Fusion Protein Comprising Factor H Domains 6 and 7 and Human IgG1 Fc as an Antibacterial Immunotherapeutic

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The emergence of antimicrobial resistance among several medically important pathogens represents a serious threat to human health globally and necessitates the development of novel therapeutics. Complement forms a key arm of innate immune defenses against invading pathogens. A mechanism of complement evasion employed by many pathogens is binding of complement inhibitors, including factor H (FH), a key downregulator of the alternative pathway. Most FH-binding bacteria engage FH through regions in FH spanned by domains 6 and 7 and/or 18 through 20. We created a chimeric protein that comprised human FH domains 6 and 7 fused to human IgG1 Fc (FH6,7/HuFc) and tested its activity as an immunotherapeutic against *Neisseria meningitidis*, which binds FH through domains 6 and 7. FH6,7/HuFc bound to meningococci and effectively blocked FH binding to bacteria. FH6,7/HuFc enhanced human C3 and C4 deposition and facilitated complement-mediated killing in a dose-responsive manner; complement activation and killing were classical pathway dependent. To investigate *in vivo* efficacy, infant Wistar rats were treated intraperitoneally (IP) with different doses of FH6,7/HuFc and challenged 2 h later with serogroup C strain 4243 given IP. At 8 to 9 h after the challenge, the FH6,7/HuFc-treated rats had >100-fold fewer CFU per ml of blood than control animals pretreated with phosphate-buffered saline (PBS) or FH18–20/HuFc, which does not bind to meningococci (*P* < 0.0001). These data provide proof of concept of the utility of FH/Fc fusion proteins as anti-infective immunotherapeutics. Because many microbes share a common binding region(s) in FH, FH/Fc chimeric proteins may be a promising candidate for adjunctive therapy against drug-resistant pathogens.

Soon after the introduction of antibiotics in modern medicine, it became evident that several bacteria had the remarkable ability to rapidly develop resistance to antimicrobials. We have now reached an era where certain infections have become resistant to all conventional antibiotics currently in clinical use (1). There is an urgent need to develop novel anti-infective agents.

The complement system forms an important arm of innate immune defenses against many microbes (2, 3). Activation of complement by one or more of the complement pathways on the microbial surface leads to deposition of C3 fragments, which "marks" these invading pathogens for uptake by professional phagocytes through complement receptors such as CR3. Opsonophagocytosis is also facilitated by interactions between the Fc domain of antibody (Ab) bound to the surface of microbes with Fc receptors (FcRs) on phagocytes. Activation of the terminal complement pathway on Gram-negative organisms may lead to insertion of the membrane attack complex (C5b-9), which can mediate bacterial killing, into the bacterial membrane.

Pathogens have evolved several mechanisms to subvert complement activation on their surfaces. Binding to host complement inhibitors such as factor H (FH), C4b-binding protein (C4BP), and vitronectin constitutes a mechanism of complement escape (4, 5). FH is a key inhibitor of the alternative pathway (AP) of complement. FH comprises 20 domains (also called short consensus repeats [SCRs]) arranged like a string of beads (6, 7). The N-terminal four domains of FH are necessary and sufficient for complement inhibition (8–10). The remaining domains are involved in binding to surfaces and for spatial conformation of the molecule (6, 11–14). Most microbes that bind human FH do so through domains 6 and 7 and/or the C-terminal domains, 18, 19, and 20 (summarized in Table S1 in the supplemental material).

Because the microbial binding domains in FH are distinct from

its complement-inhibiting domains, we explored the utility of fusing the microbial binding domains of FH with Fc to create a novel anti-infective immunotherapeutic. Our hypothesis was that the Fc portion of the bound fragment would engage C1q, activate the classical complement pathway, and confer protection against invasive infection. Conceivably, the fragment also would block binding of FH, which would increase susceptibility of the bacteria to an alternative pathway. In this study, we have provided proof of principle for the activity of a chimeric molecule that comprises FH domains 6 and 7 with human IgG1 Fc against *Neisseria meningitidis*, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial strains. *N. meningitidis* strains H44/76 (B:15:P1.7,16:ST-32) (15) and 4243 (C:2a:P1.5,2:ST-11) (16) were used in this study.

