

A Novel Sialic Acid Binding Site on Factor H Mediates Serum Resistance of Sialylated *Neisseria gonorrhoeae*

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Summary

Factor H (fH), a key alternative complement pathway regulator, is a cofactor for factor I-mediated cleavage of C3b. fH consists of 20 short consensus repeat (SCR) domains. Sialic acid binding domains have previously been localized to fH SCRs 6–10 and 13. To examine fH binding on a sialylated microbial surface, we grew *Neisseria gonorrhoeae* in the presence of 5'-cytidinemonophospho-*N*-acetylneuraminic acid, which sialylates lipooligosaccharide and converts to serum resistance gonococci previously sensitive to nonimmune serum killing. fH domains necessary for binding sialylated gonococci were determined by incubating organisms with recombinant human fH (rH) and nine mutant rH molecules (deletions spanning the entire fH molecule). rH and all mutant rH molecules that contained SCRs 16–20 bound to the sialylated strain; no mutant molecule bound to serum-sensitive nonsialylated organisms. Sialic acid was demonstrated to be the fH target by flow cytometry that showed a fourfold increase in fH binding that was reversed by neuraminidase-mediated cleavage of sialic acid off gonococci. Functional specificity of fH was confirmed by decreased total C3 binding and almost complete conversion to iC3b on sialylated gonococci. Sialic acid can therefore bind fH uniquely through SCRs 16–20. This blocks complement pathway activation for *N. gonorrhoeae* at the level of C3.

Factor H (fH)¹ is a critical regulator of the complement system, and acts as a cofactor for factor I-mediated cleavage of C3b (1–3). fH also inactivates alternative pathway convertase by dissociating Bb from the C3b,Bb complex (3–5). fH is a 150-kD single-chain glycoprotein composed entirely of 20 short consensus repeat (SCR) domains (6), each comprising 60 amino acid residues. Each SCR is characterized by four highly invariant cysteine residues and by many highly conserved amino acids folded into compact units. Three regions on fH are capable of binding C3b: SCRs 1–5, which bear the cofactor activity; SCRs 6–10; and SCRs 16–20 (7). fH has the ability to bind polyanions such as sialic acid, heparin, and most sulfated glycosaminoglycans (8). Heparin binding sites have been shown previously to reside in SCR 13 (9) and in a region spanned by SCRs 6–10 (7, 10). Sialic acid-containing surfaces enhance the interaction between C3b and fH (11, 12). fH binding proteins that may contribute to pathogenicity by inhibiting

complement-mediated destruction have been identified on several microbes. These include the streptococcal M6 protein (13, 14), the Yad A protein of *Yersinia enterocolitica* (15), and glycoproteins (gp) 120 and 41 120 of HIV-1 (16–20).

Gonorrhea remains one of the major sexually transmitted diseases worldwide. *Neisseria gonorrhoeae* causes a wide spectrum of disease including asymptomatic infection, urethritis, cervicitis, pelvic inflammatory disease, and disseminated gonococcal infection. Gonococcal strains may be classified as serum sensitive (SS) or serum resistant (SR), depending on their susceptibility to killing by normal human serum (NHS; reference 21). SS strains predominantly result in symptomatic local inflammation, whereas SR strains may cause disseminated infection in the absence of substantial local genital symptoms (22–24).

The mechanisms employed by SS strains to evade host defenses are not fully understood. Gonococci initially recovered from urethral exudates and directly tested without subculture resist the bactericidal activity of NHS, but lose this ability after serial subcultures in vitro (25). A series of elegant and detailed studies by Smith and coworkers showed that incubation and growth of gonococci in human fluids and secretions resulted in the conversion of SS gonococci to SR gonococci (26–28). A low-molecular weight dialyzable substance present in human sera (26, 27),

¹Abbreviations used in this paper: CMP-NANA, 5'-cytidinemonophospho-*N*-acetylneuraminic acid; fH, factor H; HIS, heat-inactivated serum; LOS, lipooligosaccharide; NHS, normal human serum; rH, recombinant fH; SCR, short consensus repeat; SR, serum-resistant; SS, serum-sensitive.

