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A novel factor H-Fc chimeric immunotherapeutic molecule against *Neisseria gonorrhoeae*

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Abstract

Neisseria gonorrhoeae (*Ng*), the causative agent of the sexually transmitted infection gonorrhea, has developed resistance to almost every conventional antibiotic. There is an urgent need to develop novel therapies against gonorrhea. Many pathogens, including *Ng*, bind the complement inhibitor factor H (FH) to evade complement-dependent killing. Sialylation of gonococcal lipooligosaccharide, as occurs *in vivo*, augments binding of human FH through its domains 18-20 (FH18-20). We explored the utility of fusing FH18-20 with IgG Fc (FH18-20/Fc) to create a novel anti-infective immunotherapeutic. FH18-20 also binds to select host glycosaminoglycans to limit unwanted complement activation on host cells. To identify mutation(s) in FH18-20 that eliminated complement activation on host cells, yet maintained binding to *Ng*, we created four mutations in domains 19 or 20 described in atypical hemolytic uremic syndrome that prevented binding of mutated fH to human erythrocytes. One of the mutant proteins (D to G at position 1119 in domain 19; FHD1119G/Fc) facilitated complement-dependent killing of gonococci similar to unmodified FH18-20/Fc, but unlike FH18-20/Fc, did not lyse human erythrocytes. FHD1119G/Fc bound to all (100%) of 15 sialylated clinical *Ng* isolates tested (including three contemporary ceftriaxone-

resistant strains), mediated complement-dependent killing of 10/15 (67%) strains and enhanced C3 deposition (10-fold above baseline levels) on each of the five isolates not directly killed by complement. FHD1119G/Fc facilitated opsonophagocytic killing of a serum-resistant strain by human polymorphonuclear neutrophils. FHD1119G/Fc administered intravaginally significantly reduced the duration and burden of gonococcal infection in the mouse vaginal colonization model. FHD1119G/Fc represents a novel immunotherapeutic against multidrug-resistant *Ng*.

Keywords

complement; *Neisseria gonorrhoeae*; treatment; factor H; immunotherapy; Fc fusion protein

Introduction

Antimicrobial resistance remains a major threat to public health worldwide and we are witnessing an era where several medically important microbes are becoming untreatable with antibiotics currently in clinical use. Organisms such as *Staphylococcus aureus*, *Enterococcus* spp, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and several additional members of the family Enterobacteriaceae have become resistant to most conventional antibiotics (1, 2). Similarly, *Neisseria gonorrhoeae* has also demonstrated a remarkable capacity to resist almost every antibiotic that it has encountered (3). The recent isolation of *N. gonorrhoeae* strains highly resistant to ceftriaxone, the last remaining option for empirical monotherapy, in several parts of the world represents a major public health problem (4–7). In addition to complications including pelvic inflammatory disease and its sequelae such as infertility, ectopic pregnancy and chronic pelvic pain, gonorrhea can increase the transmission and acquisition of HIV-1 infection (8, 9). Thus, spread of multidrug resistant gonorrhea represents a serious public health threat and there is an urgent need to develop novel antimicrobials, and ideally vaccines and immunotherapeutics against this pathogen.

The complement system forms a key arm of innate immune defenses against invading pathogens (10). In order to successfully establish infections in their hosts, microbes have developed mechanisms to subvert killing by complement (11). By binding of complement inhibitors, such as factor H (FH), C4b-binding protein (C4BP) and vitronectin, several pathogens, including *N. gonorrhoeae*, dampen complement activation on their surfaces (11–13). FH inhibits the alternative pathway of complement by serving as a cofactor for the factor I-mediated cleavage of C3b to the hemolytically inactive iC3b fragment (14). FH also possesses decay accelerating activity, whereby it irreversibly dissociates the Bb fragment from the alternative pathway C3 convertase, C3b,Bb (15–17). FH comprises 20 domains, also known as short consensus repeat domains (SCRs) or complement control protein domains (CCPs) that are arranged in the form of a single chain (18). The first four N-terminal domains are necessary and sufficient for complement inhibition (19). Most microbes, including *N. gonorrhoeae*, that bind FH do so through regions spanned by domains 6 and 7 and/or domains 18 through 20 (11).

In vivo, gonococci scavenge 5'-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) from the host to sialylate their lipooligosaccharide (LOS) (20, 21). The two LOS structures that can be sialylated are the nearly ubiquitously expressed lacto-*N*-neotetraose (LNT, Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-4HepI) structure and the less frequently encountered PK-like structure (Neu5Ac α 2-6Gal α 1-4Gal β 1-4Glc β 1-4HepI) (22). Sialylation of the LNT LOS structure enhances binding of the C-terminal domains 18-20 of human FH to gonococci (23, 24). This increase in FH binding is dependent on expression of gonococcal porin (PorB); replacing gonococcal PorB with meningococcal PorB abrogates Neu5Ac-mediated enhancement of FH binding (25). Several strains of *N. gonorrhoeae* also bind FH independently of LOS sialylation (26).

