Binding of Complement Factor H to Loop 5 of Porin Protein 1A: A Molecular Mechanism of Serum Resistance of Nonsialylated *Neisseria gonorrhoeae*

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Summary

Neisseria gonorrhoeae isolated from patients with disseminated infection are often of the porin (Por1A) serotype and resist killing by nonimmune normal human serum. The molecular basis of this resistance (termed stable serum resistance) in these strains has not been fully defined but is not related to sialylation of lipooligosaccharide. Here we demonstrate that Por1A bearing gonococcal strains bind more factor H, a critical downregulator of the alternative complement pathway, than their Por1B counterparts. This results in a sevenfold reduction in C3b, which is >75% converted to iC3b. Factor H binding to isogenic gonococcal strains that differed only in their porin serotype, confirmed that Por1A was the acceptor molecule for factor H. We identified a surface exposed region on the Por1A molecule that served as the binding site for factor H. We used gonococcal strains with hybrid Por1A/B molecules that differed in their surface exposed domains to localize the factor H binding site to loop 5 of Por1A. This was confirmed by inhibition of factor H binding using synthetic peptides corresponding to the putative exposed regions of the porin loops. The addition of Por1A loop 5 peptide in a serum bactericidal assay, which inhibited binding of factor H to the bacterial surface, permitted 50% killing of an otherwise completely serum resistant gonococcal strain. Collectively, these data provide a molecular basis to explain serum resistance of Por1A strains of *N. gonorrhoeae*.

Key words: *Neisseria gonorrhoeae* • factor H • porin • serum resistance

Neisseria gonorrhoeae elicit a wide range of disease syndromes that range from minimal to severe inflammation at the local genital site of infection. Gonococci causing symptomatic local inflammation (pelvic inflammatory disease, PID)[1] are predominantly sensitive to killing in vitro by nonimmune normal human serum (NHS; reference 1). In contrast, most gonococci that cause disseminated infection (DGI) in the absence of local genital symptoms are resistant to NHS killing (2, 3). Most gonococci are phenotypically serum resistant when isolated initially, but certain strains convert to a serum-sensitive phenotype with serial subculture in vitro (4); others remain stably serum resistant. Serum resistance of the serum-sensitive phenotype can be restored by the addition of 5′-cytidinemonophospho-*N*-acetyl neuraminic acid (CMP-NANA) to the growth medium (5), which results in sialylation of lipooligosaccharide (LOS; reference 6). Recent work has defined the mechanism of this conversion as direct binding of the complement regulatory protein factor H to sialylated gonococcal LOS (7). Functionally, the increase in factor H binding mediates complete conversion of bound C3b to iC3b and a decrease in total C3 bound (7; McQuillen, D.P., S. Gulati, S. Ram, A.K. Turner, D.B. Jani, T.C. Heeren, and P.A. Rice, manuscript submitted for publication). Stable serum resistance of gonococci, in most cases, also results in increased conversion of C3b to iC3b but is not explained by sialylation because many of these strains lack the appropriate acceptor site on LOS that permits sialylation.

Gonococcal Por, the major outer membrane protein, occurs in two primary immunochemical classes, Por1A and Por1B, each composed of multiple serovars (8). Por1A and Por1B differ in apparent molecular weight, susceptibility to proteolysis (9), and antibody reactivity (10). In addition to

[1] *Abbreviations used in this paper:* CMP-NANA, 5′-cytidinemonophospho-*N*-acetyl neuraminic acid; DGI, gonococci that cause disseminated infection; LOS, lipooligosaccharide; NHS, normal human serum; PID, pelvic inflammatory disease.

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being more highly sensitive to penicillin (11), thiamphenicol, and rifampin (12), strains expressing Por1A are often serum resistant and associated with DGI, whereas those expressing Por1B are more frequently serum sensitive and associated with PID (13, 14). In contrast to gonococcal LOS, which exhibits considerable antigenic and phase variation in vitro (15–17), Por is antigenically stable in vitro (10). Analysis of the structural genes for Por1A (18) and Por1B (19) suggests that there is only a single Por structural gene, composed of two alleles, in the gonococcal genome. This has been confirmed and hybrid Por1A/B genes have been constructed by transformation (20).

Microbial factor H–binding proteins that may contribute to pathogenicity have been identified as the streptococcal M6 protein (21, 22), the *Yersinia enterocolitica* YadA protein (23), and the gp41 and gp120 glycoproteins of human immunodeficiency virus 1 (24–26). We undertook the current study to define whether such a mechanism of serum resistance might be associated with gonococcal Por1A. Upon initial screening, we found that nonsialylated Por1A gonococci bound more factor H in NHS than did Por1B gonococci. Using isogenic gonococcal strains differing only in their porin type (27), we demonstrated an association between the degree of susceptibility to bacterial killing and the amount of factor H bound. Incubation of purified factor H with hybrid Por1A/B strains (20) indicated that only those mutants that contained a region representing loop 5 of Por1A bound factor H. Specificity of factor H binding was established by inhibition with a synthetic peptide that corresponded to Por1A loop 5 (28). This peptide was also able to restore killing in NHS, and thereby confirmed functional specificity of factor H binding. Collectively these data establish that the mechanism of serum resistance associated with gonococcal strains that express Por1A is due to binding of complement factor H to loop 5 of the porin. This also represents the first demonstration of a second distinct factor H binding site on the surface of a single bacterial subspecies.