leukocytes (29), erythrocytes (30–33), and guinea pig sera (30, 33, 34) renders the organism resistant to the direct killing action of NHS. This substance has been identified as the nucleotide sugar for sialic acid, 5'-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA). Purified CMP-NANA added to gonococcal growth media in nanogram amounts duplicates all of the effects of the low M_r factor with gonococci (33), including an increase in the molecular weight of their lipooligosaccharide (LOS; reference 31) that corresponds to the addition of a sialic acid residue to the LOS molecule. No concomitant alteration of any of the gonococcal outer membrane proteins has been observed (29). The acceptor site on LOS for sialic acid has been determined to be the terminal galactose of the lacto-series LOS structure (31, 35, 36) (Gal β 1 \rightarrow 4GlcNAc) that is antigenically identical to certain human glycolipids (37). The sialylation epitope is recognized by mAb 3F11, whose binding to gonococci is diminished when strains are grown in the presence of CMP-NANA. Binding is restored on neuraminidase treatment of the bacteria (36), which cleaves off sialic acid but otherwise leaves the organism intact. This effect also occurs in vivo, because gonococci from male urethral exudates fail to bind mAb 6B4, which also recognizes the sialylation epitope specifically on LOS. Again, binding is restored by neuraminidase treatment of the cells in situ (38). Electron microscopic examination of gonococci directly in urethral exudates shows a surface structure stained by ruthenium red that disappears on neuraminidase treatment (39, 40). Collectively, these data provide evidence for in vivo sialylation of gonococcal LOS. Although the gonococcal lipooligosaccharide α -2,3-sialyltransferase gene has been cloned and expressed (41), some gonococcal strains that are serum resistant do not express the Gal β 1 \rightarrow 4GlcNAc LOS acceptor site and therefore cannot be sialylated. The mechanism of this stable serum resistance is not fully understood.

Sialic acid is used as a virulence factor by several microorganisms. Capsular sialic acid of type III, group B streptococci prevents activation of the alternative complement pathway (42). The ability of the Sindbis virus to activate the alternative pathway of complement is inversely related to its host-determined envelope sialic acid content (43), which in turn is dependent on host tissue sialic acid content (44). Trypomastigotes of *Trypanosoma cruzi* express an unusual cell-surface trans-sialidase that enables the parasite to rapidly sialylate its surface when supplied with α (2 \rightarrow 3)-linked sialic acid in the form of glycoconjugates in serum or on cell surfaces. Desialylation of the trypomastigotes results in an increased ratio of C3b to the inactive molecule, iC3b, and results in increased lysis of the organism (45). A study of the bactericidal activity of the alternative complement pathway of the channel catfish (*Ictalurus punctatus*) against 15 gram-negative fish pathogens and nonpathogens found a strong correlation between the presence of sialic acid on the pathogenic strains and the absence of bactericidal activity against these strains, whereas the highly susceptible nonpathogenic strains lacked sialic acid (46).

In this study we demonstrate that sialylated gonococci

bind more fH than do their nonsialylated counterparts. This is the first clear demonstration of fH binding to sialic acid on a microbial surface and occurs through a novel sialic acid binding site on fH that was not predicted by previous in vitro experiments. This results in increased conversion of bound C3b to iC3b on the organism, which may be a mechanism for their serum resistance in vivo.

Materials and Methods

Bacterial Strains and Growth of Organisms. *N. gonorrhoeae* strain 24-1, an SS strain (47) isolated from a patient with pelvic inflammatory disease, was used in this study. Gonococci were first grown for 13 h overnight on solid media in 5% CO₂ at 37°C and then inoculated into standard gonococci liquid media and grown to mid-log phase (48).

fH and Recombinant fH Deletion Mutants. Human complement fH was purified from human plasma to a final concentration of 1 mg/ml in PBS (1). Recombinant fH (rH) and nine rH deletion mutant molecules were generated by overlap extension PCR, expressed in insect cells using the baculovirus system, and then purified by immunoaffinity chromatography (7, 49; see below). The rH molecule possesses equivalent cofactor activity to native fH purified from human plasma (49). Deletions in the nine rH mutant molecules span the entire length of the fH molecule and are shown in Fig. 1.

Sera. Pooled NHS was obtained from 11 healthy volunteers with no prior history of gonococcal infection. Heat-inactivated serum (HIS) was prepared by incubating NHS at 56°C for 30 min.

CMP-NANA and Neuraminidase Treatment. To achieve maximum sialylation of gonococci, 80 μ g of CMP-NANA was added (Boehringer-Mannheim Biochemicals, Indianapolis, IN) per ml of liquid media. In some experiments, CMP-NANA-treated organisms were treated with neuraminidase (Type V; Sigma Chemical Co., St. Louis, MO) reconstituted in 100 mM NaCl, 50 mM NaOAc, 5 mM CaCl₂, and 5 mM MgCl₂, pH 6.0 (neuraminidase buffer) to a concentration of 10 U/ml. 1.5 U of neuraminidase was added to 2×10^8 cells (final concentration: 6 U/ml in the incubation system) and incubated for 1 h at 37°C.