We have shown previously that a chimeric protein comprising human FH domains 18, 19 and 20 fused to murine IgG2a Fc (FH18-20/Fc) bound to gonococci, activated the classical pathway of complement, and resulted in complement-dependent bactericidal activity (27). Such a molecule could serve as a novel adjunctive immunotherapeutic against multidrug-resistant bacterial species, including *N. gonorrhoeae*. However, the C-terminus of FH is also critical for regulating complement activation on host cells (28, 29). Therefore, a therapeutic that uses the C-terminus of FH to anchor complement-activating Fc to the bacterial surface needs to be modified to eliminate binding to host cells. Here we describe derivation of a novel fully human FH18-20/Fc fusion immunotherapeutic molecule and characterize its activity *in vitro* against clinically relevant strains of *N. gonorrhoeae*.

Materials and methods

Bacterial strains

N. gonorrhoeae strains used in this study and their relevant characteristics are listed in Supplemental Table S1. Strains from the sexually transmitted infection clinic in Nanjing, China were chosen randomly from an initial collection of 64 male isolates that were part of an *Ng* transmission study and whose antimicrobial sensitivities are reported in Supplemental Table S1. *N. gonorrhoeae* multiantigen sequence type (NG-MAST) was determined by DNA sequencing of variable regions of *porB* and *thpB*, as previously described (30). For simplicity, an abbreviated name for the Nanjing strains has been used; the full name has been provided in Supplemental Table S1. An opacity protein (Opa)-negative mutant derivative of strain FA1090 where all 11 *opa* genes were inactivated was provided by Dr. Janne G. Cannon (University of North Carolina, Chapel Hill, NC, USA) (31). Bacteria that had been grown on chocolate agar supplemented with IsoVitalex® in an atmosphere enriched with 5% CO₂ for ~15 h at 37°C were suspended in gonococcal liquid media and grown to mid-log phase, as described previously (32). Sialylation of gonococcal LOS was achieved by adding CMP-Neu5Ac to growth media in a final concentration of 2 µg/ml. Bacteria were washed and suspended in Hanks' balanced salt solution (HBSS) containing 0.15 mM CaCl₂ and 1 mM MgCl₂ (HBSS⁺⁺) for use in binding and bactericidal assays.

Normal human serum (NHS)

Serum was obtained from normal healthy adult volunteers with no history of gonococcal or meningococcal infection who provided informed consent. Participation was approved by the

University of Massachusetts Institutional Review Board for the protection of human subjects. Serum was obtained from whole blood that was clotted for 25 °C for 30 min followed by centrifugation at 1500 g for 20 min at 4 °C. Individual sera were pooled and stored at -70 °C.

IgG and IgM depleted normal human serum (human complement)

To study the effects of the FH18-20/Fc proteins without confounding by natural anti-gonococcal antibodies present in NHS, we depleted IgG and IgM from freshly collected human serum, as described previously (33). Briefly, EDTA (final concentration 10 mM) and NaCl (final concentration 1 M) were added to freshly prepared human serum and treated sera was passed first over anti-human IgM agarose (Sigma), followed by passage through protein G-Sepharose; both columns were equilibrated in PBS containing 10 mM EDTA and 1 M NaCl. NaCl was added to minimize loss of C1q during passage of serum through the anti-human IgM column. The flow-through was collected, spin concentrated and dialyzed against PBS/0.1 mM EDTA to its original volume using a 10-kDa cutoff Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA), sterilized by passage through a 0.22-µm filter (Millipore), aliquoted and stored at -70 °C. Hemolytic activity was confirmed using a total complement hemolytic plate assay (The Binding Site Inc., Birmingham, U.K). Depletion of IgG and IgM was confirmed by dot-blot assays using alkaline phosphatase conjugated goat anti-human IgG and goat anti-human IgM, respectively. In some experiments, complement activity of serum was destroyed by heating serum at 56°C for 1 h.

Expression and purification of FH/Fc fusion proteins

Cloning, expression and purification of a chimeric protein comprising human FH (HuFH) domains 18-20 fused to mouse IgG2a Fc has been described previously (23). Briefly, the DNA encoding FH domains 18-20 was cloned into *AscI*-*NotI* sites of eukaryotic expression vector pCDNA3 containing the sequence encoding mouse IgG2a Fc (34). We created four HuFH18-20/Fc mutants using the Quickchange site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions with primers D1119G, R1182S, W1183R and R1215G (Supplemental Table S2). Where indicated, mouse IgG2a Fc was replaced by human IgG1 Fc as follows. FH domains 18-20 were amplified using primers FH18EcoRI and FH20hIgG1overlapR, and human IgG1Fc (InvivoGen) was amplified with primers FH20hIgG1overlapF and HIgG1NheI (Supplemental Table S2). The PCR products were then fused together by overlap extension PCR using primers FH18EcoRI and HIgG1NheI. The final PCR product encoding FH18-20 fused to hIgG1 was digested with *EcoRI* and *NheI* and cloned into pFUSE-hIgG1-Fc2 (Invivogen). The resulting plasmids were verified by DNA sequencing and used to transiently transfect CHO cells using lipofectin (Life Technologies), according to the manufacturer's instructions. Media from transfected cells was collected after 2 days and FH/Fc was purified by passage over protein A agarose. Protein concentrations were determined using the BCA protein Assay kit (Pierce); mass was determined by Coomassie Blue staining of proteins separated by SDS-PAGE.

