SPECIES SPECIFICITY OF *NEISSERIA GONORRHOEAE* BINDING TO FACTOR H AT COMPLEMENT CONTROL PROTEIN DOMAIN 18-20

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ABSTRACT

Neisseria gonorrhoeae infects humans in part because of the binding to its surface protein porin (Por) molecules by the complement control protein (CCP) domains 6 and 18-20 of the human complement regulator, factor H (fH). FH activity is thereby localized to the surface of *N. gonorrhoeae* and results in inactivation of the alternative pathway of complement. This project investigated the Por/fH interaction by creating species-specific sequence changes in human and chimpanzee CCP 20s in cloned fH proteins, and used flow cytometry to determine the ability of the recombinant proteins to bind *N. gonorrhoeae*. The data indicated that arginine located at position 1203 in fH CCP 20, R1203, is critical for fH-binding to *N. gonorrhoeae*.

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ACKNOWLEDGEMENTS

BACKGROUND

Gonorrhea

Gonorrhea is the second most prevalent bacterial sexually transmitted disease in the United States (Health Protection Agency, 2008). In 2007, the Centers for Disease Control and Prevention (CDC) reported 330,000 new cases of gonorrhea in the U.S., and it is believed that these cases represent only half the total estimated number (CDC 2006). Worldwide, it is estimated that there are over 60 million cases annually (Gerbase et al., 1998).

Neisseria gonorrhoeae is the causative agent of gonorrhea. *N. gonorrhoeae* typically infects the cervix, urethra, rectum, anus, or throat. Although most infected men and women probably are asymptomatic, other infected individuals experience noticeable genitourinary discharge, inflammation, and irritation (CDC, 2006). Gonorrhea is transmitted through contact with the penis, vagina, mouth, or anus of the infected individual. In addition, mothers who are infected with gonorrhea can pass the infection to their newborns during delivery. Untreated gonococcal infections can lead to further complications such as pelvic inflammatory disease (PID), an infection of the uterus and fallopian tubes. PID can create scar tissue in the fallopian tubes and block the passage of fertilized eggs to the uterus or prevent sperm from reaching the eggs. This results in complications that include ectopic pregnancy, infertility, and chronic pelvic pain (CDC, 2007). In rare cases (1% - 3%), the infection can spread to other regions of the body such as the blood, skin, and joints, a disease known as disseminated gonococcal infection (DGI) or gonococcal arthritis (Cannon et al., 1983; Sparling, 1990).

Neisseria gonorrhoeae

      N. gonorrhoeae is a gram-negative diplococcus (*Figure 1*) with its outer membrane composed of a complex mixture of phospholipids, lipo-oligosaccharide (LOS) and proteins; these are organized as an asymmetric lipid bilayer. LOS consists of lipid A and oligosaccharide chains of differing lengths. Unlike many other gram-negative enteric bacteria, the LOS of *N. gonorrhoeae* lacks repeating O-antigens (Erwin et al., 1996; Zhu et al., 2002). The core sugars of gonococcal LOS are highly variable depending on the specific genetic makeup of the organism and growth conditions used to grow the organism. This variability helps to diminish recognition by the adaptive immune system (Ram and Rice, 2008). Thus, LOS plays an essential role in the pathogenesis of *N. gonorrhoeae* (Baron et al., 1996; Zhu et al., 2002).
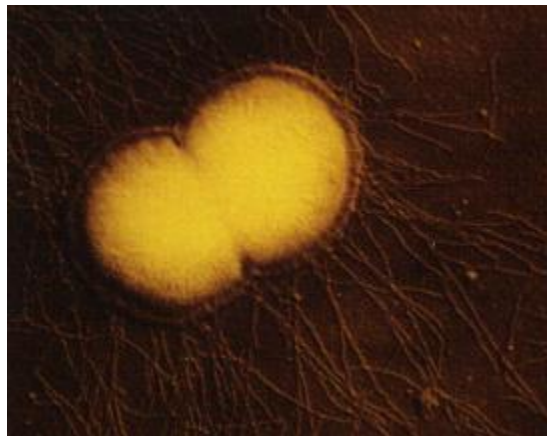

Figure 1: *Neisseria gonorrhoeae (Todar, 2005)*.

      The outer membrane of *N. gonorrhoeae* also contains a variety of proteins (Baron et al., 1996). Porin (Por) protein comprises more than 60% of all the outer membrane proteins (Blake and Gotschlich, 1986), and is 34-35 kDa in size, containing 8 transmembrane loops. The native

configuration of the protein is a homogenous trimer that functions as a selective anion channel (Douglas et al., 1981; Blake and Gotschlich, 1986).

Por can be classified into two major serotypes, Por1A and Por1B (Cannon et al., 1983; Sparling, 1990). The major difference between the Por1A and Por1B is structural; the fifth transmembrane loop of Por1B is longer than the corresponding fifth loop of Por1A by 17 amino acids, which leads to differences in molecular weight, susceptibility to proteolysis and antibody recognition (Blake et al., 1981; Sandstrom et al., 1982; Lewis et al., 2008). In addition, Por isoforms are also associated with infectious complications. Por1A strains of *N. gonorrhoeae* are more likely associated with DGI, while Por1B strains contribute to local genital infection and PID (Cannon et al., 1983; Brunham et al., 1985; Sparling, 1990).

N. gonorrhoeae Serum-Resistant and Serum-Sensitive Strains

N. gonorrhoeae strains can also be subdivided phenotypically: stable serum resistant (SSR) strains and serum sensitive strains (SS), depending on their ability to be killed by non-immune normal human serum (NHS) (Rice, 1989). SSR strains usually are Por1A types, while SS strains are Por1B (Rice, 1989). When sialic acid (a nine carbon long circular hexose with a 3 carbon extension) covalently attaches to the terminus of lacto-*N*-neotetraose containing lipo-oligosaccharide (called LNT LOS), gonococci become resistant to killing by NHS (Parsons et al., 1988; Nairn et al., 1988), so called unstable serum resistance (USR) because it can be reversed when organisms are grown on media that lack the sialic acid substrate, called cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA) (Ward et al., 1970).   Sialylation is critically important because *N. gonorrhoeae* scavenge sialic acid from their human hosts when they are growing in the body (Shell et al., 2002).

Complement System and Innate Immunity

The human body is constantly being exposed to pathogens. The immune system is a collection of mechanisms within higher organisms that protects them from pathogens and the diseases they produce. The first line of defense that a pathogen encounters after entering a potential host is the innate immune system, a non-specific, non-adaptable but redundant system. An innate response is triggered by germ-line encoded receptors of the innate immune system that recognize specific surface structural features common to many pathogens (Murphy et al., 2008).

The complement system is an arm of the human innate immune system. It consists of more than 35 fluid-phase and membrane-bound proteins in the form of inactive zymogens that normally circulate throughout the body. Complement protects against invading pathogens by triggering an enzyme-cascade whereby a protease is formed as a result of cleavage of its zymogen precursor, which then cleaves the peptide bond of another zymogen to activate more proteases. The complement cascade generates a geometrically amplified immune response (Murphy et al., 2008).

Complement Pathways

There are three distinct pathways of the complement system: (1) the classical pathway, (2) the alternative pathway, and (3) the mannose-binding lectin pathway. The pathway used depends on the form of initiation. The absolute end result of the cascade is the formation of a membrane attack complex (MAC), which is inserted into the pathogen's lipid membrane to promote osmotic lysis (*Figure 2*) (Murphy et al., 2008).