mAbs. mAbs C-5G and C-3E directed against human C3b and iC3b, respectively (reference 50; gift of Dr. Kyoko Iida, Takeda Chemical Industries, Ltd., Tsukuba, Japan), were used in whole cell ELISAs (see below) to monitor these complement components bound to gonococcal strains. mAb specific for the Bb neoantigen fragment of factor B (reference 51; Quidel, San Diego, CA) was used to detect alternative pathway convertase (C3b,Bb) on the organisms. mAb 2C3, which binds the H.8 lipoprotein antigen (38) present on all pathogenic gonococcal strains (52), was used to monitor the number of organisms deposited in each well. All mAbs in ELISAs were used at a concentration of 25 μ g/ml. mAb 3F11 (gift of Dr. Michael A. Apicella, University of Iowa, Iowa City, IA) specifically binds the nonsialylated Gal β 1 \rightarrow 4GlcNAc epitope on gonococcal LOS (53) and was used in Western blotting experiments (final concentration: 10 μ g/ml) to monitor the effects of neuraminidase treatment upon sialylated gonococci. Sialylation of LOS results in loss of 3F11 binding, which is restored after neuraminidase treatment (36).

Flow Cytometry. We used flow cytometry to quantitate binding of fH and recombinant mutant factor H molecules to gonococci. Each sample tube contained 2×10^8 bacteria recovered from liquid culture, which had been washed twice in HBSS (Sigma Chemical Co.) at room temperature and then resus-

ended in HBSS. All buffers were passed through a 0.22- μ m filter (Millipore Corporation, Bedford, MA) to exclude particles that could interfere with flow cytometry. The bacterial suspension (90 μ l) was incubated for 10 or 30 min at 37°C with 10 μ l NHS, or 10 μ l HIS, or 5 μ g purified fH (1 mg/ml in PBS). Suspensions were centrifuged at 10,000 g for 2 min and pellets were washed once with HBSS before adding anti-fH antibody used for detecting fH (see below).

To demonstrate that the sialic acid residue on LOS was the acceptor molecule for fH, we treated organisms with neuraminidase after they had been incubated with fH. Sialylated organisms (2×10^8) recovered from liquid culture were resuspended in 100 μ l of neuraminidase buffer, and then 5 μ g of purified human fH was added. The mixture was incubated for 30 min at 37°C, then 1.5 U of type V neuraminidase, or neuraminidase buffer alone (positive control) was added, followed by incubation for 1 h at 37°C. 24-1 NANA treated with neuraminidase before the addition of fH (neuraminidase was removed by washing, ensuring that fH was not desialylated) and 24-1 treated with fH alone were used as the negative controls.

Surface-bound fH was monitored by the addition of affinity-purified polyclonal rabbit anti-fH (final dilution of 1:100 of a 0.26 mg/ml stock solution) for 30 min at room temperature, followed by FITC-labeled anti-rabbit IgG (Sigma Chemical Co.; final dilution of 1:100). The bacteria were resuspended in 1 ml HBSS and applied to a fluorescence-activated cell sorter (FACScan[®], Becton Dickinson Immunocytometry Systems, San Jose, CA) and 50,000 events were counted.

The region of fH responsible for binding to sialylated gonococcal LOS was determined by incubating 2.5 μ g of each of the fH mutant molecules with gonococci, followed by detection with rabbit anti-fH as described above. All recombinant proteins were purified from the medium supernatant by application to an anti-fH-Sepharose immunoadsorbent column using the same anti-fH reagent (7, 49). This confirmed that the anti-fH antibody used in these experiments bound all of the rH mutant proteins.

Western Blots. fH binding to gonococci after incubation with serum or purified fH was also detected by Western blotting. After gonococci were harvested and washed, 4×10^8 cells suspended in 180 μ l of HBSS were opsonized with 20 μ l of serum or 10 μ g of purified fH for 30 min at 37°C. 180 μ l of the organism-containing mixtures were then layered onto 20% sucrose in HBSS and centrifuged at 10,000 g for 3 min to separate free fH from that bound to organisms. The pellets were harvested, resuspended in 35 μ l of 1% SDS, and boiled for 30 min. After centrifugation (10,000 g for 10 min), 25 μ l of the supernatant was carefully pipetted out, and protein concentration was determined by the bicinchoninic acid assay (BCA assay kit; Pierce Chemical Co., Rockford, IL). Samples containing equal amounts of protein were digested (0.3 M Tris HCl [pH 6.8], 2% SDS, 12.5% glycerol), boiled for 5 min, separated by 10% SDS-PAGE, and transferred onto an Immobilon PVDF transfer membrane (Millipore; reference 54). Nonspecific binding sites were blocked with 1% (wt/vol) natural nonfat dry milk in PBS. Western blots were then incubated with polyclonal sheep anti-human fH (The Binding Site, Birmingham, UK; final dilution: 1:1000) in PBS containing 1% BSA for 1 h at room temperature. Specificity of binding was identified with alkaline phosphatase-conjugated anti-sheep IgG antibody (Sigma Chemical Co.), and visualized after the addition of substrate (55).