Antibodies

Sheep anti-human C3c-FITC was obtained from AbD Serotec (cat. # AHP031F), anti-mouse IgG FITC and anti-human IgG FITC were from Sigma. Both antibodies (Abs) were used at a dilution of 1:200 in HBSS⁺⁺ and 1% bovine serum albumin (BSA) (HBSS⁺⁺/BSA) in flow cytometry assays.

Flow cytometry

Binding of FH/Fc to bacteria and C3 fragments deposited on bacteria were measured by flow cytometry as described previously (35). Data were acquired on a LSRII flow cytometer and data were analyzed using FlowJo software.

Serum bactericidal assay

Serum bactericidal assays were performed as described previously (36). Bacteria that had been harvested from an overnight culture on chocolate agar plates were grown in gonococcal liquid media supplemented with CMP-Neu5Ac (2 µg/ml) from an OD_{600nm} of ~0.1 to the mid-log phase (OD_{600nm} ~0.25). Approximately 2000 colony forming units (CFUs) of *N. gonorrhoeae* were incubated with human complement in the presence or the absence of the FH/Fc fusion protein (concentration indicated for each experiment). The final volume of the bactericidal reaction mixture was 150 µl. Aliquots of 25-µl reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay (t₀) and again after incubation at 37°C for 30 min (t₃₀). Survival was calculated as the number of viable colonies at t₃₀ relative to t₀.

Hemolytic assay

Lysis of human erythrocytes was measured using a method similar to one described previously (37). Freshly isolated human red blood cells (RBCs) (5×10^6) were incubated with 7 µg/ml anti-CD59 monoclonal antibody (mAb) (clone MEM43; Abcam) at 4°C for 20 min and then mixed, on ice, with NHS derived from the homologous donor (final NHS concentration 40%), gelatin veronal buffer (GVB), 5 mM MgCl₂ and 5 mM EGTA and the indicated concentrations of FH/Fc. Mixtures were then transferred to a 37°C water bath and incubated for 20 min. GVB-EDTA (200 µl) was added to a final concentration of 10 mM EDTA to block further complement activation and the samples were immediately centrifuged at 4°C and the OD_{410nm} of the supernatants were determined. Background lysis (anti-CD59-treated RBCs plus buffer alone) was subtracted from each reading and the results were expressed as OD_{410nm}.

Opsonophagocytosis assay

Heparinized venous blood was obtained from a healthy adult volunteer in accordance with a protocol approved by the Institutional Review Board. Polymorphonuclear leukocytes (PMNs) were isolated using Mono-Poly® resolving medium (MP Biomedicals, LLC) according to the manufacturer's instructions. Isolated PMNs were washed and suspended in HBSS without added divalent cations, counted, and diluted to 1×10^7 /ml in HEPES-buffered RPMI-1640 medium supplemented with L-glutamine and 1% heat-inactivated fetal bovine serum (FBS). To measure survival of gonococci in the presence of PMNs, an

Opacity protein (Opa)-negative mutant of *N. gonorrhoeae* strain FA1090 that was grown in media containing 2 µg/ml CMP-Neu5Ac to sialylated LOS was added to 1×10^6 PMNs at a multiplicity of infection (MOI) of 10 (10 bacteria to 1 PMN). Opa-negative *N. gonorrhoeae* was used because select Opa proteins serve as ligands for human carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3) that is expressed by PMNs and results in phagocytosis (38). FHD1119G/HuFc was added at a concentration of 16.7 µg/ml, followed by 10% human complement (prepared as described above). Bacteria plus PMNs and 10% NHS (Ab intact) was used as a positive control for killing. The reaction mixtures were incubated for 60 min at 37°C in a shaking water bath. Bacteria were serially diluted and plated at 0 and 60 min on chocolate agar plates. Percent survival of gonococci in each reaction was calculated as a ratio of CFUs at 60 min to CFUs at the start of the assay (0 min).