Construction of FH6,7/human Fc (FH6,7/HuFc) and FH18–20/HuFc. Overlap extension PCR was used to create DNA encoding FH domains 6 and 7 fused to human IgG1 Fc, as follows. All primers are listed in Table S2 in the supplemental material. FH domains 6 and 7 were amplified by PCR using primers FH6EcoRI and HuIgG1overlapR using a plasmid previously created to express FH6,7/mouse IgG2a Fc (17). Hu-
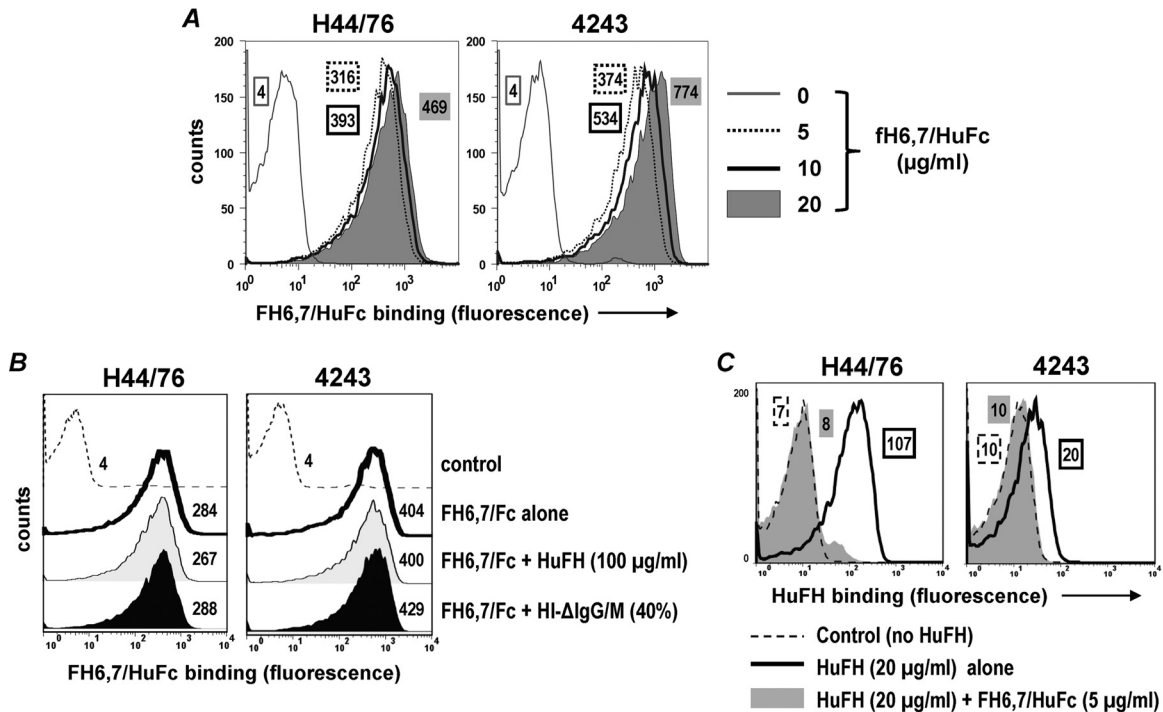
FIG 1 Binding of FH6,7/HuFc to meningococci. (A) *N. meningitidis* strains H44/76 (left) and 4243 (right) were incubated with increasing concentrations of FH6,7/HuFc, and binding was measured by flow cytometry. Numbers in boxes (boxes are shaded or outlined to correspond to the relevant graph) indicate the median fluorescence of the entire bacterial population. Fluorescence is on a $\log_{10}$ scale. (B) FH6,7/HuFc binds to meningococci in the presence of pure human FH and human serum. Strains H44/76 and 4243 were incubated with FH6,7/HuFc (10 µg/ml) in either the absence or presence of increasing concentrations (3 µg/ml to 100 µg/ml) of purified human FH or heat-inactivated IgG- and IgM-depleted normal human serum (HI-ΔIgG/M; 5% to 40%, containing ~25 to 200 µg/ml of FH). Bound FH6,7/HuFc was detected by flow cytometry with anti-human IgG FITC. For simplicity, only data obtained with the highest concentrations of FH or serum are shown. Controls were bacteria incubated with anti-human IgG FITC. Numbers alongside each histogram are as described for panel A. One representative experiment of two reproducible repeats is shown. (C) FH6,7/HuFc can inhibit binding of human FH to meningococci. Strains H44/76 and 4243 were incubated with purified human FH (20 µg/ml) in either the absence or the presence of increasing concentrations (5 µg/ml to 40 µg/ml) of FH6,7/HuFc. FH bound to bacteria was detected by flow cytometry with anti-FH MAb followed by anti-mouse IgG FITC (specific for the Fab fragment). For simplicity, only data obtained with the lowest FH6,7/HuFc concentration tested are shown. Controls were reaction mixtures that lacked human FH (but contained FH6,7/HuFc, anti-FH, and the FITC conjugate). Numbers alongside each histogram are as described for panel A. Results of one representative experiment of two reproducible repeats are shown.