ELISA. We measured C3b, iC3b, factor Bb, and C3 binding to gonococci opsonized with NHS by ELISA. 2×10^8 organisms were incubated with 10 μ l NHS (10 min at 37°C) as described

for flow cytometry. Reactions were stopped after 10 min by washing three times with ice cold HBSS containing 5 mM PMSF (Calbiochem Corp., La Jolla, CA) in a refrigerated microcentrifuge (National Labnet Co., Woodbridge, NJ). Organisms were resuspended in 200 μ l of the same buffer, and 50 μ l of each sample was applied per well of a 96-well U-bottomed polystyrene microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) for 3 h at 37°C. The plates were washed with PBS containing 0.05% Tween 20. Primary antibodies were diluted in PBS, and secondary antibodies were diluted in PBS/0.05% Tween 20. Total C3 bound to organisms was detected using polyclonal goat IgG fraction to human complement C3 (Organon Teknica-Cappel, Durham, NC; final concentration of specific antibody: 7 μ g/ml) followed by anti-goat IgG alkaline phosphate conjugate (Sigma Chemical Co.). C3b, iC3b and factor Bb bound to organisms were separately measured by mAbs directed against these components, followed by anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co.). To normalize the measurement of complement components per unit of organism, we determined H.8 gonococcal antigen concentration coated to wells as a measure of gonococcal density using mAb 2C3 (38), followed by anti-mouse IgG alkaline phosphatase conjugate.

Results

Binding of fH to Gonococci. Direct binding of fH to 24-1 and 24-1 NANA was demonstrated by incubation under three conditions: in NHS, HIS, or pure fH. Flow cytometric analysis of fH binding to 24-1 and its sialylated derivative (24-1 NANA) demonstrated significantly (fourfold) greater deposition of fH on 24-1 NANA when incubated with NHS. Results are shown for a 30 min incubation period in NHS to allow for maximal shedding of C3b-bound fH by sensitive 24-1 gonococci (see below for results at 10 min of incubation), thereby permitting a better estimate of the amount of fH binding directly to the organisms. The difference in fH binding was even more pronounced in the

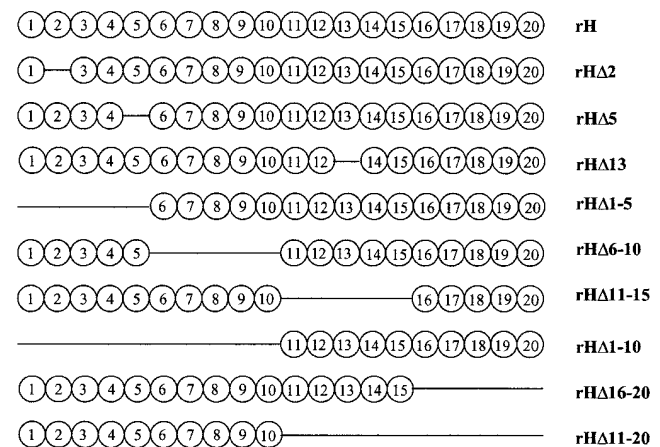


Figure 1. Schematic representation of rH and the nine deletion mutant molecules used in this study. Each circle represents an individual SCR. rH and rH constructs lacking the indicated domains were generated by PCR, expressed in insect cells using the baculovirus system, and purified for use in this study (7, 49).