Mouse vaginal colonization model

Use of animals in this study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School. Female BALB/c mice 6–8 weeks of age (Jackson Laboratories) in the diestrus phase of the estrous cycle were started on treatment (that day) with 0.5 mg Premarin (Pfizer) in 200 µl of water given subcutaneously on each of three days; −2, 0 and +2 days (before, the day of and after inoculation) to prolong the estrus phase of the cycle and promote susceptibility to *Ng* infection. Antibiotics (vancomycin, colistin, neomycin, trimethoprim and streptomycin) ineffective against *Ng* were also used to reduce competitive microflora (39). Mice (n=26) were infected (Day 0) with 1.5×10^6 CFU of strain F62. One group of mice (n=14) was treated daily with 12 µg FHD1119G/mouse IgG2a Fc (1.5 mg/ml in PBS) intravaginally while the remaining 12 mice were given a corresponding volume of PBS (vehicle controls). A construct containing mouse IgG2a Fc (distinct from human IgG1 Fc used in the bactericidal and opsonophagocytosis assays) was used to maintain species congruity between Fc and its cognate FcR. The therapeutic was administered locally because initial experiments with systemic administration of FH/Fc to wild-type mice had resulted in the generation of anti-human FH Ab. Finally, 10 µg of CMP-Neu5Ac was also administered locally to all mice daily to ensure LOS substitution with Neu5Ac. Mice possess an enzyme called CMP-N-acetylneuraminic acid hydroxylase (CMAH) that converts CMP-Neu5Ac to CMP-N-glycolylneuraminic acid (CMP-Neu5Gc) (40–42) and mice therefore express both CMP-Neu5Ac and CMP-Neu5Gc. In contrast, humans make only CMP-Neu5Ac. Both CMP-sialic acids can serve as a substrate for gonococcal LOS sialyltransferase (43), and therefore, in mice gonococcal LNT LOS can be substituted with either Neu5Ac or Neu5Gc, while in humans it is exclusively substituted with Neu5Ac. Similar to LNT LOS substituted with Neu5Ac, Neu5Gc-substituted LNT LOS also renders gonococci complement-resistant (43). Administering CMP-Neu5Ac to mice may result in more 'human-like' LNT LOS substitution with Neu5Ac.

Experiments that compared clearance of *N. gonorrhoeae* in independent groups of mice estimated and tested three characteristics of the data (44): Time to clearance, longitudinal

trends in mean $\log_{10}$ CFU and the cumulative CFU as area under the curve (AUC). Statistical analyses were performed using mice that initially yielded bacterial colonies on Days 1 and/or 2. Median time to clearance was estimated using Kaplan-Meier survival curves; times to clearance were compared between groups using a log-rank test. Mean $\log_{10}$ CFU trends over time were compared between groups using a linear mixed model with mouse as the random effect using both a random intercept and a random slope. A cubic function in time was determined to provide the best fit; random slopes were linear in time. A likelihood ratio test was used to compare nested models (with and without the interaction term of group and time) to test whether the trend differed over time between the two groups. The mean AUC ($\log_{10}$ CFU versus time) was computed for each mouse to estimate the bacterial burden over time (cumulative infection); the means under the curves were compared between groups using the nonparametric rank sum test because distributions were skewed or kurtotic.

Results

Selection of the FHD1119G/Fc as the lead FH/Fc therapeutic molecule

We had shown previously that a chimeric molecule comprising FH domains 18, 19 and 20 fused to murine Fc bound to and mediated complement-dependent killing of *N. gonorrhoeae* strains F62 and 252 (27). The C-terminal domains of FH (domains 19 and 20) bind to C3b/C3d (19, 45), heparin/heparan sulfate-containing surfaces (46), and endothelial cells (47) and protects host cells from complement attack. If left unmodified, the C-terminal domains of FH (domains 19 and 20) in FH/Fc would compete with binding and function of the full-length FH on human cells. Competition for binding and function of full-length FH by recombinant FH molecules comprising domains 19 and 20 was shown on RBCs treated with anti-CD59 (erythrocytes treated with anti-CD59 rely on binding of FH to regulate complement activation and hemolysis; (37)). Therefore, we sought to define and utilize mutations in FH18-20 that eliminated complement activation on host cells while maintaining the ability to bind to and mediate killing of *N. gonorrhoeae*.

Atypical hemolytic uremic syndrome (aHUS) is a condition that results from 'over-activity' of the alternative pathway of complement. Mutations of FH that diminish FH binding to glycosaminoglycans and/or C3 fragments on host cells are important in the development of aHUS (reviewed in references (48–50)). Ferreira et al introduced some of these mutations into recombinant molecules that comprised FH domains 19 and 20, and examined whether the mutant molecules prevented full-length human FH from protecting anti-CD59-treated human RBCs from complement-mediated hemolysis (37). As expected, the wild-type FH 19–20 out-competed the full-length FH and resulted in hemolysis. Four mutant molecules, D1119G (domain 19), R1182S, W1183R and R1215G (the latter three in domain 20) did not interfere with the normal function of native FH (37). Our choice of mutations was guided by the aHUS mutations in FH domains 19 and 20 that were characterized by Ferreira and colleagues (37). FH18-20/murine IgG2a Fc proteins that contained these individual mutations were expressed in CHO cells and purified from tissue culture supernatants; the molecular masses and relative purity of the proteins were determined by SDS-PAGE stained with Coomassie Blue (data not shown). Bacteria were grown in media containing CMP-

Neu5Ac, which results in sialylation of LNT LOS, similar to sialylation that occurs *in vivo* (20). We compared binding of FH18-20/Fc mutant proteins to sialylated gonococci with binding of the wild-type (WT) FH18-20/Fc (Fig. 1A). Two mutant proteins, FHD1119G/Fc and FHR1182S/Fc showed similar binding to sialylated *N. gonorrhoeae* strain F62 compared to the wild-type protein (FH18-20/Fc). The FH/Fc molecules bearing the W1183R or R1215G mutations showed weak or no binding to sialylated strain F62, respectively (Fig. 1A). FH/Fc mutated proteins were tested for complement-dependent killing of sialylated strain F62 (Fig. 1B). Consistent with our previous work (27), wild-type molecule (FH18-20/Fc) killed strain F62 in a dose-responsive manner (Fig. 1B); FHD1119G/Fc and FHR1182S/Fc each killed sialylated strain F62. Neither FHR1183R/Fc or FHR1215G/Fc, killed sialylated strain F62 at the maximal concentration tested (6.7 µg/ml).