The first pathway, the *classical pathway*, is initiated as a result of binding of C1q of the C1-complex, which is also comprised of two zymogens C1r and C1s, to either the surface of the foreign antigen, or to IgM and IgG antibodies that have interacted with foreign antigens. This process activates the C1s of the complex to cleave C4 into C4b. This reaction exposes the internal thioester bonds in C4b allowing the formation of covalent ester or amide bonds with hydroxyl or amino groups on target surfaces. C1s also cleaves C2 into C2a, which binds to C4b molecules to form the classical pathway C3-convertase (C4b2a). Furthermore, this C3-convertase cleaves C3 into C3b, which in turns binds to the C3-convertase to make C5-convertase (Murphy et al., 2008).

The *alternative pathway* is initiated by the spontaneous and continuous hydrolysis of circulating C3 to produce $C3(H_2O)$. Factor B then binds to the $C3(H_2O)$ to form $C3(H_2O)B$, which is the substrate for factor D. Factor D cleaves the complex into $C3(H_2O)Ba$ (inactive) and $C3(H_2O)Bb$ (also known as the fluid-phase C3-convertase). $C3(H_2O)Bb$ continues to cleave C3 to yield C3a and C3b. This reaction exposes the internal thioester bonds in C3b (like C4b) allowing the formation of covalent ester or amide bonds with hydroxyl or amino groups on target surfaces (Gordon and Hostetter, 1986). However, if the C3b does not bind to the target surface, hydrolysis deactivates the complex. The bound C3b further binds to circulating factor B to form a complex and is then further cleaved by factor D to yield the alternative C3-convertase, C3bBb. The cleavage of C3 by the alternative C3-convertase initiates an amplified positive feedback loop. The binding of C3 to the alternative C3-convertase also results in the formation of the C5-convertase (C3bBbC3b) (Murphy et al., 2008).

The *lectin mannose pathway* is homologous to the classical pathway. The mannose binding lectin (MBL) binds to sugar residues in polysaccharides on the surfaces of

microorganisms. MASP-1 and MASP-2, which are analogous to C1s and C1r of the C1 complex in the complement system, are activated when they bind to surface sugars on organisms. MASP-2 cleaves C4 and C2 into C4a, C4b, C2a, and C2b. C4b covalently binds to the surface target. C2b binds to C4b to form the classical pathway C3-convertase. This C3-convertase cleaves C3 into C3b, which binds to more C3-convertase to form C5-convertase (Murphy et al., 2008).

The three cascades merge and can proceed, ultimately to form the membrane attack complex (MAC) that is responsible for the killing of target pathogens. C5-convertase binds to the target membrane and is then cleaved to yield C5a, which acts as an inflammatory mediator by activating members of the G-protein-linked receptors (Murphy et al., 2008, pg 251). C5b is also formed and binds to the target membrane. This site of binding also accommodates the binding of other terminal complement components (Muller-Eberhard, 1988). C6 and C7 bind to C5b, which alters its confirmation to render it hydrophobic, allowing it to enter the membrane of the target pathogen. C8 binding to C5b6,7 creates a transmembrane channel which disrupts the outer membrane of the pathogen. Up to 16 molecules of C9 then bind to the C5b-8 complex to complete the pore structure of the MAC. The pore permits the flow of ions and small molecules, disrupts the pathogen's homeostasis and eventually leads to the destruction of the pathogen by osmotic lysis (Murphy et al., 2008).
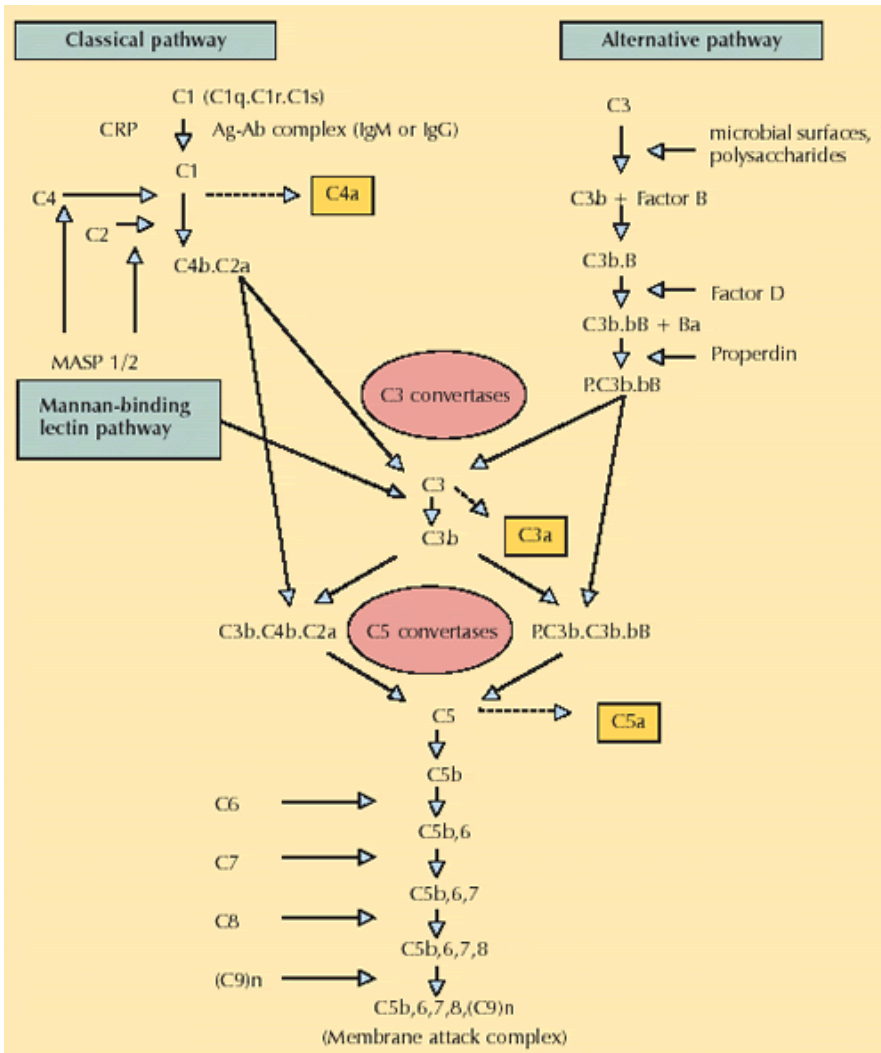
Figure 2: **Complement Pathways of the Human Innate Immune System (Legrand et al., 2008).**

Complement Regulatory Proteins

The complement system is highly regulated by a number of different proteins that protect host cells from inappropriate activation of the immune system. Although many regulatory proteins have been identified, we will focus on a few proteins known to be important in *N. gonorrhoeae* infection. Two host regulatory proteins in particular that have been observed to play a role in the pathogensis of *N. gonorrhoeae* are: (a) C4b-binding protein and (b) factor H.

C4b-binding protein (C4BP) is a multimeric plasma glycoprotein comprised predominantly of seven α chains and a single β chain. The α chains are each 70 kDa in size and are composed of eight complement control protein (CCP) domains. The β chain is 45 kDa in size and is composed of 3 CCP domains. C4BP regulates the classical pathway by acting as a cofactor for factor I, which mediates cleavage of C4b thereby halting the formation of classical pathway C3 and C5 convertases. Furthermore, C4BP also binds to classical pathway C3 and C5 convertases that are already formed and accelerates their decay (Scharfstein et al., 1978; Hillarp and Dahlback, 1990; Ogata et al., 1993; Hillarp et al., 1997).