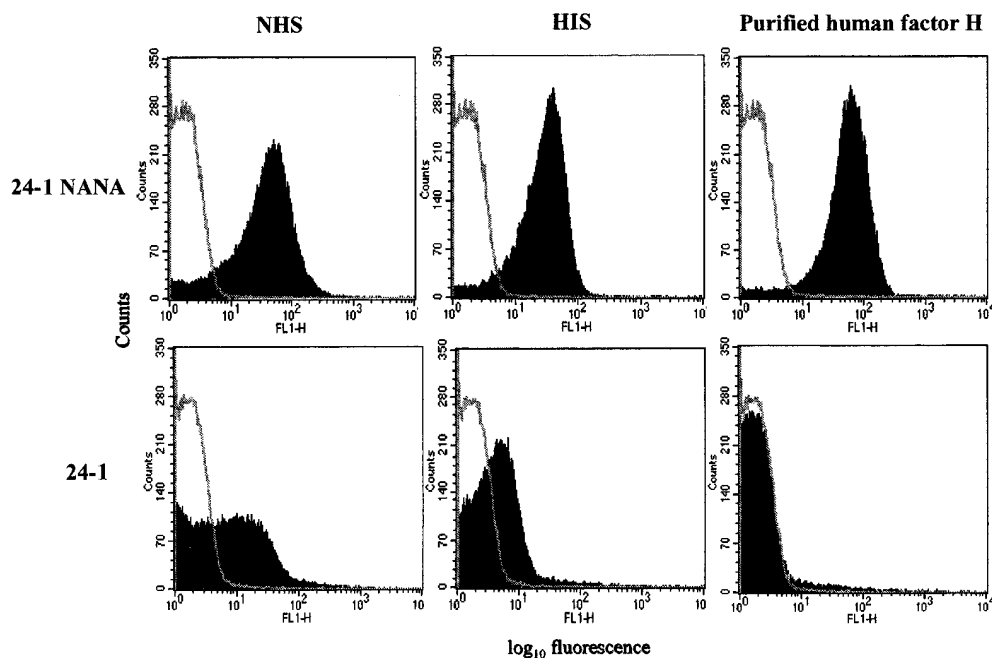


Figure 2. Flow cytometric quantitation of fH binding (using affinity-purified polyclonal rabbit anti-human fH) to sialylated strain 24-1 NANA (*top*) and parent strain 24-1 (*bottom*) when opsonized for 30 min with pooled NHS (10 μ l), HIS (10 μ l), or purified human fH (5 μ g). 24-1 NANA showed significantly greater fH binding under all three conditions of opsonization.

absence of intervening C3b when the organisms were incubated with HIS or pure fH. Here, 24-1 showed no significant binding of fH directly. Fig. 2 depicts one such experiment representative of three replications. The specificity of the binding of affinity-purified polyclonal rabbit anti-fH was confirmed by incubating the bacteria with fH-depleted serum, which yielded a reduction in the mean fluorescence intensity to background isotype control levels (data not shown).

fH binding on the surface of the two strains was also studied by Western blotting (Fig. 3), which confirmed the results obtained by flow cytometry. A visual comparison was made by loading equal amounts (10 μ g) of protein in each lane. Again, 24-1 bound fH only when opsonized with NHS, and the intensity of the band was qualitatively less than the 24-1 NANA lanes.

C3 and iC3b Deposition on Gonococci. To demonstrate the functional consequences of fH binding, we quantitated deposition of C3b (maximal at 10 min [data not shown]), iC3b, factor Bb, and total C3 on the two strains (Fig. 4) using whole cell ELISAs. To normalize for the numbers of organisms that were deposited in each well, we used mAb 2C3 to quantitate the amount of H.8 antigen present, which showed an almost identical OD_{410nm} for both strains (data not shown). To correlate the binding of C3 and its components at 10 min of opsonization with fH binding, we also measured fH binding at this time point by flow cytometry. Similar to the results shown in Fig. 2 at 30 min, there was no detectable binding of pure fH at 10 min. However, at 10 min of opsonization in NHS, 24-1 bound 40% more fH, which by 30 min had diminished because of loss of C3b-bound fH from the surface of the organism. fH binding remained unchanged when 24-1 NANA was opsonized with NHS or pure fH at either time point.

Almost all the C3 on 24-1 NANA existed in the form of iC3b. This was in sharp contrast to 24-1, where almost all the C3 was in the form of C3b. The total C3 bound by 24-1 was fourfold greater than that by 24-1 NANA. Together with the observation that factor Bb binding to 24-1 was \sim 10-fold greater than that on 24-1 NANA, these findings suggest that increased binding of fH to 24-1 NANA results in highly efficient conversion of C3b to iC3b, and limits total C3 deposition by inactivating the alternative pathway C3 convertase (C3b,Bb).

Localization of the Site in fH that Binds to Sialylated Gonococcal Strain 24-1. We determined the region of human fH that bound to 24-1 NANA using rH and nine mutant fH proteins lacking domains or groups of domains (Fig. 1). The binding of rH and the nine mutant protein molecules to 24-1 NANA were determined by flow cytometry using affinity-purified polyclonal rabbit anti-human fH followed by FITC-conjugated anti-rabbit IgG used as the detector system. rH, in addition to all rH mutant molecules, except rH Δ 11-20 and rH Δ 16-20, bound to 24-1 NANA (Fig. 5). This suggests that SCRs 16–20 were essential for the binding of fH to 24-1 NANA. The experiment was repeated using 1.25 μ g of each of the mutant molecules, and similar results were obtained. Neither rH nor any of the rH mutants showed significant binding to 24-1 (histograms not shown). Compared to intact rH, four of the rH mutant

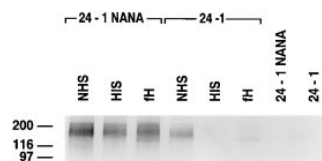


Figure 3. Western blot confirming increased fH binding to 24-1 NANA compared to 24-1 (detected by sheep anti-human fH), when incubated with NHS, HIS, or purified fH.