The killing curves for sialylated strain F62, in particular for the D1119G mutant and wild-type molecule and the mutant, were steep – i.e., small differences in FH/Fc concentration resulted in dramatic increases in complement-dependent killing. To confirm apparent superiority of function of FHD1119G/Fc mutant over FHR1182S/Fc mutant, we used strain 252 that is intrinsically more resistant to killing by complement than F62 (36, 51). As shown in (Fig. 1C), only the D1119G mutant and the wild-type molecule possessed activity against sialylated strain 252. Thus, FHD1119G/Fc represented our lead molecule and was studied further.

FHD1119G/Fc does not cause hemolysis

Having identified the D1119G mutant (FHD1119G/Fc) as the molecule with the best bactericidal activity among the mutants tested, we next asked whether this mutation eliminated toxicity to host cells, as measured by the human RBC lysis assay described by Ferreira et al (37). Complement-mediated lysis of human RBCs was measured in the presence of NHS and FHD1119G/Fc or the control wild type FH18-20/Fc (Fig. 2). Two FHD1119G/Fc chimeric molecules were tested – one that contained mouse IgG2a Fc and a second containing human IgG1 Fc. The latter was developed for use in studies with human PMNs (see below) and in anticipation of possible use as a therapeutic antimicrobial in humans. Fig. 2 shows lysis of RBCs when mouse FH18-20/Fc (WT) protein was added to the reaction mixture (positive control); FHD1119G/Fc proteins (mouse or human Fc) did not cause measurable lysis over baseline levels (controls with buffer alone) at any concentration tested (0 – 66.7 µg/ml). FHD1119G/mouse IgG2a Fc and FHD1119G/human IgG1 Fc exhibited similar bactericidal activities against three different strains of *N. gonorrhoeae* (Supplemental Fig. S1). FHD1119G/human IgG1 Fc was used in subsequent experiments.

Binding of FHD1119G/human IgG1 Fc to *N. gonorrhoeae*

A clinically useful anti-bacterial immunotherapeutic should possess activity against a wide repertoire of clinically relevant strains. We next tested binding of FHD1119G/human IgG1 Fc to 15 clinical isolates of *N. gonorrhoeae* (listed in Supplemental Table S1), including three contemporary ceftriaxone-resistant isolates: CTXr(Sp) (4), H041 (7), and NJ-60 and two isolates with elevated MICs to ceftriaxone (NJ-44 and NJ-48). All strains tested for binding of D1119G/Fc were grown in media supplemented with 2 µg/ml CMP-Neu5Ac to sialylate LOS, as occurs *in vivo* (20, 21). Although binding of FHD1119G/HuFc varied

across strains, it was seen to all sialylated strains that were tested in a flow cytometry assay ($\approx$ 6 to 346-fold fluorescence increase over control values; Fig. 3). Representative histogram tracings from a flow cytometry experiment are shown in Supplemental Fig. S2.

Bactericidal activity of FHD1119G/Fc against *N. gonorrhoeae*

FHD1119G/Fc was tested for complement dependent killing of 15 sialylated clinical isolates of *N. gonorrhoeae* (Fig. 4). Ten of the 15 strains showed from 0% to <50% survival (50%–100% killing) compared to baseline survival (bacteria plus human complement (normal human serum depleted of IgG and IgM) alone) (Fig. 4), while the remaining 5 strains (FA1090, CTXr (Sp), NJ-11, NJ-19 and NJ-26) survived >50% in the presence of FHD1119G/Fc (marked with an “#” in Fig. 4). It is worth noting that the amount of binding of FHD1119G/Fc did not correlate with bacterial killing.

C3 fragment deposition mediated by FHD1119G/Fc on *N. gonorrhoeae* resistant to direct complement-dependent lysis

Complement-dependent opsonophagocytosis may also contribute to clearance of gonococci in humans. We sought to determine whether the 5 isolates that resisted direct killing by complement (mediated by insertion of the membrane attack complex [C5b-9]), also resist deposition of C3. Sialylated bacteria were incubated either with human complement (normal human serum depleted of IgG and IgM) alone, or complement plus FHD1119G/Fc; C3 fragments deposited on bacteria were measured by flow cytometry (Fig. 5). Minimal C3 was deposited on these strains in the presence of complement alone (similar to baseline antibody conjugate control levels). Addition of FHD1119G/Fc markedly increased C3 fragment deposition. Increases ranged from 13- to 88-fold above levels seen with complement alone. These data suggest that resistance of these strains to direct complement-dependent killing may be the result of a block in complement function distal to C3 deposition.