Of particular interest to this project, *factor H* (fH) is a complement protein that regulates the alternative complement pathway. FH acts as a cofactor for factor I, which inactivates C3b to iC3b through cleavage, thereby halting the formation of alternative pathway C3 and Ç5 convertases. Furthermore, fH accelerates the decay of these C3 and C5 convertases by competing with the binding of factor B to C3b in the C3 convertases (C3bBb). Thus, fH is an essential regulating tool that terminates the alternative cascade from proceeding (Murphy et al., 2008). The fH regulatory protein (*Figure 3*) consists of 20 CCP domains (Knapp et al., 1984); each is comprised of approximately 60 amino acid residues and are cysteine-rich (Smith et al., 1995) and therefore internally cross-linked. In the plasma, the normal concentration of human fH is approximately 500 µg/ml. To exhibit full complement activity in cervical mucus, only 10% of the concentration present in normal undiluted human serum is needed (Ward et al., 1992).
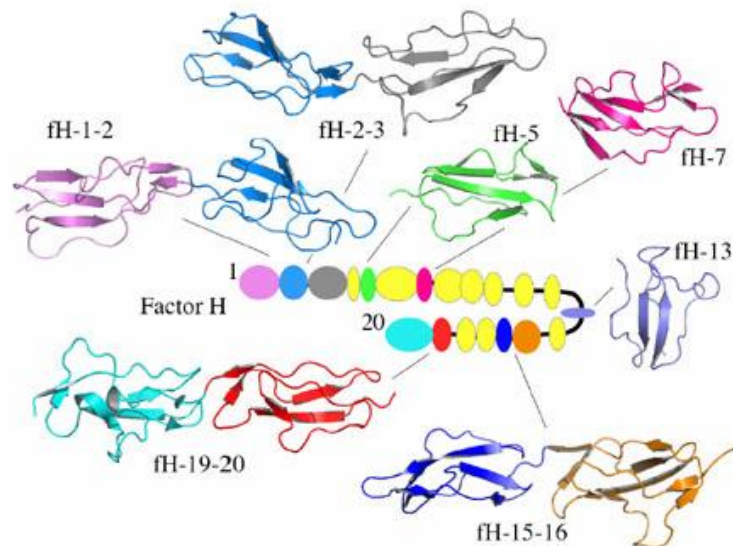
***Figure 3*: Structure of 20 CCPs of factor H (Barlow, 2008).**

Factor H Binding to Microorganisms

Microbial pathogens have evolved many different means for protection against natural complement killing. One method of protection involves the binding of bacteria to host regulatory proteins such as fH to suppress the alternative pathway of complement. Organisms that are known to use fH-binding include group A (Horstmann et al, 1988) and group B streptococci (Areschoug et al., 2002; Jarva et al., 2004), *Streptococcus pneumoniae* (Jarva et al., 2002), *Yersinia enterocolitica* (China et al., 1993), HIV-1 (Pinter et al., 1995; Pinter et al., 1995; Sadlon et al., 1994), *Borrelia burgdorferi* (Hellwage et al., 2001), *B. afzelii* (Wallich et al., 2005), *B. hermsii* (Hovis et al., 2006), *Leptospira interrogans* (Verman et al., 2006), West Nile virus (Chung et al., 2006), and *N. meningitidis* (Madico et al., 2006).

N. gonorrhoeae binds human regulatory proteins such as fH and allows this bacterium to infect, survive, and cause disease (Arko, 1989; Lewis et al., 2008). The 5th loop of the Por 1A protein binds fH (Ram et al., 1998). Por 1B proteins binds fH weakly, however, after sialylation of LNT LOS, binding between sialylated Por1B strains and fH increases dramatically (Ram et

al., 1998). For these "unstable" SR strains, both Por1B protein and sialylation are required for binding of fH (Madico et al., 2007).

To analyze the binding of *N. gonorrhoeae* with recombinant fH proteins, our laboratory constructed different human fH CCP domains fused to the N-terminus of the Fc portion of mouse IgG2a (fH/Fc). Fc serves as a tag to help detect fH proteins that bind to the bacterial Por protein when labeled (or conjugated) anti-Fc antibodies are used for detection. Fc tags also permit symmetric comparison of binding among different fH CCP domains (Ngampasutadol et al., 2008). Two particular regions of human fH; CCP 6 and CCPs 18-20, have been shown to bind to gonococci. Por1A strains (stable serum resistant" [SSR]) bound both CCP domains, while sialylated Por1B stains ("unstable serum resistant" [USR]) bound only the CCPs 18-20 domain (Ngampasutadol et al., 2008).

Lack of Animal Models and Species Specificity of *N. gonorhoeae*

The human is the only natural reservoir for *N. gonorrhoeae*. There are few animal models for *N. gonorhoeae* infection, and this limitation in simulating gonococcal syndromes in humans has restricted our understanding of the pathogenesis of gonorrhea. The only animal species other than humans that can be readily infected experimentally is the chimpanzee (*Pan troglodytes*). Chimpanzees sustain implanted urethral infections for 3-6 weeks. Lower animal species, including baboons and monkeys cannot be infected experimentally (Arko, 1989; Arko et al., 1976).

Our laboratory has examined the species specificity of immune interactions between vertebrates and *N. gonorrhoeae*. We have found that sera from lower animals such as rats and rabbits kill *N. gonorrhoeae*. However, when human sera are added to rat and rabbit sera, gonococci withstand killing. This may indicate that in lower animals, *N. gonorrhoeae* does not

evade serum complement, a possible explanation for the lack of natural (or experimental) infection with this organism. In addition, binding between *N. gonorrhoeae* and non-human fH has revealed variable binding avidity. While human fH binds to Por1A strains, chimp fH binds only weakly, and rhesus does not bind at all. Upon comparing the primary amino acid structures of human, chimp, and rhesus fH, differences in sequences were apparent between human and chimp in CCP 6, and CCPs 19-20, which could explain the difference in binding avidity (Ngampasutadol et al., 2008).

PROJECT PURPOSE

N. gonorrhoeae infects humans in part due to the binding of its surface protein Por to human factor H (fH), resulting in inactivation of the alternative complement pathway. Serum-resistant (SR) Por1A bacterial strains bind fH at complement control proteins (CCP) domains 6 and 18-20. Serum-sensitive (SS) Por1B strains do not bind to fH unless they are sialylated, whereupon they bind to CCP domains 18-20.

Furthermore, the structure and function of complement regulatory proteins are largely conserved across species (Kemper et al., 2000), however, only human fH, but not fH from other primates bind directly to gonococci. Consistent with direct human fH binding, unsialylated Por1A gonococci resist killing by human complement, but not by complement from other primates, rodents or lagomorphs; adding human fH to these heterologous sera restores serum resistance (Ngampasutadol et al., 2008). Understanding the basis for the human-specific binding of fH to gonococci may advance our understanding of the host-specificity of this infection. Such studies may also aid in the development of compounds that block fH-gonococcal interactions and may provide therapeutic options against this pathogen that has rapidly become resistant to multiple antibiotics.

In this project, we have exploited the selective binding of gonococci to human fH molecules to identify amino acids that are important in the interactions. We have generated recombinant fH CCPs 18-20/Fc molecules with chimpanzee mutations on a human background and analyzed the binding of the recombinant proteins to *N. gonorrhoeae* by flow cytometry.

METHODS

Bacterial Strains

Two gonococcal strains were used in this project: Por1A strain 252 and Por1B strain F62. These strains were chosen for their ability to bind to fH, but not to C4BP, in order to avoid potentially confounding effects due to the binding of the latter complement regulatory protein and its effect upon the functional assays that we used.

Recombinant fH/murine Fc Fusion Proteins

Using site-directed mutagenesis, we created recombinant fH CCPs 18-20/Fc molecules that contained chimpanzee mutations upon a human background, and then examined the binding of the recombinant molecules, or loss thereof, to *N. gonorrhoeae*. Human and chimpanzee fH SCR 20 differ by 11 amino acids. We created mutant proteins that targeted seven of the 11 amino acids that define differences between human and chimpanzee SCRs 20 (some mutant proteins contained mutations at two positions). Additionally, we created recombinant fH/Fc proteins that consisted of human CCPs 16-19 and chimpanzee 20, and replaced chimpanzee amino acids with human amino acids at positions 1200 and 1203; I1200V and N1203R. The recombinant fH CCPs were fused at the C-terminus to the N-terminus of the Fc portion of mouse IgG2a. The Fc region served as a tag, allowing for the detection of the fusion molecules in flow cytometry when using anti-mouse IgG and also permitted symmetric comparisons of binding among the different CCP constructs.