Materials and Methods

Bacterial Strains. 23 strains of *N. gonorrhoeae* (this paper; references 2, 29–35) that were initially screened for factor H, total C3, C3b, iC3b, and factor Bb binding in NHS are listed in Table 1. Serological classification of the gonococcal strains was performed using mAbs directed against Por (8). Subsequent measurements of factor H binding to the following mutant gonococcal strains were conducted using purified factor H instead of NHS (see below). Isogenic strains that differed only in their porin structures were constructed from strains FA19 (Por1A) and MS11 (Por1B; reference 27; see summary in the tabular portion of Fig. 2). Strain FA6564 had Por1A porin reintroduced into an FA19 (Por1A) background, whereas the isogenic pair FA6571 had Por1B porin introduced into a Por1A background. Strain FA6616 had Por1B porin reintroduced into an MS11 (Por1B) background, and the isogenic mutant FA6611 had a Por1A porin introduced into a Por1B background. Nine strains that contained Por1A/B porin hybrid molecules (20) were used to localize the factor H–binding region in the porin molecule. The hybrid strains were analyzed by

Table 1. *Gonococcal Strains Surveyed for Factor H Binding*

Strain	Por subclass	Reference
15253	1A	29, 30
24-1	1B	31
WG	1B	32
MS11	1B	33
FA19	1A	34
F62	1B	35
NRL no. 31772	1A	2
252	1B	This paper
339063	1A	This paper
273043	1A	This paper
442089	1A	This paper
401082	1A	This paper
269041	1B	This paper
156001	1B	This paper
274045	1A	This paper
255034	1B	This paper
336062	1B	This paper
256036	1B	This paper
179008	1A	This paper
374073	1A	This paper
422083	1B	This paper
398079	1B	This paper
150002	1B	This paper

colony hybridization by using 10 oligonucleotides that were specific for either Por1A or Por1B gene sequences, to assess which portions of the hybrid Por genes contained Por1A sequences and which contained Por1B sequences (20).

Growth of Strains. The 23 strains of *N. gonorrhoeae* (Table 1), and the Por1A/B hybrid mutant strains were grown for 13 h (5% CO_2, 37°C) on solid media supplemented with Isovitalex equivalent (36). Nonpiliated, transparent organisms were lifted off the plates with a sterile cotton swab, washed twice in HBSS, and resuspended in the same buffer for use in flow cytometry. This method enabled us to screen a large number of strains at the same time, to allow for a more meaningful and accurate interstrain comparison. For all other experiments, gonococci were first grown on solid media as described above, then inoculated into liquid media and grown to mid-log phase (36).

Sera and Complement Reagents. Pooled nonimmune NHS was obtained from 11 healthy volunteers with no prior history of gonococcal infection and used for initial screening of 23 gonococcal strains (Table 1). Human factor H was purified from human plasma to a final concentration of 1 mg/ml in PBS (37) and used for binding studies with the Por1A/B hybrid and isogenic mutant strains described above.

Antibodies. Factor H bound to the organism surface was detected in flow cytometry experiments using affinity-purified rabbit polyclonal anti-factor H. This antibody was used at a final dilution of 1/100 of a stock solution of 0.26 mg/ml (7). mAbs C-5G and C-3E, directed against human C3b and iC3b respectively (gifts of

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Dr. Kyoko Iida, Takeda Chemical Industries, Ltd., Tsukuba, Japan; reference 38), and a mAb specific for the Bb neoantigen fragment of human factor B (39; Quidel, San Diego, CA) were used in whole cell ELISAs (see below) to monitor these complement components bound to gonococcal strains. mAb 2C3, that binds the H.8 lipoprotein antigen that is present on all pathogenic gonococcal strains (40, 41), was used to monitor the number of organisms in the reaction mixtures, thereby permitting calculation of the density of the measured complement component on gonococci. All mAbs were used at a final concentration of 25 μg/ml in PBS. Total C3 bound to the organism surface was detected with goat polyclonal anti-human C3 (Organon Teknika-Cappel, Durham, NC; final concentration of specific antibody, 7 μg/ml in PBS). All the above antibodies have been described in detail previously (7). mAbs 3C8 and 5.51 that are specific for loop 5 of Por1B (28) were used in dot-blot assays to characterize the Por1A/B hybrid mutants. Only those hybrids (class 4 and class 9) that contained Por1B loop 5 reacted with these mAbs; the class 7 and class 8 hybrids did not react and therefore were determined to contain Por1A loop 5.

DNA Techniques and Porin Sequencing. Genomic DNA was extracted from gonococcal strains 15253, 273043, 442089, 401082, 339063, and the class 4 hybrid using the phenol-chloroform extraction method as described previously (42). The porin gene was amplified from these templates by PCR using primers 33 and 34 that have been previously described (43, 44). After purification of the PCR product, the region encompassing loop 5 was sequenced using two internal primers, 5′-TTCGGGCAAAAATCACAGC (sense oligonucleotide), and 5′-CGGAAGCGTACAGGGCATCA (anti-sense oligonucleotide), using an Applied Biosystems sequencer at the DNA core facility at Boston University.