FHD1119G/Fc enhances complement-dependent killing by PMNs

Having shown that FHD1119G/Fc augments C3 deposition on gonococci, we examined whether opsonophagocytic killing by human PMNs was also facilitated. Select gonococcal opacity proteins (Opa) can engage human CEACAMs (38, 52). CEACAM3 that is expressed by human PMNs can facilitate uptake and killing of gonococci through Opa in a complement and FcR independent manner (38). To eliminate Opa-CEACAM3 interactions, we used an Opa-negative derivative of strain FA1090 where all 11 *opa* genes had been inactivated (31). Similar to wild-type FA1090, the isogenic Opa-negative (Opa⁻) mutant of FA1090 also resisted direct complement-dependent killing (>100% survival; data not shown). FHD1119G/Fc increased killing of FA1090 Opa-negative in the presence of active complement and PMNs (Fig. 6). Complement alone or FHD1119G/Fc alone did not facilitate PMN-dependent killing of bacteria, indicating that complement and Fc together were required for opsonophagocytic killing of Opa-negative gonococci by human PMNs *in vitro*.

FHD1119G/Fc decreases the burden and duration of gonococcal infection in the mouse vaginal colonization model

The efficacy of FHD1119G/mouse IgG2a Fc was tested in the mouse vaginal colonization model. The rationale for the use of mouse Fc, intravaginal administration and the concomitant use of CMP-Neu5Ac have been discussed in Materials and Methods. Mice were infected with strain F62 and administered either 12 μ g of FHD1119G/Fc daily intravaginally for 7 days (n=14 mice) or PBS as a vehicle control (n=12). As shown in Fig. 7, the group that received FHD1119G/mouse IgG2a Fc cleared the infection faster (Fig. 7A; median time to clearance was 5 days, versus 7 days in the control group; $P=0.05$; clearance times were compared between groups using a log-rank test). Mixed model analysis indicated significant quantitative differences in gonococcal colonization trends between the two groups comparing FHD1119G/Fc with PBS-treated groups ($P<0.0001$; Fig. 7B). A significant difference in the Mean Areas Under the Curve (mean AUCs) ($\log_{10}$ CFU versus time) between the treated and control groups was also observed ($P=0.011$; Fig. 7C).

Discussion

Resistance of pathogens to many of the currently available antimicrobial agents poses a major threat to human health worldwide. The Centers for Disease Control and Prevention (CDC) has proclaimed that *N. gonorrhoeae* is one of three organisms (together with *Clostridium difficile* and carbapenem-resistant Enterobacteriaceae) where resistance to antimicrobials represents an “urgent threat” to human health (5). The “Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*” recently published by the WHO emphasizes the need for novel approaches to prevent and treat gonorrhea (53). Newer modalities of treatment whose mechanism(s) of action differ from those of conventional agents provide hope that drug-resistance may be deterred when traditional mechanisms of selection are circumvented (3). Sialylation of gonococcal LOS is an important component of gonococcal pathogenesis, which occurs in humans (20, 21) and also during experimental infection of mice (54). Gonococcal mutants that are incapable of LOS sialylation following deletion of the LOS sialyltransferase (*lst*) gene are less virulent in the mouse model of vaginal colonization (54). Sialylation of LOS facilitates evasion of gonococcal killing by the alternative and classical pathways of complement and may also augment bacterial resistance to killing by cationic peptides (55).

We have shown previously that LOS sialylation enhances FH binding through C-terminal domains of FH (24, 27). We have also shown that a chimeric molecule comprising FH domains 18-20 fused to mouse IgG2a Fc mediates complement-dependent killing of sialylated gonococci (27). Killing of gonococci by FH/Fc is classical pathway dependent and occurs at Fc concentrations well below that required to block FH binding to bacteria (27). However, because the C-terminal domains of FH plays a key role in “self–nonself” discrimination (28, 29), the use of a FH18-20/Fc molecule with an unmodified FH has the capacity to bind to human cells and activate complement; this is revealed in our experiments of complement-dependent lysis of anti-CD59-treated RBCs by unmodified FH18-20/Fc (Fig. 2). FH domains 19 and 20 interact with C3 fragments and glycosaminoglycans respectively, to limit complement activation on host cells (28, 29). Therefore, it was

necessary to introduce a mutation in the FH fragment to abrogate toxicity. To achieve this, we capitalized on prior work that characterized select mutations in FH domains 19 and 20 that have been described in individuals with aHUS (37). We focused on four FH mutations that did not interfere with full-length FH's inhibition of lysis of human erythrocytes and selected a mutant, FHD1119G/Fc that showed activity that was comparable to FH18-20/Fc activity against gonococci but did not exhibit complement-dependent lysis of human RBCs.

It is worth noting that although FHD1119G/Fc did not cause direct complement-mediated killing of five of 15 tested isolates (Fig. 4), it enhanced C3 deposition on the five isolates (Fig. 5) and resulted in opsonophagocytic killing of an Opa-negative mutant derived from one of these strains (Fig. 6). The reason(s) for the resistance of these five strains to direct complement-dependent killing despite the observed enhanced C3 deposition is not clear. Possible (and not mutually exclusive) explanations include insufficient C5 convertase formation and/or prevention of effective C5b-9 formation, for example by binding vitronectin (56–59). The relative roles of membrane attack complex-mediated bacterial killing versus opsonophagocytosis in clearance of gonococci *in vivo* remains to be elucidated.