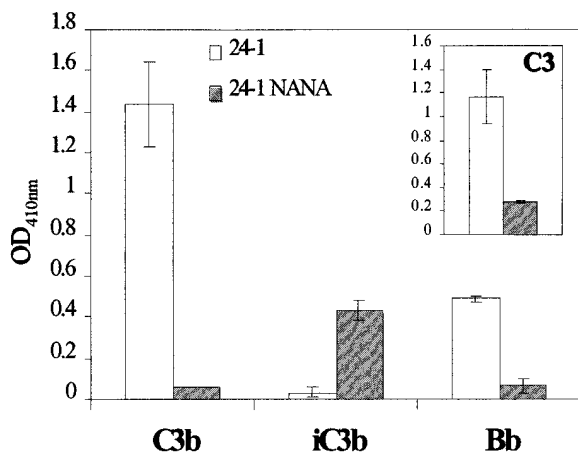


Figure 4. Quantitation of C3b, iC3b, factor Bb, and total C3 binding to 24-1 and 24-1 NANA after incubation in NHS for 10 min, determined by whole cell ELISA. Each bar represents the mean \pm SD of two experiments. The ratio of C3b to iC3b binding was significantly greater for 24-1 than 24-1 NANA, which bound almost no C3b. 24-1 NANA bound \sim 10-fold less Bb and 4-fold less total C3 than 24-1 (*inset*).

protein molecules (rH Δ 5, rH Δ 13, rH Δ 1-5, and rH Δ 11-15) showed more binding to 24-1 NANA (twofold or greater as measured by median fluorescence intensity).

We attempted to inhibit the binding of purified human fH to 24-1 NANA by preincubating fH with an excess of heparin or *N*-acetylneuraminic acid (Type VI; Sigma Chemical Co.) at room temperature for 30 min, followed by the addition of this mixture to the organisms. No decrease in fH binding to the organism surface was seen by the flow cytometry assay (data not shown). This provides further evidence that a site distinct from the previously defined polyanion binding sites on fH is responsible for binding to 24-1 NANA.

Characterization of Sialic Acid as the Gonococcal Target for fH. The role of sialic acid in the binding of fH was further investigated by neuraminidase treatment of 24-1 NANA both before and after the organisms were incubated with purified human fH. This was followed by flow cy-

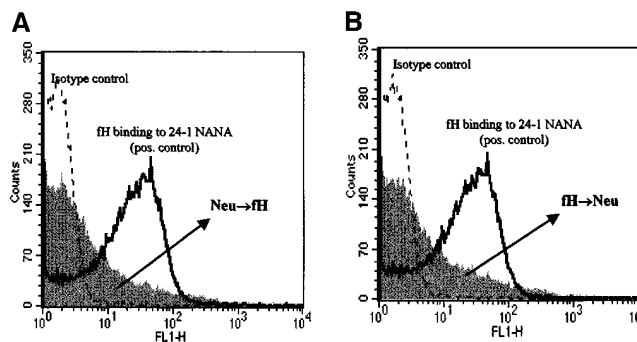


Figure 6. Specificity of fH binding to sialic acid is shown by neuraminidase treatment of 24-1 NANA both before (A, Neu \rightarrow fH) and after (B, fH \rightarrow Neu) incubating the organisms with pure fH. In both experiments, fH binding is reduced to near background levels. The solid lines represent fH binding to 24-1 NANA (positive control), the shaded areas represent fH binding to neuraminidase-treated samples, and the dotted lines show isotype controls. No binding of fH above isotype control levels was seen with 24-1 (data not shown).

tometry to detect fH bound to bacterial cells. Fig. 6 shows that fH binding was reduced to near background levels in both experiments. Neuraminidase treatment of 24-1 NANA both before (Fig. 6 A) or after (Fig. 6 B) fH treatment resulted in remarkably similar histogram plots.

The specificity of neuraminidase treatment on bacterial cells was confirmed by SDS-PAGE electrophoresis and Western blotting of the samples used for flow cytometric analysis, followed by probing with mAb 3F11, which selectively recognizes the sialylation site (in the unsialylated state) on gonococcal LOS (Gal β 1 \rightarrow 4 GlcNAc residue; reference 36). Sialylation of this LOS epitope results in loss of 3F11 binding, which is restored with neuraminidase treatment. The 24-1 NANA sample that had been treated with fH but not with neuraminidase showed a barely visible band (Fig. 7, lane 1). In contrast, 3F11 reacted strongly with the LOS of neuraminidase-treated samples (Fig. 7, lanes 3 and 4).