Synthetic Peptides. Synthetic peptides encompassing the putative exposed regions of the Por1A molecule were synthesized using tBOC protocols, as described (28). The loop 1, 3, and 5 peptides used in this study correspond to the previously described peptides 2, 3, and 4, respectively (28). The peptides were synthesized with an NH_2-terminal cysteine. Peptides were dissolved in 1% DMSO (vol/vol) in HBSS (to yield a 2 mg/ml stock solution). 50 μg and 100 μg of each peptide were used in flow cytometry and bactericidal experiments respectively. An equivalent amount of DMSO was included in all control reactions to ensure that the results were not influenced by DMSO.

Flow Cytometry. We used flow cytometry to quantitate binding of factor H to gonococci. This procedure has been described in detail elsewhere (7). In brief, each sample tube contained 2×10^8 bacteria washed twice in HBSS and resuspended in 90 μl of the same buffer. All buffers were passed through a 0.22-μm filter to exclude particles that could interfere with flow cytometry. Bacterial suspensions were incubated initially for 30 min with 10 μl NHS or 5 μg purified human factor H (1 mg/ml in PBS). In certain experiments 50 μg of each synthetic peptide was used in the suspension to attempt competition for factor H binding to bacteria. Suspensions were centrifuged at 10,000 *g* for 1 min, and pellets were washed once with HBSS. Surface-bound factor H was detected using anti-factor H antibody (incubated for 30 min at room temperature), followed by FITC-labeled anti–rabbit IgG (final dilution 1/100; Sigma Chemical Co.). The bacteria were resuspended in 1 ml HBSS, applied immediately to a fluorescence-activated cell sorter (FACScan®; Becton Dickinson Immunocytometry Systems, San Jose, CA), and 50,000 events were counted.

ELISA. C3b, iC3b, factor Bb, and total C3 bound to gonococci opsonized with NHS were measured by whole cell ELISA (7).

Serum Bactericidal Assay. Susceptibility of 23 gonococcal strains to complement-mediated killing by nonimmune NHS was surveyed with a serum bactericidal assay (final serum concentration of 6.7%; reference 36; see Fig. 1). The isogenic porin mutant strains (see Fig. 3) were subjected to NHS at a concentration of 33.3%. In bactericidal assays used to study the effect of the porin loop peptides, the peptides (100 μg) were first added to the organism suspension, followed by the addition of NHS to a final concentration of 33.3%. A higher concentration of NHS was used here because strain FA19 is completely serum resistant, whereas strain MS11 is only partially serum resistant (27). Routinely included as a control was NHS heat inactivated (56°C for 30 min) to ensure that reduction in colony counts at time zero was not the result of agglutination.

Statistical Methods. Factorial analysis of variance with Fisher's Protected Least Significant Difference (45) was performed using the Statview 4.51 statistical analysis package (Abacus Concepts Inc., Berkeley, CA).

Results

Identification of Por1A as the Acceptor Molecule for Factor H on Nonsialylated N. gonorrhoeae. Using flow cytometry we screened 23 strains of *N. gonorrhoeae* that had been incubated with 10% NHS (30 min) for factor H binding. The factor H binding observed after opsonization of organisms lifted off solid media correlated well with factor H binding to the same strains grown in liquid media on other occasions. The forward scatter profiles did not indicate aggregation of organisms. Factor H may directly bind to the bacterial surface, or may only be indirectly associated with the bacteria through C3b that is bound to the bacterial outer membrane. The 30-min time point was chosen to allow for dissociation of any factor H bound only to C3b that eventually would be processed further to iC3b, thus providing a better estimate of factor H directly bound to the organism surface. As seen in Fig. 1, Por1A strains bound significantly greater amounts of factor H as judged by a greater median $\log_{10}$ fluorescence intensity ($P = 0.0125$) on these strains. A functional counterpart of factor H binding was examined by measuring iC3b and C3b deposited on the organism surface by whole-cell ELISA. We chose a 10-min time point of opsonization because kinetic studies have shown that maximal deposition of C3 components onto gonococci occurs within 5 to 10 min of opsonization (46; McQuillen et al., manuscript submitted for publication). Por1A strains bound significantly less total C3 than Por1B strains (optical density = 0.449 ± 0.092 vs. 0.917 ± 0.123; $P = 0.0089$). Total iC3b bound was not significantly different between Por1A and Por1B strains ($P = 0.5294$, not shown) but the total C3b as well as the factor Bb bound to Por1A strains was significantly lower than for Por1B strains (Fig. 1; $P = 0.0011$ and $P = 0.0018$, respectively). Therefore, the ratio iC3b/(iC3b+C3b) was significantly higher for Por1A strains (Fig. 1; $P = 0.0125$), suggesting highly efficient conversion of C3b to iC3b on these strains. These observations also support dissociation of the alternative pathway C3 convertase (C3b,Bb) by factor H, thereby limiting the total amount of C3 deposited on the organism.

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Collectively these data suggested that Por1A may act as a gonococcal surface acceptor for factor H.