To create the recombinant fH/Fc proteins, mutant oligonucleotide "primers" that contained the desired base changes were purchased from Invitrogen. cDNA coding for human

CCPs 18-20 cloned into a eukaryotic expression vector pcDNA3 (Invitrogen) already containing the Fc fragment of mouse IgG2a was used as the template. Mutations were introduced using the QuikChange site directed mutagenesis kit (Stratagene). We used Polymerase Chain Reaction (PCR) to create and amplify fH/Fc plasmids that contained the desired mutations. The PCR products were then treated with restriction endonuclease *Dpn I*, which is specific for methylated and hemimethylated DNA. *Dpn I* digests the parent DNA template that lacks the desired mutations. We transformed the mutated fH/Fc DNA into XL1-gold ultracompetent *E. coli* cells. The transformed *E. coli* cells were plated on LB- agar plates containing ampicillin (100 µg/ml) and the plates were incubated overnight at 37°C. Several colonies were picked from the plates, and DNA from individual colonies (clones) was extracted using a QIAprep Spin Miniprep Kit (QIAGEN), following the manufacturer's instructions. All mutations were confirmed by automated DNA sequencing.

To express the recombinant proteins, we transfected Chinese hamster ovary (CHO) cells with the fH/Fc constructs using lipofectin (Invitrogen Life Technologies), according to the manufacturer's instructions. Transfected CHO cells were incubated at 37°C for two days and supernatants containing the recombinant fH proteins were collected and concentrated using Amicon Ultra (Mllipore).

Flow Cytometry

Bacteria were grown for 10-12 h in 5% CO_2 on chocolate agar plates supplemented with Isovitalex equivalent. Bacteria were then suspended in Hanks' balanced salt solution (HBSS) containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ ($HBSS^{2+}$) and the concentrations of bacterial cells were adjusted to 3×10^8 cells/ml. Sialylation of gonococci was achieved by adding CMP-NANA

in growth media to a final concentration of 2 µg/ml of CMP-NANA. Bacteria (10^8 organisms)

were incubated with concentrated supernatants containing 0.5 µg of recombinant fH/Fc protein

in a final reaction volume of 100 µl for 20 min at 37°C. The cells were centrifuged at 8×10^3 rpm

for 5 minutes and the supernatants were aspirated. FITC-labeled goat anti-mouse IgG (Sigma) at

a dilution of 1:100 in 1% BSA/HBSS^{2+} was added and the mixtures incubated at 37°C for 20

minutes. Again, the cells were centrifuged at 8×10^3 rpm for 5 minutes and the supernatants were

aspirated. Finally, we resuspended the cells in 500 µl of 1% paraformaldehyde in HBSS^{2+}. Using

flow cytometry (LSRII flow cytometer; BD biosciences), we detected recombinant fH/Fc protein

bound to bacteria using FITC-conjugated anti-mouse IgG (Sigma-Aldrich). Flowjo data analysis

software (www.TreeStar.com) was used to analyze the binding of recombinant fH protein to

bacteria.

ELISA Assay

We used ELISA assays to measure bacterial binding domains in fH CCPs 18-20 that

bound to C3b and heparin. First, we investigated the binding to C3b. We coated microtitre plates

with human C3b (Complement Technology, Inc.) at concentrations of 10 µg/ml, then incubated

the plates overnight at 4°C. After "blocking" non-specific binding sites on the plates with PBS-

0.05% Tween 20, we added 10 and 100-fold serial dilutions of concentrated supernatants that

contained the recombinant fH proteins, and the plates were incubated for 1 hour at 37°C. After

washing the plates, secondary antibody consisting of anti-mouse IgG conjugated with alkaline

phosphatase was added to the wells at a 1/1000 dilution in PBS-0.05% Tween 20 and the plates

incubated at 37°C for 30 minutes. After a final wash, *p*-Nitrophenyl phosphate (pNPP) (Sigma)

substrate was added and the development of color was measured (405 nm) at 15 minute intervals using a microplate reader.

To detect the binding of heparin to fH/Fc proteins, microtitre plates were first coated with Strepavidin (Strep) at concentrations of 10 µg/ml. Following incubation as described above, solutions containing Heparin-Biotin at concentrations of 10 µg/ml were added for 1 hour at 37°C to affix heparin via Strep-Avidin binding. Non-specific binding sites were blocked with PBS-0.05% Tween 20. 10 and 100-fold serial dilutions of concentrated supernatants (contaning fH/Fc) were added, and the plates incubated for 1 hour at 37°C. A second antibody, anti-mouse IgG alkaline phosphatase (diluted 1/1000 in PBS-0.05% Tween 20), was added and the plates were incubated for 30 minutes at 37°C. Finally, to develop color, pNPP substrate was added, and the development of color (405 nm) was measured at 30 minute intervals using a microplate reader.

RESULTS

Complement forms a key arm of innate immune defenses against gonococcal infection. *N. gonorrhoeae* evades the human complement system through binding to complement regulatory proteins such as C4BP and fH. Gonorrhea is a disease that is restricted to humans. Gonococcal strains that bear Por1A (Por 1A strains) bind fH at complement control protein (CCP) domains 6 and 18-20, while sialylated Por1B strains bind to CCPs 18-20. Only human fH, but not fH from other primates, bind directly to gonococci. Consistent with direct human fH binding, Por1A gonococci resist killing only by human complement, but not by complement from other primates, rodents or lagomorphs. Understanding differences in the primary structure between human and chimp fH at the binding CCP 18-20 domains and the effects these differences have on fH-binding will provide insights into the pathogenesis of gonoccocal infection.

Previously our laboratory reported that Por1A *N. gonorrhoeae* and sialylated Por1B gonococcal strains bind to human fH strongly (Ram et al., 1998). The binding site(s) on fH were defined by creating fusion proteins (fH/Fc) that consisted of contiguous fH CCPs with the fH C-terminus fused to the N-terminus of the Fc portion of mouse IgG2a. The fusion proteins that were created covered the full spectrum of human fH CCPs. Using flow cytometry and FITC-labeled goat anti-mouse IgG as detector antibody, we found that a Por1A strain bound to CCP 6 and CCPs 18-20, while a Por1B strain required sialylation to bind to CCPs 18-20. Non-sialylated Por1B strains did not bind fH/Fc fragments; negative control (*Figure 4*) (Ngampasutadol et al., 2008).
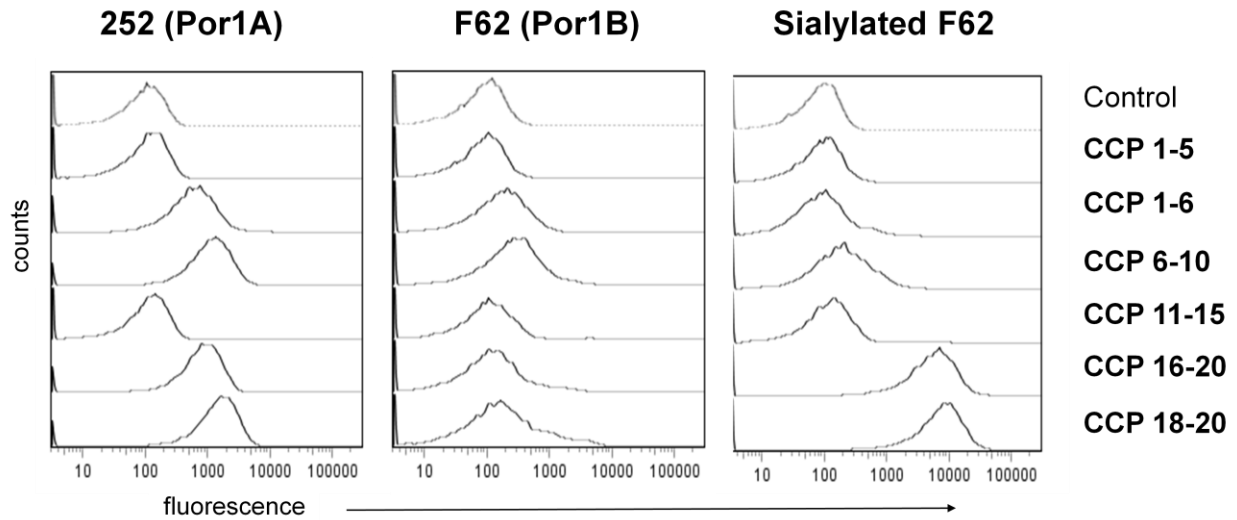
252 (Por1A)   **F62 (Por1B)**   **Sialylated F62**