FHD1119G/Fc showed activity against gonococci in the mouse vaginal colonization model and represents a promising initial step in the search for novel therapeutics against gonorrhea that is rapidly becoming multidrug-resistant. We acknowledge that further studies to evaluate the safety of FH/Fc as well as its efficacy against other strains of gonorrhea are necessary.

Notably, Meri et al (60) showed that the D1119G mutation in FH domain 19 did not affect binding to several microbes, including *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Bordetella pertussis*, *Streptococcus pneumoniae* and *Candida albicans*, suggesting that FHD1119G/Fc may also enhance complement activation and possess therapeutic activity against these pathogens. In particular, *P. aeruginosa* and *C. albicans* have been cited by the CDC as microbes where drug-resistance represents a “serious” threat level (5). Activity of FHD1119G/Fc as an adjunctive treatment in these infections merits study.

In this study we have focused on LOS sialylation, a key virulence mechanism of gonococci, to design a novel FH/Fc fusion protein that possesses bactericidal activity (either direct killing by complement or through opsonophagocytosis) against a wide array of gonococcal isolates *in vitro*. In order to develop resistance to this agent, gonococci would have to lose the ability to sialylate LOS and bind to FH. Loss of the ability to sialylate LOS would decrease complement resistance and resistance to cationic peptides, which may diminish bacterial fitness and pose a barrier to the development of drug-resistance, which may not be simply overcome by the traditional microbial mechanisms of “escape mutations” (3). Accordingly, gonococci that lack the ability to sialylate their LOS (*lst* deletion mutants) are out-competed by the parent strain in the mouse vaginal colonization model (54, 61).

Four genes that are involved in core glycan extensions of gonococcal LOS (*lgtA*, *lgtC*, *lgtD* and *lgtG*) possess homopolymeric tracts and therefore are subject to phase variation (62). In some instances, phase variation may lead to loss of expression of the LNT LOS structure

beyond the lactosyl structure that is linked to the core heptose (as an example, *lgtA* “OFF” (62)). LNT is the major sialic acid acceptor and loss of its expression may not be favored *in vivo*. Compelling evidence for the importance of sialylation of LOS *in vivo* was provided by Schneider and colleagues who inoculated two human male volunteers intraurethally with a phase variant of strain MS11 that *in vitro* (before inoculation) expressed only lactose from HepI, which itself does not sialylate. Bacteria recovered from these subjects at the onset of leukorrhoea all reverted to expressing the extended LNT LOS structure (63). In the same study, *N. gonorrhoeae* recovered from 100% of 36 men at the time they sought medical attention expressed the LNT LOS (63). An ongoing analysis of over 60 minimally passaged gonococcal strains recovered recently from subjects attending an STD clinic in Nanjing, China (10 randomly selected isolates were used in this study), has shown that every isolate expresses the LNT LOS, as defined by binding to mAb 3F11 (64). The importance of LOS sialylation in *N. gonorrhoeae* pathogenesis provides confidence to target this important virulence factor for developing therapeutics. Resistance – in this instance, the inability of *N. gonorrhoeae* to sialylate LOS and bind to FH/Fc – would occur at a cost in fitness.

In summary, we have designed a novel FH/Fc fusion protein that shows promising activity both *in vivo* and *in vitro* against diverse *N. gonorrhoeae* isolates. Additional studies to evaluate this molecule against other microbes that bind to a similar region in FH are warranted. These data may provide a novel approach to combat multidrug resistant pathogens that pose a threat to the health of humans worldwide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviation used

FH	factor H
LOS	lipooligosaccharide
WT	wild type
NHS	normal human serum
CMP-Neu5Ac	5'-cytidinemonophospho- <i>N</i> -acetylneuraminic acid
LNT	lacto- <i>N</i> -neotetraose
HBSS	Hanks' balanced salt solution
PMN	polymorphonuclear neutrophil

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Figure 1.

Binding to and bactericidal properties of FH18-20/murine IgG2a Fc and its derivatives containing point mutations in the FH region against *N. gonorrhoeae* of wild-type. **A.** Binding of WT FH18-20/murine IgG2a Fc and mutant proteins to *N. gonorrhoeae* strain F62 grown in media containing CMP-Neu5Ac (2 $\mu\text{g/ml}$) to sialylate its LOS. The D1119G mutation is located in FH domain 19; R1182S, W1183R and R1215G mutations are in FH domain 20. Each FH/Fc protein (10 $\mu\text{g/ml}$) was incubated with sialylated strain F62 and bound FH/Fc was detected by flow cytometry. Numbers alongside each histogram represents the median fluorescence of the entire bacterial population. Control reaction mixtures lack FH/Fc. **B** and **C.** Bactericidal activity of FH18-20/murine IgG2a Fc and mutant proteins directed against sialylated *N. gonorrhoeae*. Sialylated strain F62 (**B**) and sialylated strain 252 (**C**) were each incubated with varying concentrations of each FH/Fc molecule (X-axis), followed by the addition of human complement (normal human serum (NHS) depleted of IgG and IgM). Survival of bacteria at 30 min relative to bacterial counts at the beginning of the assay (t_0 min) is shown on the Y-axis (mean $\pm$ SD of at least 2 independently performed experiments).