To confirm this hypothesis, we examined factor H binding to isogenic gonococcal mutant strains differing only in their porin type. These isogenic mutants were generated from strains FA19 (Por1A) and MS11 (Por1B; reference 27; tabular portion of Fig. 2). Pure factor H bound only to strains that contained a Por1A porin (strains FA6564 and FA6611), independent of the background (Fig. 2). This suggests that the Por1A molecule is the acceptor for factor H. The functional significance of factor H binding was established by a serum bactericidal assay. Resistance to the bactericidal activity of NHS (33.3%) correlated well with the porin type of the strain (Fig. 3), with Por1A-bearing strains FA6564 and FA6611 being completely serum resistant, whereas Por1B containing strains FA6571 and FA6616 being serum sensitive to the same degree.

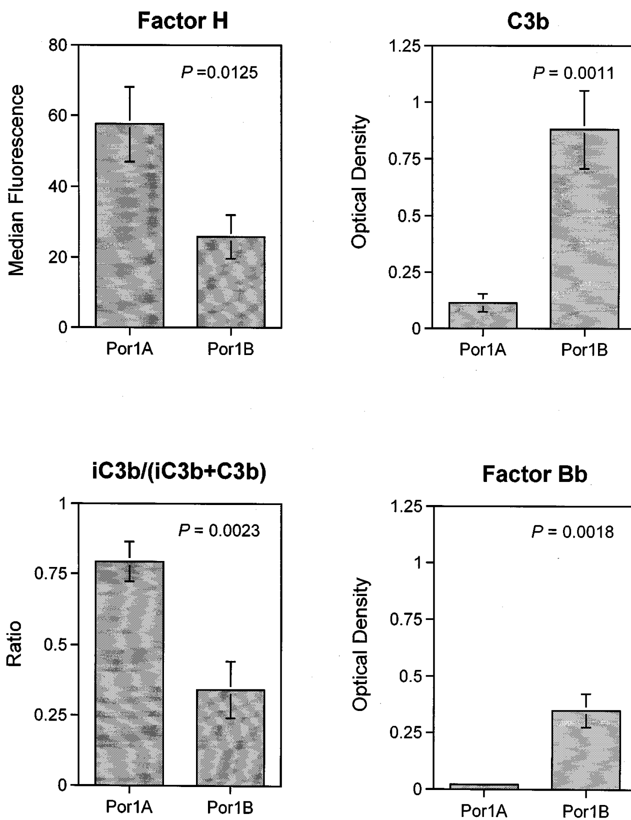
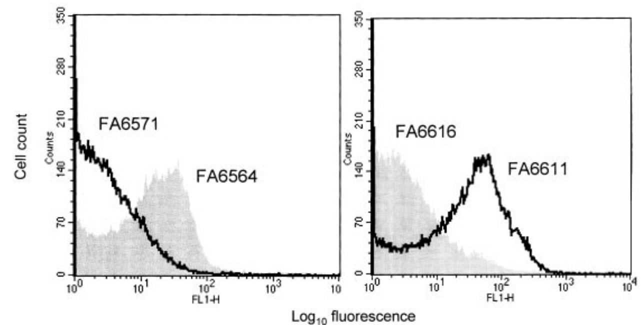


Figure 1. Binding of factor H, C3b, and factor Bb to gonococci. 23 strains (10 Por1A, 13 Por1B; Table 1) of *Neisseria gonorrhoeae* were incubated in 10% nonimmune NHS (30 min for factor H binding, and 10 min for other complement components). Factor H binding was measured by flow cytometry and is expressed as median $\log_{10}$ fluorescence. Because this was a screening survey, factor H measurement was obtained once for each strain and several strains were measured twice to control for potential experimental variation. Total C3, C3b, iC3b, and Bb were measured by ELISA (duplicate experiments); data (*C3b* and *Bb*) are expressed as optical density. The ratio of iC3b/(*iC3b*+*C3b*) was calculated and is also shown. All data shown represent mean ± SEM; significance was calculated by factorial analysis of variance with Fisher's Protected Least Significant Difference (45).

Localization of the Specific Region of Por1A Where Factor H Binds. To localize the specific Por1A region necessary for factor H binding, we studied the binding of pure factor H to nine Por1A/B hybrid mutant strains (20) by flow cytometry. The four hybrid strains that enabled us to localize loop 5 as the region necessary for factor H binding are shown in Fig. 4. The class 9 hybrid porin has been sequenced previously (47), and contains a Por1A sequence to amino acid 144, where a crossover to MS11 (Por 1B) occurs. Downstream reversion to a Por1A sequence takes place in a conserved region between Por1A amino acids 208 and 213. Thus, the exposed regions of loops 4 and 5 are of a Por1B type, whereas all the other loops are of a Por1A type. The class 4 hybrid consists of loops 1 through 4 that belong to Por1A, and loops 5 through 8 that belong to Por1B; the crossover event occurs at amino acid 175 of Por1A. mAbs 3C8 and 5.51, which are both specific for Por1B loop 5, were used in dot-blot assays to further characterize the structure of the hybrid strains (28). Both the class 4 and class 9 hybrids bound to these mAbs, whereas the class 7 and class 8 hybrids did not react (data not shown). This confirmed that Por1B loop 5 was present in the class 4 and class 9 hybrids, but not in the class 7 and class 8 hybrids. The class 9 hybrid did not bind factor H, which narrowed down the binding region to loops 4 and 5. The class 4 hybrid did not bind factor H either, and therefore ruled out Por1A loop 4 as the factor H–binding region. The factor H binding domain in Por1A was narrowed down to a region spanned by amino acids 175 and