man IgG1 Fc was amplified from pFUSE-hIgG1-Fc2 with primers HuIgG1overlapF and HuIgG1NheI-R. The two PCR products were then fused together by PCR with flanking primers FH6EcoRI and HuIgG1NheI-R. Similarly, FH18−20/HuFc was created by amplifying domains 18 to 20 with primers FH18EcoRI and HuIgG1NheI-R from a plasmid used to create FH18−20/mouse IgG2a Fc (18). Human IgG1 Fc was amplified as described above, and the two PCR fragments were ligated together with primers FH18EcoRI and HuIgG1NheI-R. The final PCR products were digested with EcoRI and NheI and cloned into the corresponding sites in pFUSE-hIgG1-fc2, and the plasmid sequences were verified by DNA sequencing. CHO cells were transfected using Lipofectin (Life Technologies), and the FH/HuFc fusion proteins were purified from cell culture supernatants over protein A-agarose as described previously (17, 18).

IgG- and IgM-depleted human serum. Human serum was obtained by phlebotomy from normal healthy adult volunteers in accordance with a protocol approved by the University of Massachusetts Institutional Review Board for the protection of human subjects. Serum was obtained by allowing blood to clot at 22°C for 30 min followed by centrifugation at 1,500 × g for 20 min at 4°C. To study the effects of FH6,7/HuFc without confounding by anti-meningococcal Ab present in human serum, we depleted IgG and IgM from freshly collected human serum, as described previously (19). Briefly, EDTA (final concentration, 10 mM) and NaCl (final concentration, 1 M) were added to freshly prepared human serum and passed first over anti-human IgM agarose (Sigma) that had been

equilibrated in phosphate-buffered saline (PBS)–10 mM EDTA–1 M NaCl, followed by passage through protein G-Sepharose equilibrated in the same buffer. NaCl was added to minimize C1q depletion during passage of serum through the anti-human IgM column. The flowthrough was collected, spin concentrated, and dialyzed to its original volume using a 10-kDa-cutoff Amicon Ultra-15 centrifugal filter device (Millipore) against PBS–0.1 mM EDTA, sterilized by passage through a 0.22-µm filter (Millipore), and stored at −70°C. Hemolytic activity was measured, and serum was aliquoted and stored as described earlier. Depletion of IgG and IgM was confirmed by dot blot assays. In some experiments, complement activity of serum was destroyed by heating serum at 56°C for 1 h. C1q-depleted serum was from Complement Technology, Inc. (Tyler, TX). IgG and IgM present in C1q-depleted serum were removed as described above; the only exception was that the addition of NaCl to prevent C1q loss was not necessary.

Infant rat complement. Infant rat pups were sacrificed at 8 to 9 days of age by cardiac puncture to prepare pooled complement as described previously (20). The infant rat protection was performed in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (36). The protocol was approved by the Children's Hospital & Research Center at Oakland Institutional Animal Care and Use Committee.