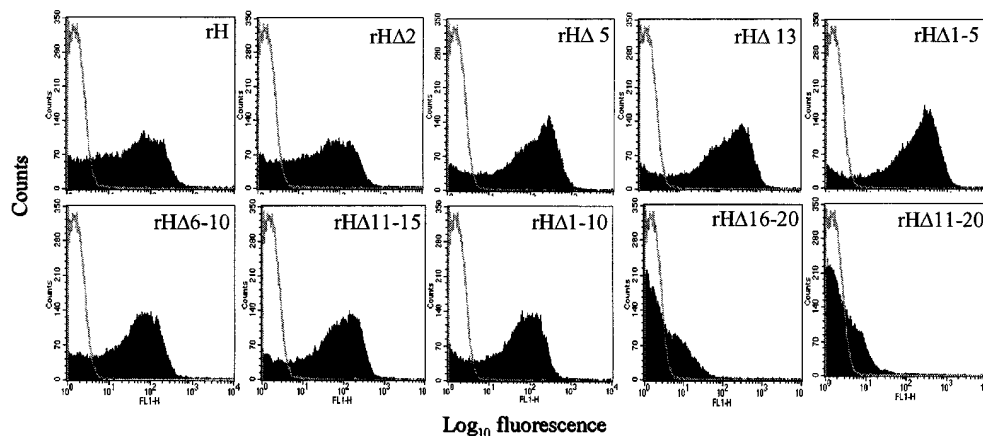


Figure 5. Binding of rH and the nine rH mutant molecules to 24-1 NANA in a flow cytometry assay. All molecules that contained SCRs 16-20 bound to the organism. Only rH Δ 16-20 and rH Δ 11-20 did not show significant binding, suggesting that SCRs 16-20 are essential for fH binding to 24-1 NANA.

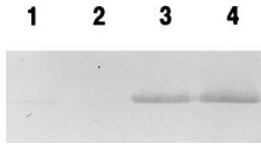


Figure 7. Specificity of neuraminidase treatment of gonococcal LOS. Samples used in flow cytometry (Fig. 6) were centrifuged, and then the pellets were harvested, digested, and subjected to electrophoresis on a 15% SDS-polyacrylamide gel followed by Western

blotting. Blots were probed with mAb 3F11 that specifically recognizes the sialylation epitope (Gal β 1 \rightarrow 4GlcNAc) on LOS. Binding of 3F11 is abolished when LOS is sialylated, and is restored with neuraminidase treatment. Lane 1: 24-1 NANA incubated with fH alone; lane 2: empty; lane 3: 24-1 NANA treated with neuraminidase before incubation with fH (Neu \rightarrow fH); lane 4: 24-1 NANA treated with neuraminidase after incubation with fH (fH \rightarrow Neu).

Discussion

Sialic acid is an essential component of eukaryotic cell surfaces that plays an important role in preventing destruction of host tissue by constant low grade activation of the alternative complement pathway. The affinity of C3b for factor B remains constant on both activator and nonactivator surfaces; activator surfaces are characterized by a low affinity of C3b for fH (56). Sialylation of eukaryotic membrane surfaces enhances the interaction between C3b and fH, resulting in highly efficient cleavage of C3b to iC3b by factor I. Desialylation of red cell membranes results in their conversion from nonactivators to activators of the alternative complement pathway (12) as a result of a reduction in fH binding (57). Certain fluid-phase polyanions enhance binding of fH to C3b attached to activating surfaces, which may be a mechanism of discrimination of host cells and tissues from foreign cells that activate the alternative pathway (58).

In vivo modification by sialylation of the terminal lactosyl residue in *Neisseria gonorrhoeae* (and other *Neisseria* species) LOS is crucial in converting SS strains that possess the Gal β 1 \rightarrow 4GlcNAc epitope to an SR phenotype. A recent study indicated that the sialylation of LOS and capsule of *N. meningitidis* facilitates the formation of iC3b (59). Direct binding of fH was proposed as the most likely explanation but was not demonstrated, and organism-specific endogenous complement-regulatory or fH-like activity, which has been seen in other microorganisms, was not excluded (60–67).

In our study, using flow cytometry as well as Western blotting, we have shown that in vitro sialylation of gonococcal LOS by growth in the presence of CMP-NANA results in increased binding of fH. This in turn leads to highly efficient conversion of C3b to iC3b, as evidenced by minimal C3b deposition on 24-1 NANA (Fig. 4). In addition, the total C3 detected on 24-1 NANA was significantly (fourfold) less than on its nonsialylated variant because the alternative pathway C3 convertase (C3b,Bb) had been inactivated. This was confirmed by demonstrating a minimal amount of factor Bb on 24-1 NANA, which limited the accumulation of C3b on the surface of this strain. Fig. 2 shows minimal binding of fH to 24-1 when incubated with NHS, but not with HIS or purified fH. This likely represents fH binding to the abundant amount of C3b deposited

on the organism. However, this limited degree of fH binding to C3b may not be functionally significant because very little iC3b is detected on 24-1 (Fig. 4), and because 24-1 is exquisitely sensitive to the bactericidal activity of nonimmune NHS (47).