Strain	Background	Porin type reintroduced
FA6564	FA19 (Por1A)	FA19 (Por1A)
FA6571	FA19 (Por1A)	MS11 (Por1B)
FA6616	MS11 (Por1B)	MS11 (Por1B)
FA6611	MS11 (Por1B)	FA19 (Por1A)

Figure 2. Binding of purified factor H (5 μg) to isogenic porin mutant strains, measured by flow cytometry (one representative experiment of two performed). Strain FA6564 (Por1A porin in a Por1A background) bound factor H, while its isogenic mutant FA6571, differing only in the porin molecule present (Por1B), did not. Similarly, FA6616 (Por1B porin type in a Por1B background) did not bind factor H, while its isogenic pair FA6611 (Por1A porin type in a Por1B background) bound factor H. Thus, factor H binds only to strains bearing the Por1A subclass, independent of the background.

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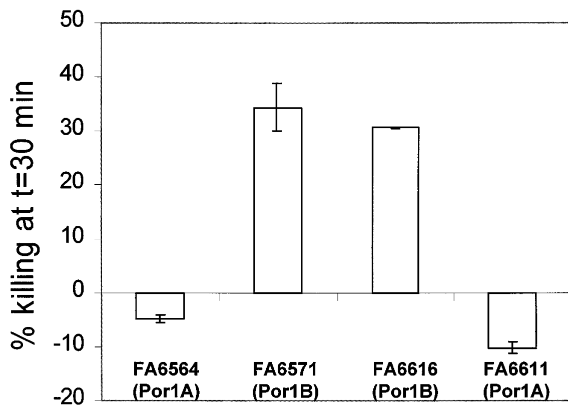

Figure 3. Functional significance of factor H binding. Isogenic porin mutant strains were subjected to the bactericidal action of 33% NHS. Strains bearing a Por1B molecule (*FA6571* and *FA6616*) were susceptible to killing by NHS, while those that did not (*FA6564* and *FA6611*) resisted killing. This effect was independent of the background structure of the strains. Data shown represent mean ± SD of duplicate experiments.

208. The only exposed region in this part of the Por1A molecule was loop 5. Thus, it was inferred that Por1A loop 5 was essential for factor H binding. This is further supported by the fact that the class 7 and class 8 hybrids, both of which contain Por1A loop 5, bound factor H (Fig. 4). Results obtained with five other Por1A/B hybrid strains were consistent with those obtained above; only those hybrids that contained the fifth Por1A loop bound factor H (data not shown).

To confirm that loop 5 was the factor H–binding region in Por1A by a second independent method, we used a synthetic 25-mer peptide that encompassed the putative exposed region of Por1A loop 5 to inhibit the binding of purified factor H to strain FA6564. The amino acid sequence of the loop 5 peptide is CVQYAGFYKRHSYTTEKHQVHRLV. The segment that corresponds to the surface exposed region is underlined (amino acids 179–187). Two other 25-mer peptides encompassing the exposed regions of Por1A loop 1 and loop 3 were used as controls to impart specificity to the assay (28). 50 μg of each peptide was used to inhibit the binding of 5 μg of purified factor H. Only the loop 5 peptide was able to inhibit factor H binding to FA6564 to any significant degree (Fig. 5), validating the fact that loop 5 is the factor H binding region in Por1A. The six COOH-terminal amino acids in the loop 5 peptide are unlikely to be relevant in binding of fH because they are identical to this region in the Por1B structure.

Using the Por1A loop 5 peptide, we next attempted to inhibit fH binding to five additional Por1A strains (15253,