Control
CCP 1-5
CCP 1-6
CCP 6-10
CCP 11-15
CCP 16-20
CCP 18-20

counts

fluorescence

Figure 4: **Binding of fH/Fc Fusion Proteins to *N. gonorrhoeae*. Flow cytometric analysis was used to identify fH binding domains by incubating *N. gonorrhoeae* in supernatants containing fH/Fc fusion proteins and then using FITC-labeled goat anti-mouse IgG for detection. In the left panel, Por1A strain 252 bound to fH/Fc fusion proteins at 1-6, 6-10, 16-20 and 18-20 (as evidenced by a shift to the right of the control binding patterns). Shown in the middle panel, Por1B strain F62 did not bind any of the fH/fc fusion proteins; after sialylation (right panel), F62 bound to CCPs 16-20 and 18-20. In all graphs, the y-axis represents the number of events and the x-axis represents units of fluorescence. (Ngampasutadol et al., 2008).**

Only Human Factor H CCP 18-20/Fc Binds to N. gonorrhoeae

To validate the use of the fH/Fc fusion proteins to define the CCPs in fH that bind

gonococci, we first tested the binding of human, chimpanzee *(Pan troglotydes),* and rhesus

(*Rhesus macaque)* monkey fH CCPs 18-20/Fc fusion proteins to *N. gonorrhoeae* by flow

cytometry. Consistent with our prior observations that only human fH binds *N. gonorrhoeae,* as

seen in *Figure 5*, human CCPs 18-20/Fc bound to gonococcal strains 252 and sialylated F62.

Chimpanzee CCPs 18-20/Fc and rhesus CCPs 18-20/Fc showed no binding to *N. gonorrhoeae*.
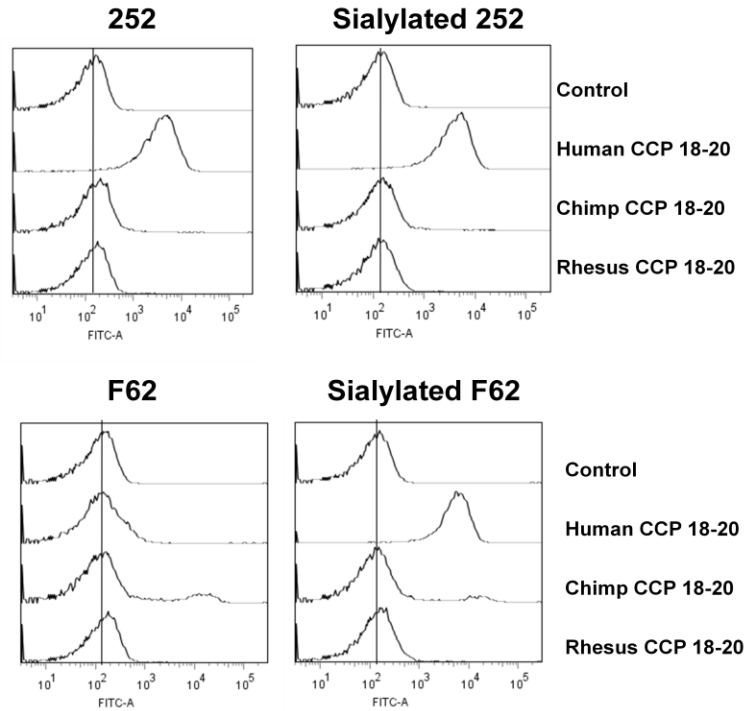
Figure 5: Factor H/Fc CCP 18-20 of Human, Chimpanzee, and Rhesus Binding to Unsialylated and Sialylated Forms of *N. gonorrhoeae* Por1A and Por1B strains, 252 and F62 respectively. 252 and sialylated 252 showed binding to human fH/Fc CCP domains 18-20 (upper panel). F62 (lower left, unsialylated) showed no binding to the human, chimpanzee, or rhesus fH/Fc CCP 18-20. After sialylation of F62 (lower right), the strain showed binding to human fH/Fc CCP 18-20 (lower right panel). In all graphs, the y-axis represents the number of events and the x-axis represents the units of fluorescence. "Control" – no protein added (negative control).

Sequence Comparison of fH CCPs 18-20

We compared the amino acid sequences of CCPs 18-20 of fH of three primate species, namely human, chimpanzee, and rhesus (*Figure 6*). These species were chosen because previous studies have shown that only human fH binds to Por1A and Por1B gonococci, while chimpanzee and rhesus monkey fH do not bind to either Por1A or Por1B gonococci. Furthermore, it is known that the binding site for both Por1A and sialylated Por1B gonococci in human fH resides within CCPs 18-20.

Figure 6 shows a number of differences between human, chimpanzee and rhesus CCPs 18-20 amino acid sequences. These differences may occur as a result of the distance between humans and the other species in the evolutionary chain. In CCP 18, the human and chimpanzee share identical amino acid sequences, however, differences exist in CCP 19 (2 amino acids) and 20 (11 amino acids). In the mutagenesis experiments, we chose to change individual (and

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sometimes 2 at a time) human amino acids to the corresponding chimpanzee counterpart(s). Seven recombinant mutants of fH (amino acid changes at positions; 1176, 1184, 1187, 1200, 1203, 1210 and 1213) were constructed and expressed.

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CCP18 Human   DTSCVNPPTV QNAYIVSRQM SKYPSGERVR YQCRSPYEMF GDEEVMCLNG NWTEPPQCK
      Chimp   .......... .......... .......... .......... .......... .........
      Rhesus  .....D..S. K..H...... ...A...... .E........ .......V.. .........

CCP19 Human   DSTGKCGPPP PIDNGDITSF PLSVYAPASS VEYQCQNLYQ LEGNKRITCR NGQWSEPPKC L
      Chimp   .......... .......... .........T .........E .......... .......... .
      Rhesus  .......... .......... .......D.. ...K.D... .......... .....D.... .