fH binds sialic acid, heparin, and certain other polyanions such as dextran sulfate, chondroitin sulfate A, and carrageenan (types III and IV). Little or no binding occurs with chondroitin sulfate C, keratan sulfate, hyaluronic acid, colominic acid (bacterial polysialic acid), or polyaspartic acid (8, 9). These data demonstrate that the interaction between fH and polyanions is specific and in the case of sulfated polyanions may depend upon the number, orientation, and polymeric arrangement of sulfate groups. Sialic acid containing gangliosides, when incorporated into liposomes, inhibit the alternative complement pathway. More than 90% of this inhibitory activity is lost after removal of the terminal C9 carbon from the polyhydroxylated tail of sialic acid or conversion of its carboxyl group to a hydroxymethyl group (68). When incorporated into liposomal membranes, the monoganglioside GM1 and sialosylparagloboside inhibit activation of the alternative pathway of complement, an effect that is reversed by neuraminidase treatment. Increased binding of radiolabeled fH to liposome-bound C3b has been observed in the presence of GM1 (69). Polyanion binding regions on fH have been previously defined using heparin (7, 9, 10). The first heparin binding site on fH was identified as a positively charged arginine-rich site in SCR 13 (9). Subsequently, using the same rH mutant molecules that we have used in this study, a second higher-affinity binding site was identified between SCRs 6 and 10 (7). Another recent study (10), using fH SCR deletion mutant proteins expressed in Chinese hamster ovary cells, identified a heparin binding domain in SCR 7. The possibility that additional heparin binding sites (other than SCR 13) exist within SCRs 10–20 was raised in that study because a double deletion of SCRs 7 and 13 from the full-length fH showed >97% binding to heparin-agarose and an elution profile similar to that of a single deletion of SCR 7.

Deletion mutations of complement regulatory proteins organized in SCR domains have been used in other studies to define binding regions of fH and other complement regulatory proteins (7, 70–73). This approach is justifiable because of the apparent independent folding of the 20 domains of fH and their linear arrangement. Using rH mutant proteins that were expressed in the baculovirus system, we have identified SCRs 16–20 as containing a novel sialic acid binding site on fH. Four rH mutant molecules (rH Δ 5, rH Δ 13, rH Δ 1-5, and rH Δ 11-15) showed greater binding than did rH. This phenomenon has also been observed with rH Δ 1-5, rH Δ 16-20, and rH Δ 11-20 in a recent study that used rH and six rH mutant molecules to identify SCRs 6–10 as the region in fH that bound to M protein of *Streptococcus pyogenes* (72). The increased binding of certain rH mutants compared to rH may be due to differences in size and/or charge of the mutant molecules. Sialic acid, when configured onto gonococcal LOS, binds a region on fH not

predicted by other in vitro experiments. Furthermore, these results impart another biological function to the COOH-terminal end (SCRs 16–20) of the fH molecule, which previously was believed to contain only a C3b binding site (7). Binding of the COOH-terminal end of fH to sialic acid on gonococcal LOS might enable the highly elongated and flexible fH molecule (74) to interact efficiently with nearby C3b molecules deposited on the bacterium through the NH₂-terminal cofactor activity-bearing domains (SCRs 1–5) as well as through the second C3b-binding region (SCRs 6–10).

We confirmed that sialic acid was the receptor for fH by neuraminidase treatment both before and after incubation of the bacteria with fH. In both instances the ability to bind fH was lost, suggesting that in this system sialic acid is essential for the binding of fH. If a second receptor had been involved in binding fH, residual fH should have been detected when incubation with fH was followed by neuraminidase treatment. In our experiments using sialylated organisms,

all of the bound fH was associated with sialic acid. This is the first unequivocal demonstration of fH binding to sialic acid on a microbial surface.

Sialylation of neisserial LOS can contribute to pathogenicity in several other ways. These include decreased phagocytosis of gonococci or meningococci by human neutrophils (75–78), decreased ability to bind IgM and IgA (59), and decreased ability to stimulate an oxidative burst in neutrophils and to adhere to neutrophils in the absence of complement and antibody (78).

In this study we have defined the mechanism of serum resistance of sialylated gonococci. However, other gonococcal strains (predominantly isolated from patients with disseminated gonococcal infection) remain SR in in vitro assays (termed stable serum resistance) without the addition of CMP-NANA (79–81). Using flow cytometry, we have shown that most of these stable SR strains also bind significant amounts of fH (our unpublished data), suggesting that another acceptor site(s) for fH may also exist.

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