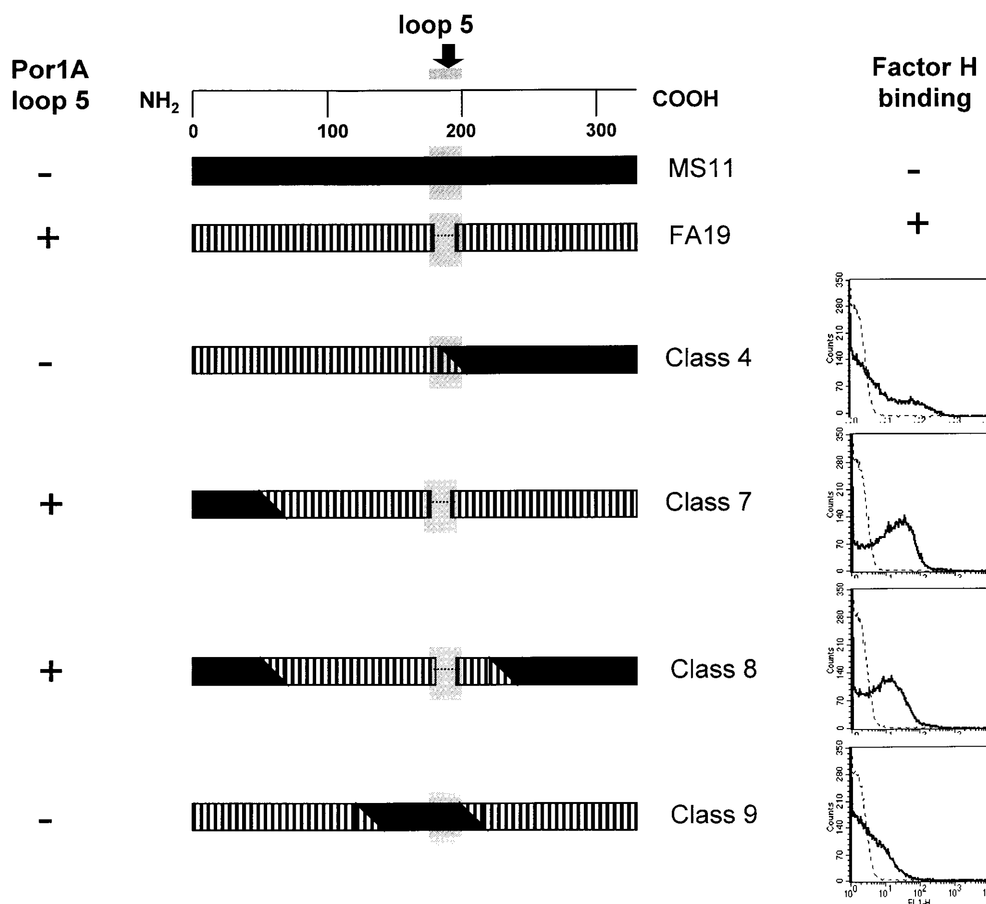

Figure 4. The specific porin loop of Por1A that binds factor H was determined by incubating factor H (5 μg) with Por1A/B hybrid strains (20) followed by flow cytometry (data shown here for four hybrid strains; one representative experiment of two performed). The grey shaded area indicates the location of loop 5. Only those mutants that displayed a region that contained loop 5 of the Por1A molecule bound factor H, thus establishing this as the Por region required for factor H binding. The dotted line within the Por1A molecule represents the amino acid segment (present in Por1B of MS11) that is deleted in Por1A of FA19.

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273043, 442089, 401082, and 339063) that directly bound pure fH. We were unable to demonstrate inhibition of fH binding to any of these strains with this peptide. In each case the sequence of putative surface exposed region of the Por loop 5 had an amino acid sequence of RHNYTTEKH (amino acids 179–187). This differed from the exposed region of loop 5 of FA6564 (RHSYTTEKH), where an Asn residue replaces Ser at amino acid 181 (underlined).

Functional Correlation of Por1A Loop 5 Binding of Factor H. To evaluate the functional impact of factor H binding to serum resistant strain FA6564 (a strain completely resistant to NHS), we attempted to block binding of factor H to the organism in the presence of NHS. A bactericidal assay was performed using strain FA6564 that was first suspended in buffer containing (or lacking) loop 5 peptide (100 μg), then combined with NHS (final concentration 33.3%). Having previously established that only loop 5 peptide inhibited factor H binding to the organism, we used only loop 1 peptide as a control in this experiment. Additional controls included organisms incubated with NHS alone, loop 5 peptide alone, heat-inactivated NHS plus loop 5 peptide, and NHS plus loop 1 peptide. Killing in the presence of the peptides was compared with killing at the corresponding time point with NHS alone (percentage killing, Fig. 6). The reaction mixture containing NHS and loop 5 peptide showed 37% killing even at time zero, which increased to 52% at 30 min. No significant killing was seen under any other conditions of incubation, including NHS alone at either time point. Loop 5 peptide did not deplete hemolytic complement as evidenced by the ability of NHS plus loop 5 peptide to lyse sensitized sheep erythrocytes. Similar results were obtained when loop 5 peptide was preincubated with NHS (30 min on ice) before incubation with the organisms (48% killing at time zero and 66% killing at 30 min). The addition of loop 5 peptide presumably prevented factor H (in NHS) from binding to the organisms. This in turn converted strain FA6564 to a complement-activating surface that resulted in killing of this strain. These data underscore the importance of factor H in mediating serum resistance.

Discussion

The association between local genital inflammation observed during infection with *Neisseria gonorrhoeae* and the degree of resistance to serum killing is well established (1). The several molecular mechanisms responsible for this relationship have begun to be delineated over the past decade and a half. Isolates that are sensitive to killing by NHS in vitro can be converted to a resistant phenotype by sialylation of the terminal lactosyl residue of their LOS (5, 48). Recent work in our laboratory demonstrated that presence of this terminal sialic acid residue permits direct binding of factor H, accounting for the resultant conversion to a resistant phenotype (7). Factor H, a critical regulator of the alternative complement pathway, acts as a cofactor for factor I–mediated cleavage of C3b to iC3b and facilitates dissociation of Bb from the alternative pathway C3 convertase (C3b,Bb; references 37, 49, 50). Functionally, the increased binding of factor H to sialylated serum-sensitive gonococci (7) results in complete conversion of C3b to iC3b on these isolates and decreased total C3 binding (7; McQuillen, D.P., S. Gulati, S. Ram, A.K. Turner, D.B. Jani, T.C. Heeren, and P.A. Rice, manuscript submitted for publication).