CCP20 Human   HPCVISREIM ENYNIALRWT AKQKLYSRTG ESVEFVCKRG YRLSSRSHTL RTTCWDGKLE YPTCAKR
      Chimp   .......... .K.......K ..E.I..... .....I..N. ....PS..A. ..A....... ....V..
      Rhesus  .A....P... .K...T.K.K .EE....... .P...R.RS. .Y...N..P. ....Q....K .......
                          ↑          ↑  ↑              ↑   ↑          ↑   ↑
                         1176      1184 1187         1200 1203      1210 1213
```

Figure 6: **Amino Acid Sequences of Human, Chimpanzee and Rhesus fH at CCPs 18-20 Domains. Dots indicate similarities in chimpanzee /rhesus sequences to those in humans.**

Recombinant fH CCPs 18-20/Fc Mutant Proteins Binding to N. gonorrhoeae

Examining differences in amino acid sequences within the known region of binding, the CCPs 18-20 domain, of human, chimp, and rhesus fH pinpointed particular amino acids that likely are involved in fH binding to *N. gonorrhoeae*. We created human-to-chimpanzee mutations at amino acids 1176, 1184/1187 (double mutation), 1200/1203 (double mutation), 1200, 1203 and 1210/1213 (double mutation) in the CCP 20 domain of fH/Fc CCPs 18-20, and measured binding by flow cytometry of the modified fH/Fc CCP18-20 molecules to *N. gonorrhoeae.* We examined binding to a Por1A strain, named 252; sialylation of strain 252 had little effect on binding of the recombinant fH/Fc CCP 18-20 molecules (*Figure 7*). Strain 252, sialylated or not bound strongly to native fH/Fc CCPs 18-20 and all recombinant fH/Fc molecules tested except for those that contained the R1203N mutation (*Figure 7)*.
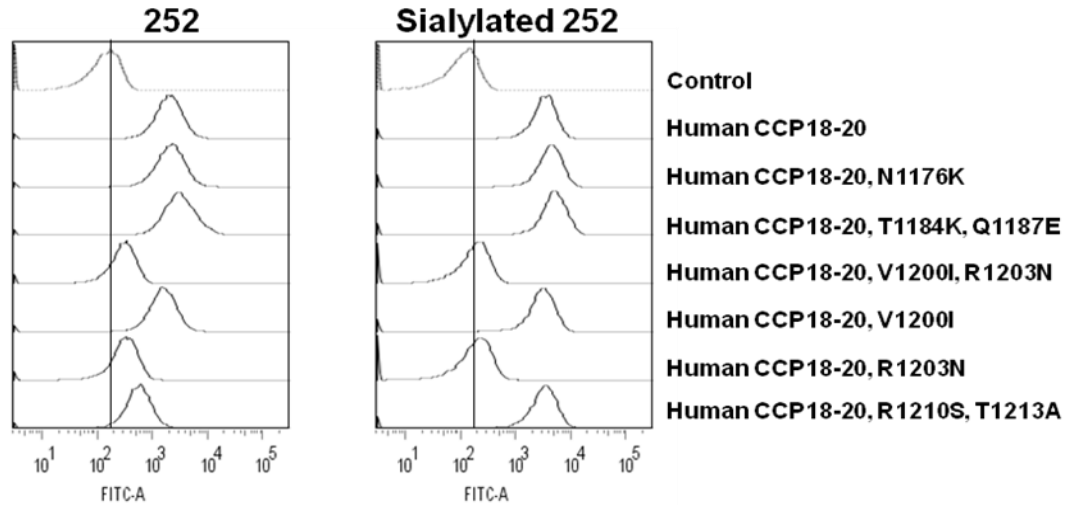
252   **Sialylated 252**

Control

Human CCP18-20

Human CCP18-20, N1176K

Human CCP18-20, T1184K, Q1187E

Human CCP18-20, V1200I, R1203N

Human CCP18-20, V1200I

Human CCP18-20, R1203N

Human CCP18-20, R1210S, T1213A

FITC-A   FITC-A

Figure 7: **Recombinant Human fH Binding to** *N. gonorrhoeae* **Por1A Strain, 252. Binding of fH/Fc CCPs 18-20 to strain 252 (sialylated, right panel; or not, left panel) was unaffected by chimpanzee-like changes in amino acid at position(s) 1176, 1184, 1187, 1200, 1210 and 1213, but required position 1203 be maintained for full binding. Binding of human fH to sialylated strain 252 was enhanced (to normal levels) when amino acids at positions 1210 and 1213 were changed to the corresponding chimpanzee amino acids. In all graphs, the y-axis represents the number of events and the x-axis represents units of fluorescence. "Control"-no protein added (negative control); Human fH/Fc CCP 18-20-full binding (positive control).**

We also examined the binding of recombinant fH proteins to a Por1B gonococcal strain, named F62. As expected, unsialylated strain F62 did not bind human fH/Fc CCPs 18-20 (*Figure 8*). After sialylation however, gonococcal strain F62 bound strongly to "native" human fH/Fc CCPs 18-20 and to all recombinant fh/Fc CCPs 18-20 molecules except the two that contained the mutation at amino acid position 1203.
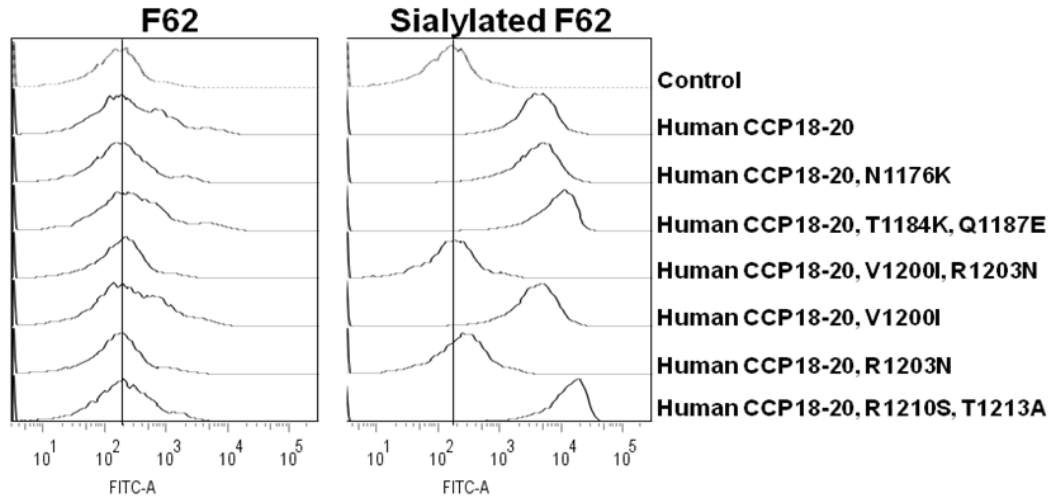
25

F62 **Sialylated F62**

Control
Human CCP18-20
Human CCP18-20, N1176K
Human CCP18-20, T1184K, Q1187E
Human CCP18-20, V1200I, R1203N
Human CCP18-20, V1200I
Human CCP18-20, R1203N
Human CCP18-20, R1210S, T1213A

FITC-A   FITC-A

Figure 8: **Recombinant Human fH/Fc CCPs 18-20 Binding to F62, a Por1B Strain of *N. gonorrhoeae*. F62 (unsialylated, left panel) showed no binding to "native" human fH/Fc CCP 18-20, or to any of the recombinant fH/Fc proteins (left panel). After sialylation of F62 (right panel), all factor H recombinant proteins bound to sialylated F62, except for those that contained the mutation at amino acid position 1203 (right panel). In all graphs, the y-axis represents the number of events, and the x-axis represents units of fluorescence. "Control"-no protein added (negative control); Human fH/Fc CCP 18-20-full binding (positive control).**

To further determine the significance of the amino acid at position 1203 in binding to gonococci, we constructed a reverse mutation by replacing the amino acid asparagine (N) at position 1203 in CCP 20 of chimpanzee fH with the human counterpart, arginine (R), N1203R. We then examined the binding of the modified fH/Fc CCPs 18-20 (human CCPs 18-19 linked to chimpanzee CCP 20) to *N. gonorrhoeae*. The importance of the arginine (R) substitution at the 1203 position in CCP 20 is shown in *Figure 9* by restoration of binding to both Por1A and Por1B sialylated gonococcal strains of a human CCPs 16-19, chimp CCP 20/Fc construct when the amino, asparagine (N) at the 1203 position in the chimpanzee CCP 20 portion of the construct was changed to the human amino acid, arginine (R).