Figure 2.

Complement-mediated lysis of anti-CD59 treated RBCs by FH/Fc fusion proteins. Increasing concentrations (indicated on the X-axis) of wild type FH18-20/murine IgG2a Fc or FHD1119G/Fc (either mouse (Mo) IgG2a Fc or human (Hu) IgG1 Fc) were added to anti-CD59-treated human RBCs in GVB/Mg⁺⁺EGTA followed by the addition of homologous NHS to a concentration of 40%. The reaction mixture was then incubated at 37°C for 20 min. Cold GVB/EDTA was added, samples were centrifuged and OD_{410nm} was measured to determine degree of hemolysis (indicated on the Y-axis). Each experiment was performed in duplicate and results indicated; each point represents the mean (SD).

Figure 3.

Binding of FHD1119G/human Fc to strains of sialylated *N. gonorrhoeae*. Binding of FHD1119G/human IgG1 Fc (10 µg/ml) was measured by flow cytometry. The Y-axis represents median fluorescence (mean [SD] of 2 independently performed experiments). 'Control' represents a reaction mixture that lacked FH/Fc. Strains that are resistant to ceftriaxone (MIC >0.25 µg/ml) are indicated by solid black bars, strains that show elevated MICs to ceftriaxone (MICs 0.125 µg/ml and 0.25 µg/ml) by hatched bars and ceftriaxone-sensitive isolates (MICs <0.125 µg/ml) by grey shaded bars.

Figure 4.

Complement-dependent bactericidal activity of FHD1119G/Fc against *N. gonorrhoeae*. Sialylated *N. gonorrhoeae* strains (same strains as in Fig. 4) were incubated with FHD1119G/human IgG1 Fc (33.3 μ g/ml; grey bars) or buffer alone (controls; black bars), followed by the addition of 20% (v/v) human complement for 30 min at 37 °C. Percent survival of bacterial counts at 30 min relative to counts at the beginning of the assay (t_0 min) is shown on the Y-axis (mean $\pm$ SEM of at least 2 separately performed experiments). Strains that resisted killing by FHD1119G/Fc plus complement (>50% survival of CFU at 30 min) are indicated (“#”).

Figure 5.

C3 fragment deposition on 5 sialylated strains that resisted killing by complement: CTXr (Spain), FA1090, NJ-11, NJ-19 and NJ-26 (strains indicated by “#” in Figure 4). Sialylated strains were incubated with FHD1119G/human IgG1 Fc (33.3 $\mu\text{g}/\text{ml}$) and 10% (v/v) human complement. Sialylated F62 served as a positive control. C3 fragments (C3b/iC3b) deposited on bacteria in the presence of FHD1119G/Fc were detected by flow cytometry (histograms shown with a solid black line). C3 fragment deposition on bacteria incubated with complement alone shown by the grey shaded histogram and antibody conjugate controls (bacteria plus anti-C3c FITC) by the dashed histogram. The number next to each histogram represents median fluorescence. One representative experiment of two reproducible repeat experiments is shown.

Figure 6.

Opsonophagocytic killing of the sialylated Opa-negative mutant of *N. gonorrhoeae* FA1090 by FHD1119G/human Fc and complement. Opa-negative FA1090 grown in media containing CMP-Neu5Ac to sialylate LOS (10^7 CFU) was incubated with FHD1119G/human Fc (FHD1119G/HuFc; 16.7 $\mu\text{g}/\text{ml}$) and 10% (v/v) human complement, followed by the addition of 10^6 freshly isolated human PMNs for 60 min at 37 °C (MOI 10:1). Bacterial survival at 60 min relative to t_0 is shown on the Y-axis (mean ($\pm$ SD) of 4 independently performed experiments). Controls included reactions where complement was heat-inactivated (indicated by “-” in the “Active complement” row), or where FHD1119G/Fc was omitted. *, $P < 0.05$; **, $P < 0.01$ (ANOVA).

Figure 7.

FHD1119G/mouse IgG2a Fc reduces the duration and burden of gonococcal infection in the murine vaginal model of gonococcal colonization. Two groups of Premarin-treated wild-type BALB/c mice were infected with 1.5×10^6 CFU of *N. gonorrhoeae* strain F62 and given either 12 μ g FHD1119G/mouse IgG2a Fc (n=14) or a corresponding volume of PBS (n=12) as a vehicle control, daily for the duration of the experiment. Each mouse also received 10 μ g of CMP-Neu5Ac daily as described in the Materials and Methods. Vaginal swabs were obtained daily to quantify *Ng* CFUs. **A.** Kaplan-Meier analysis of time to clearance. **B.** Colonization of bacteria (log₁₀ CFU) measured daily. **C.** Bacterial burdens consolidated over time (Area Under the Curve [log₁₀ CFU] analysis) for the two groups.