Although *N. gonorrhoeae* clearly can use sialylation as a means to evade the bactericidal action of the complement system, unquestionably there are additional mechanisms involved in serum resistance. Strains isolated from patients with DGI remain predominantly serum resistant in vitro without the addition of CMP-NANA to the growth medium (1, 3). In addition, gonococci expressing Por1A are more commonly serum resistant and associated with DGI, whereas those expressing Por1B are more frequently serum sensitive and associated with PID (14). Factor H–binding proteins that may contribute to pathogenicity have been identified on several microorganisms. The M6 protein of *Streptococcus pyogenes* selectively binds factor H and mediates inactivation of surface bound C3b (21, 22). *Yersinia enterocolitica* that express YadA protein bind factor H and degrade surface bound C3b to iC3b faster than those not expressing YadA protein (23). Finally, gp41 (25) and gp120 (26) glycoproteins of human immunodeficiency virus 1 have been shown to bind factor H. Recombinant gp120 coated on the surface of CD4 cells also mediates cleavage of C3b (24). We undertook this study to define whether a similar mechanism was responsible for the observed association of gonococcal Por1A with serum resistance.

Initial screening of 23 clinical and laboratory isolates indicated that nonsialylated gonococci that expressed Por1A

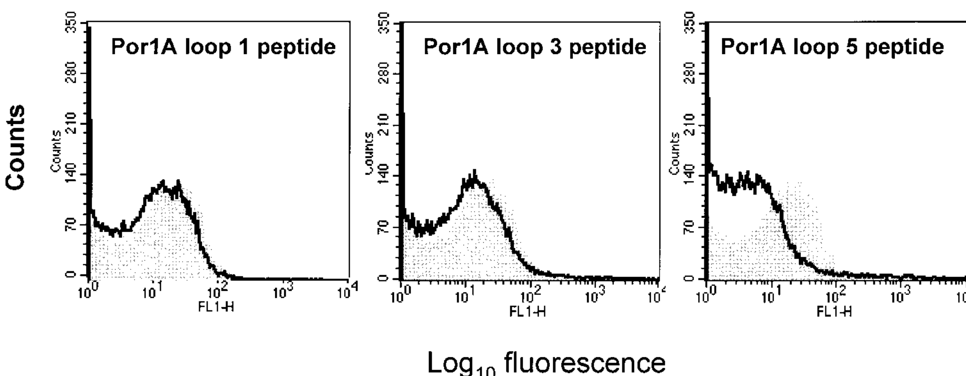

Figure 5. Synthetic 25-mer peptides (50 μg each) corresponding to three of the putative exposed loops of the Por1A molecule (28) were incubated with strain FA6564 followed by addition of factor H (5 μg). Factor H binding was measured by flow cytometry (one representative experiment of two performed). Only the peptide corresponding to loop 5 inhibited factor H binding, providing additional proof that loop 5 of Por1A is critical for factor H binding.

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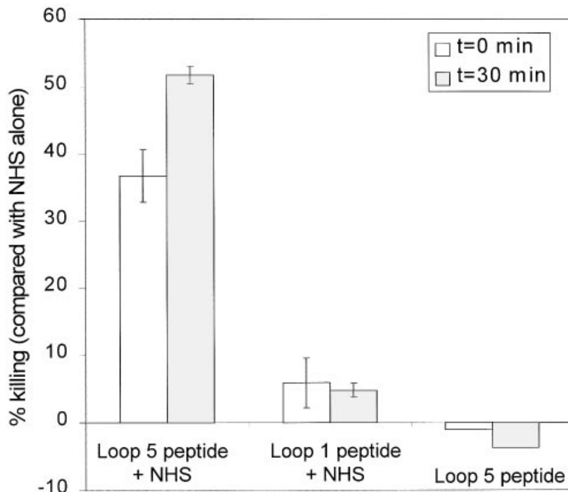

Figure 6. Functional importance of factor H in determining serum resistance. An inhibition serum bactericidal assay was performed. 100 μg of 25-mer peptides corresponding to Por1A loop 1 and loop 5 were added to the organisms (FA6564) before addition of NHS. Killing in the presence of the peptides was compared with killing at the corresponding time point with NHS alone (results of duplicate experiments shown, mean ± SD). Significant killing (35%) was seen even at time zero in the presence of loop 5 peptide, which increased to 50% at 30 min. No killing was observed with the loop 5 peptide in the absence of NHS, or with loop 1 peptide in the presence of NHS. Data shown represent mean ± SD of duplicate experiments.

bound more factor H in NHS than did gonococci expressing Por1B ($P = 0.0125$; Fig. 1). Functionally, this resulted in increased conversion of C3b to iC3b manifested as an increase in the iC3b/(iC3b+C3b) ratio ($P = 0.0023$; Fig. 1) and decreased susceptibility to killing by NHS of Por1A strains (Fig. 3). Sialylation occurs to a variable degree in vivo (40), perhaps as a result of sialidases secreted by other organisms that may be present in genital secretions (51–53). Under such conditions, the presence of a second binding target for factor H would confer protection against complement-mediated killing in the desialylated state.