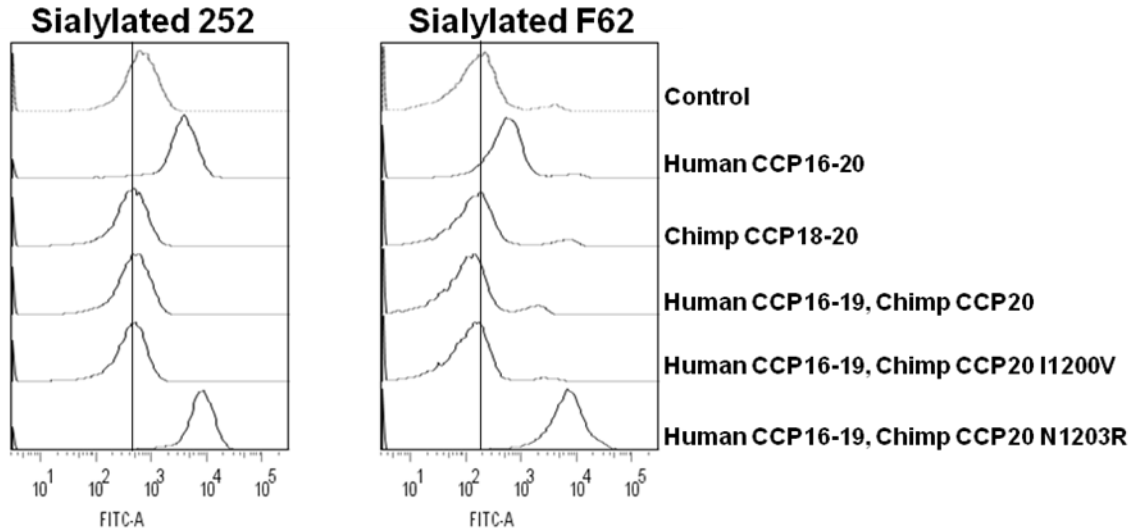
Figure 9: **fH Derived CCP Binding to *N. gonorrhoeae*. Human CCP 16-20 (second profile) binds to both sialylated 252 (Por 1A) and sialylated F62 (Por 1B); positive control. Chimp CCP 18-20 (third profile) did not bind to either organism; negative control. CCP 16-20 constituted with Human CCP 16-19 and Chimp CCP 20, the latter bearing a N1203R substitution bound to both organisms (bottom [sixth] profile). First, fourth and fifth profiles show additional negative controls. No protein was present in the first profile, marked Control. In all graphs, the y-axis represents the number of events and the x-axis represents units of fluorescence.**

Factor H Binding to C3b and Heparin

Human fH also contains binding sites for C3b, a protease in the complement immune system, and heparin. Previous studies showed that human fH binding to C3b is localized to fH CCP 1-4, CCP 6-8 and CCP 19-20; fH binds heparin to at CCP 6-8 and 19-20 (Figure 10) (Jokiranta et al., 2000; Prodinger et al., 1998; Blackmore et al., 1998).
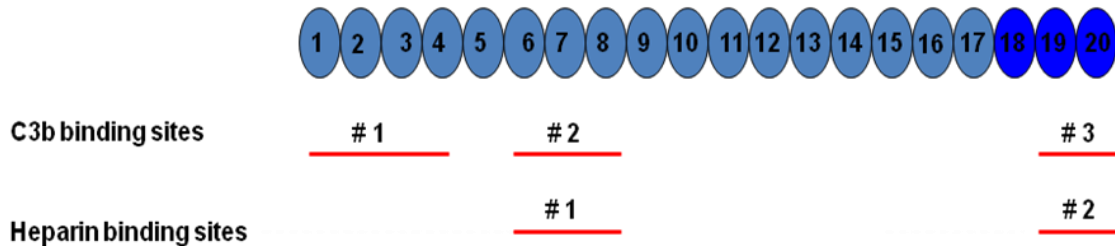

Figure 10: **Human fH Binding Sites on C3b and Heparin. FH binds C3b at three different sites; CCP 1-4, CCP 6-8 and CCP 19-20. FH binds Heparin at two sites: CCP 6-7 and CCP 19-20. (Jokiranta et al., 2000; Prodinger et al., 1998; Blackmore et al., 1998).**

FH binding sites on C3b and heparin correspond to certain fH binding sites on *N. gonorrhoeae.* For example, using flow cytometry, we observed that heparin blocked binding of fH/Fc derived CCPs 16-20 to *N. gonorrhoeae* (*Figure 11*), indicating that heparin and *N. gonorrhoeae* share some of the same binding sites on fH.
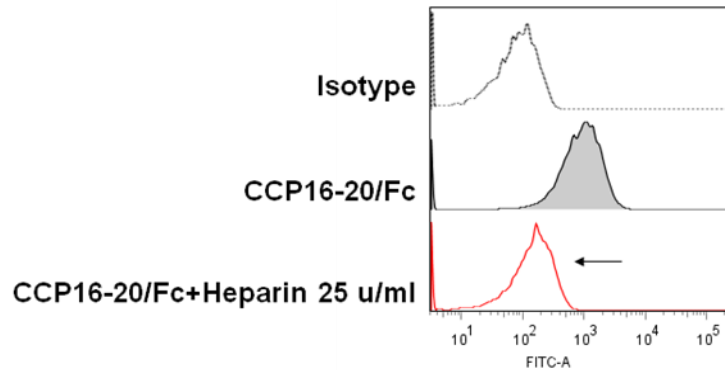

Figure 11: **Heparin Blocks Binding of Human fh/Fc CCP 16-20 to *N. gonorrhoeae*. Heparin and *N. gonorrhoeae* share a binding site within the CCP 19-20 portion of the CCP 16-20 construct. In these graphs, the y-axis represents the number of events and the x-axis represents units of fluorescence.**

To validate the hypothesized specificity of C3b and heparin binding to CCP 20, and to more precisely map the location of the binding sites within CCP 20, we examined binding to C3b (F*igure 12a)* and heparin (*Figure 12b)* of recombinant fH/Fc CCP 18-20 proteins, which contained CCP 20 mutations (single and double) at amino acid positions: 1176, 1184/1187, 1200, 1203 1200/1203, and 1210/1213. Using ELISAs we found that the decreases in binding of the recombinant proteins to C3b and heparin were similar for both molecules except that changes at 1184 and 1187 (together) more effectively diminished binding to C3b than heparin. The single mutation at position 1203, R1203N (the human to chimp mutation), or the double mutation R1210S and T1213A decreased binding to C3b and heparin the most.
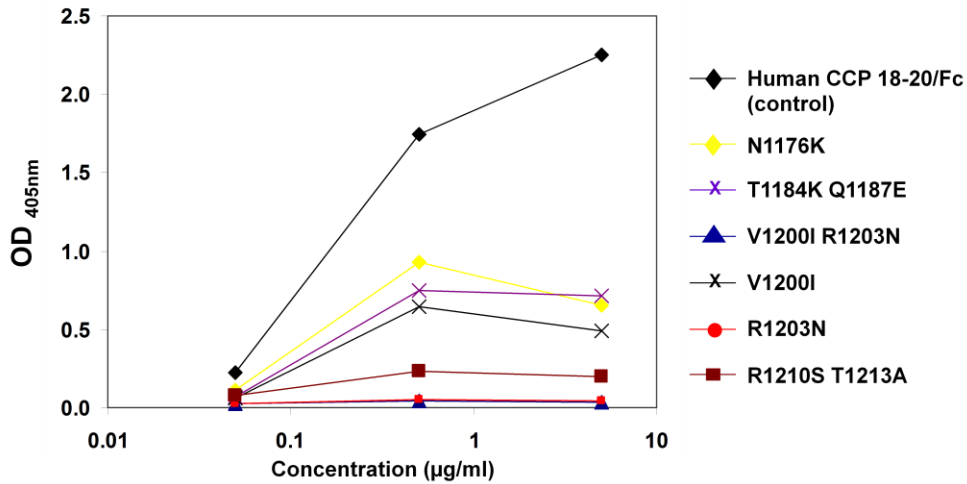
(a) C3b binding
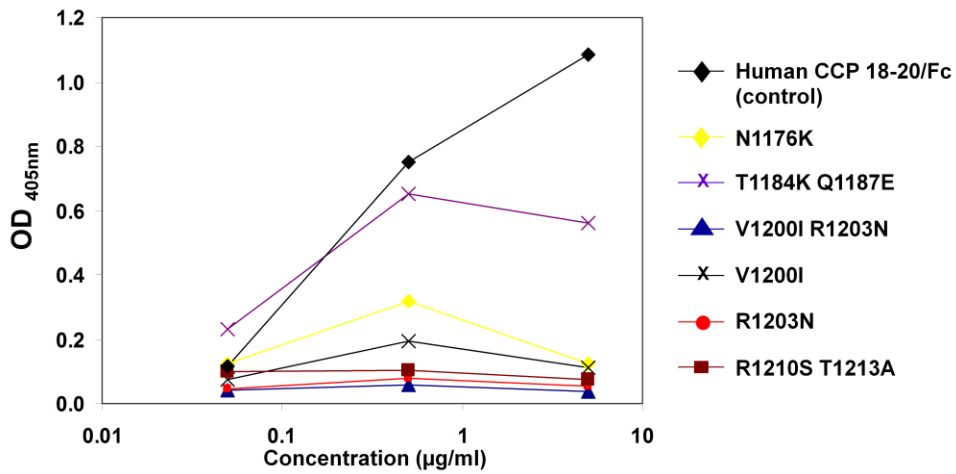
(b)Heparin binding

***Figure 12*: ELISAs Measuring the Binding of Recombinant fH/Fc CCP 18-20 to C3b and Heparin. Recombinant fH containing mutation R1203N almost completely eliminates fH-binding to both C3b and heparin. The binding of recombinant fH was detected through ELISA assay using alkaline phosphatase-conjugated goat anti-mouse IgG and p-Nitrophenyl phosphate (pNPP) as substrate.**

DISCUSSION

N. gonorrhoeae infects humans by evading the complement system, an arm of the human innate immune system. Binding to regulatory proteins such as C4BP and fH allows the bacterium to evade the classical and alternative pathways respectively, thereby preventing complement mediated killing by normal human serum (NHS) (Ram et al., 2001; Ram et al., 1998; Ram et al., 1998). *N. gonorrhoeae* exhibits species specificity by infecting humans naturally and (sometimes) chimpanzees experimentally (Arko, 1989; Arko et al., 1976) but no other vertebrate species, including baboons, monkeys, rabbits, and guinea pigs (Arko, 1989). Gonococci are able to attach to, damage and invade the oviduct mucosa of chimpanzees but not the oviduct mucosa of baboons, suggesting that, from a chronologic standpoint, susceptibility to gonococcal infection begins at an "evolutionary watershed", which occurs between baboons and chimpanzees (or between monkeys and great apes) (McGee et al., 1990). Past studies that have examined killing of gonococci by non-human animal sera have shown that, except for chimpanzee C4BP, which binds to Por 1B gonococci, complement regulators in these sera fail to bind to *N. gonorrhoeae*. This results in unrestrained killing of gonococcal organisms by non-human serum. (Ngampasutadol et al., 2005).

