in classical pathway activation also reflected in, for example, higher levels of rhesus C4 binding to bacteria. Neisserial LOS binds C3 and C4 (68, 69) and it is possible that sialylation of LOS may prevent binding of C3 and C4. However, sialylation in the experiments described was performed using low concentrations (2 μg/ml) of CMP-NANA, which results in <5% of the total LOS becoming sialylated (70). Classical pathway regulation by gonococcal LOS sialylation is not secondary to a decrease in NHS-derived Ig binding to bacteria (71) or because of an increase in C4BP binding (72). Therefore, a unifying explanation of how sialylation of gonococci reduces C4 binding to organisms in chimpanzee (but not rhesus) serum is not evident presently. A previous report has suggested that gonococcal LOS sialylates regulates the classical pathway by decreasing C1q binding to bacteria-bound IgM (72).

Other mechanisms that may involve increased susceptibility to human gonococcal infection may invoke, for example, other human complement regulatory proteins such as CD46 and fH binding by neisserial species. Recently, as in the case with piliated *N. meningitidis*, we have shown that select fH-binding strains of *N*. *meningitidis* (73), although the complement regulatory function of this protein has not been specifically implicated in this enhanced susceptibility.

The specific species of complement regulatory protein binding to Neisseria may also serve to explain why rabbit complement is more bactericidal than human complement when vaccine-induced Abs are tested for their complement-dependent killing activity (74, 75). Our preliminary data suggests that fH binding to *N. meningitidis* is also restricted to humans (our unpublished observations). This suggests that human complement may be more relevant to use in the evaluation of meningococcal vaccine candidates.

In conclusion, we have identified the HuH CCPs that bind to gonococci, and have shown that direct binding of fH to gonococci is restricted to HuH. These data, in part, provide an explanation for the restriction of serum resistance of *N. gonorrhoeae* to humans separate from that seen with direct binding of C4BP to *N. gonorrhoeae*. Knowledge of factors that permit this neisserial species to survive in nonhuman hosts may lead to development of animal models that will better simulate human disease.

**Acknowledgments**

We thank Dr. Hanna Jarva for providing mAb 90X.

**Disclosures**

The authors have no financial conflict of interest.

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