Por1A was determined to be the target for factor H by the use of isogenic gonococcal strains differing only in their porin molecules. Fig. 2 demonstrates that only those strains containing a Por1A porin molecule bound factor H, independent of the background of the strain. Factor H binding correlated well with an increased resistance to the bactericidal action of NHS (Fig. 3). The moderate degree of serum resistance observed with these Por1B strains can probably be explained by the fact that both parent strains bind significant amounts of C4b-binding protein (our unpublished observations). A prior study demonstrated that FA6616 was almost completely killed (<10% survivors) by 30% serum, whereas 30% of FA6571 colonies survived at this concentration of serum (27). The differences in the sensitivities between FA6616 and FA6571 that were observed in the earlier study using 30% serum (27) were not seen in our bactericidal assay at this concentration, but were evident when we used 66.7% NHS (data not shown). At this higher serum concentration, ~40% of FA6571 colonies survived, whereas <10% of FA6616 colonies survived, consistent with results of the earlier study. A possible explanation is a difference in the bactericidal method used in the two studies. In the current study, ~2,000 CFU of *N. gonorrhoeae* were incubated with 33% serum, whereas in the previous study, only 200–300 CFU were exposed to the effects of 30% serum. This may have permitted more complete and efficient opsonization in the earlier study, due to greater numbers of C3 molecules present per organism, an effect observed with erythrocytes (46). At a serum concentration of 66.7%, the ratio of C3 to organisms more closely approximated the opsonization conditions of the prior study. Finally, incubation of purified factor H with the hybrid strains (20) indicated that only those mutants that contained a region spanning loop 5 of Por1A bound factor H (Fig. 4). All of the porin hybrid strains in this study are hypersusceptible to the killing activity of NHS (27). This may explain the extreme rarity of such strains in nature (54), although Por1A/B hybrids can be isolated in vitro after DNA transformation (55). Therefore, we could not perform bactericidal assays to correlate factor H binding with serum resistance of these Por1A/B hybrid strains.

Specificity of factor H binding was established by inhibition of purified factor H binding with a synthetic peptide corresponding to Por1A loop 5 (28). Killing of FA6564 in NHS was observed only in the presence of this peptide, thereby confirming functional specificity of factor H binding. The effects of inhibiting the binding of factor H to strain FA6564 when incubated with 33.3% NHS are seen in Fig. 6. These results suggested almost immediate deposition of C3b and insertion of membrane attack complex, resulting in impaired viability of the organism, which was manifested as a reduction in colony counts (when compared with the same number of organisms incubated with NHS alone) at time zero as well as at 30 min. Control experiments ensured that this reduction in colony counts was the result of immediate killing and not of agglutination. This degree of killing is impressive because strain FA6564 is completely resistant to the bactericidal activity of even 66% NHS (data not shown). FA6564 also binds C4b-binding protein (our unpublished data), which downregulates the classical pathway (56). This effect may protect the organism against complete killing when factor H binding to the organism is inhibited with the Por1A loop 5 peptide. Finally, the sequence of loop 5 of Por1A in strain FA19 (27) lacks the chymotrypsin cleavage site (9) present in loop 5 of Por1B in strain MS11 (20). The exposed amino acid sequence of loop 5 in Por1A (27) is relatively conserved among strains of *N. gonorrhoeae* that express Por1A (by GenBank search) and appeared to confer the ability to bind factor H. The inability of the FA19 Por1A loop 5 peptide, even when used at a 500-fold molar excess, to inhibit fH binding to Por1A strains that express a different loop 5 sequence may be because the porin molecules with different sequences bind to distinct, or separate, regions on fH. Taken together, these data establish that the serum resistance associated with gonococcal strains expressing Por1A results from binding of complement factor H to loop 5 of

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the porin. Based on prior published gonococcal Por1A sequences and the sequences in this study we have derived a consensus sequence for fH binding, which is RH-[SN]-[YF]-TTEKH. These data also represent the first demonstration of a second, distinct factor H binding site on the surface of a single bacterial subspecies.

Gonococcal porin is universally expressed and undergoes minimal antigenic variation (10). These properties have led to the intense investigation of the porin molecule as a potential vaccine candidate. Two previous studies, which used porin as an immunogen, demonstrated that the Por1A molecule did not elicit as potent an immune response as Por1B (47, 57, 58). Subsequently, the same synthetic peptides that were used in this study were used to elicit an immune response in rabbits. Interestingly, the Por1A loop 5 peptide elicited an antibody response in only one of two immunized rabbits, despite the prediction that this loop was likely to be highly immunogenic (28). In addition, the antibody directed against this loop was not bactericidal. These phenomena have not been fully explained. In contrast, the other Por1A peptides tested elicited a good bactericidal antibody response. Because loop 5 is highly conserved across Por1A strains generally (GenBank search), the construction of an effective porin vaccine may require an immune response that nullifies binding of complement downregulators such as factor H (and perhaps C4b-binding protein, as seen in our preliminary unpublished data) to these Por1A sequences in order to permit a beneficial effect of immune antibody.

Although the binding of factor H is critical in mediating serum resistance of most strains of *N. gonorrhoeae*, certain non-sialylated serum resistant strains exist that do not bind factor H, yet bind a significant amount of C3b (our unpublished observations). Other mechanisms such as blocking antibodies, which can block killing while activating complement (59, 60), or regulation of membrane attack complex insertion may be important in mediating serum resistance of these strains.

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