In this project, we investigated the binding of *N. gonorrhoeae* to the human regulatory protein, fH. Two regions of human fH were shown to bind to *N. gonorrhoeae*, CCP 6 and CCPs 18-20. Our experiments revealed that while Por1A strain 252 bound at both fH binding domains, sialylated Por1B strain F62 bound only at domains within CCPs 18-20. Thus, we focused our studies on CCPs 18-20 because both major types of *N. gonorrhoeae* (Por 1A and Por 1B) utilize this binding region. We also elicited the details of the previously reported (Ngampasutadol et al.,

2008) species specificity of *N. gonorrhoeae* (Por1B strain F62) binding to fH of human, chimpanzee and rhesus. We found that sialylated Por 1B strain F62 bound to human fH CCP 18-20, but did not bind to chimpanzee or rhesus fH, suggesting fundamental differences that dictate binding may exist between the human, chimpanzee and rhesus fH structure in domains CCPs 18-20.

Human and chimpanzee fH display different amino acids at 2 positions in CCP 19, and at 11 positions (1176, 1184, 1187, 1189, 1200, 1203, 1209, 1210, 1213, 1217 and 1229) in CCP 20 (none in CCP 18). Because the 2 positions that differ between human and chimpanzee CCP 19 are the same in human and rhesus, yet rhesus fH also does not bind to *N. gonorrhoeae*, we reasoned that these amino acids are not involved in binding to *N. gonorrhoeae*.

We created recombinant fH/Fc proteins that spanned selected binding regions in CCPs 18-20, focusing on CCP 20 where we had reasoned the binding domains exist. We used Fc fusion proteins to facilitate symmetric detection of fH CCPs because we had shown in earlier experiments (not shown) that exposure of the CCPs was sometimes cryptic after binding, thereby creating difficulty in recognizing the bound fragment(s). Having created single (in some cases double) amino acid substitutions (human substituted by chimpanzee) at 7 of the 11 disparate positions in CCP 20, we found that a R1203N, mutation (substitution of arginine by asparagine) was the single most important substitution that abrogated pre-existing binding of human fh/Fc CCPs 18-20 to *N. gonorrhoeae*. To validate this finding, we performed the reverse substitution, replacing asparagine at position 1203 in the chimpanzee CCP 20 with arginine, the amino acid present in human CCP 20, and showed restoration of binding to *N. gonorrhoeae* of a construct composed of fH/Fc Human CCP 18-19 and Chimpanzee CCP 20.

We also investigated the binding of C3b and heparin, both known to bind fH domains within CCPs 19-20, to pinpoint the specificity of fH binding of each of these molecules. Similar to fH binding to *N. gonorrhoeae*, fH binding to C3b and heparin occurred predominantly through the interaction of arginine at position 1203 in CCP 20. Together these results lead us to propose that arginine, located at position 1203 forms the critical motif (structure) that permits the interaction of fH with a variety of target molecules.

The mutation at 1203 from arginine to asparagine (a basic polar to a neutral polar), we believe, modifies and distorts the structure of fH to prevent proper binding to *N. gonorrhoeae* at domain CCP 20. The data suggests that arginine, a basic amino acid, at the 1203 position plays a significant role is holding together the tertiary structure of fH to create a binding region for *N. gonorrhoeae,* C4BP, heparin, and possibly other substances that utilize that binding region. By substituting asparagine, a polar neutral amino acid, for arginine, the base (positive) charge is removed, which distorts the structure of fH at binding CCP domain 20. An appropriate next step will be to create a crystal structure to analyze how the mutation at position 1203 from arginine to asparagine affects the structure of the fH protein as a whole.

In conclusion, we have identified amino acid R1203 in human fH as critical for fH binding to *N. gonorrhoeae*. A better understanding of the specific contribution of arginine at this site to the overall structure of fH will be required to define the interaction of fH at this site with its target molecule(s) on the surface of *N. gonorrhoeae*.

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