Antibodies and purified complement proteins. Anti-human C3c conjugated to fluorescein isothiocyanate (FITC) (Abcam) and anti-human Ig-FITC (Sigma) were used in flow cytometry assays. Human FH
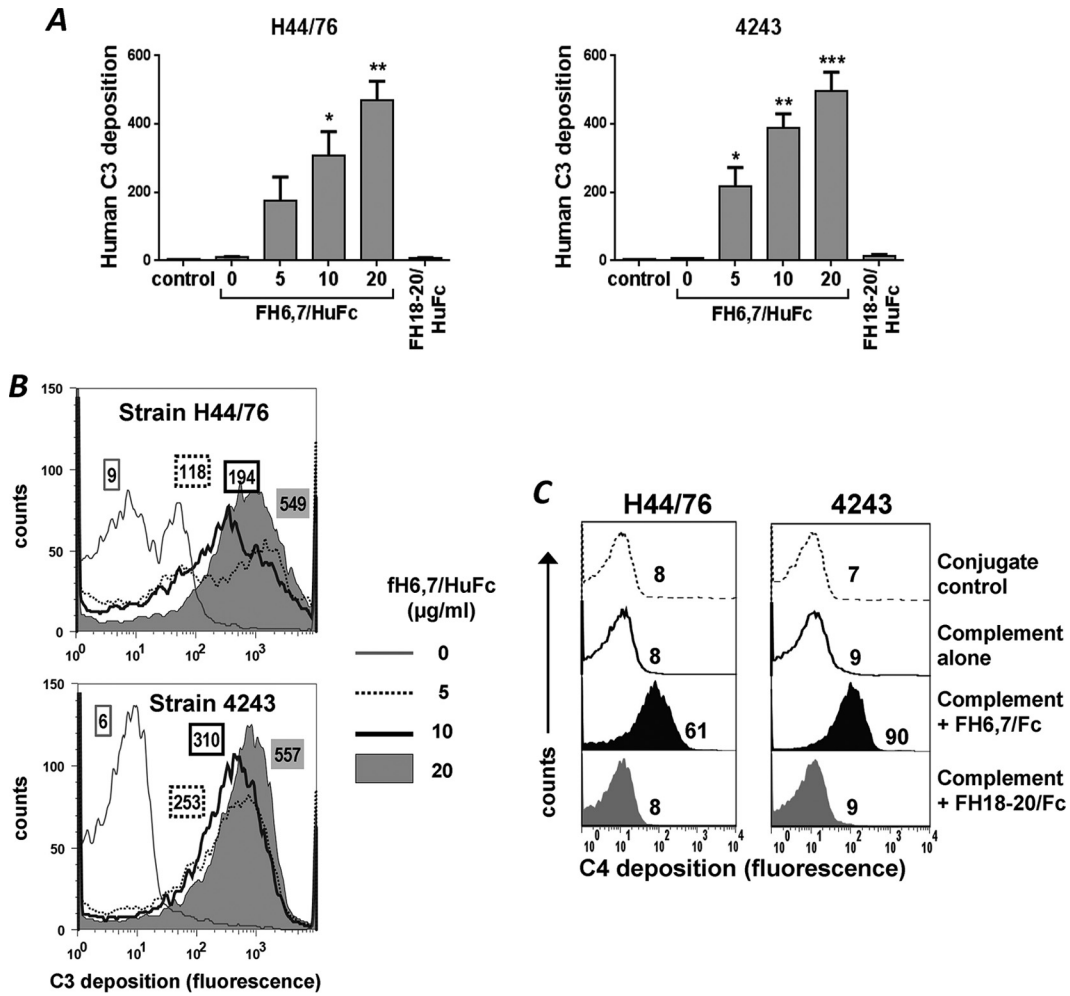
FIG 2 FH6,7/HuFc enhances C3 and C4 fragment deposition on meningococci. Strains H44/76 and 4243 were incubated with human complement (normal human serum depleted of IgG and IgM) in the absence or presence of increasing amounts of FH6,7/HuFc, and the amount of C3 deposited on bacteria (median fluorescence) was measured by flow cytometry. FH18–20/HuFc (20 µg/ml) was used as a negative control. (A) Data are means and standard errors of the means (SEM) from 3 separate experiments. (B) Histogram tracings from one representative experiment. For simplicity, a representative histogram with negative-control protein FH18–20/HuFc is shown in Fig. S3 in the supplemental material. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, determined by analysis of variance (ANOVA) compared to C3 deposition mediated by complement alone (0 µg/ml FH6,7/HuFc). (C) FH6,7/HuFc activates the classical pathway on meningococci. Bacteria were incubated with human complement in the absence or the presence of FH6,7/HuFc or negative-control protein FH18–20/HuFc (both at 20 µg/ml), and C4 fragment deposition was measured by flow cytometry. Results of one representative experiment of two reproducible repeats are shown. The numbers alongside the histograms in panel B are as described for Fig. 1.

bound to bacteria was detected with an anti-human FH monoclonal antibody (MAb) (catalog no. A254 [anti-human FH MAb 2]; Quidel) followed by anti-mouse IgG FITC specific for mouse Fab (Sigma). Rat C3 deposition on bacteria was detected with goat anti-guinea pig C3 (MP Biomedical/Cappel)-FITC, which reacts with rat C3, as described previously (20). Human C4 deposited on bacteria was measured with polyclonal chicken anti-human C4 biotin (Cedarlane Laboratories) followed by Extravidin-FITC (Sigma). Antibodies against group C capsule and MAb P1.2 against the PorA expressed by strain 4243 were used as positive controls for protection in the infant rat challenge assay. MAb against factor Bb (catalog no. A227; Quidel) was added to serum at a concentration of 100 µg/ml to block the alternative pathway of complement (21). Purified C1q and purified human FH were from Complement Technology, Inc. (Tyler, TX).

Flow cytometry. Binding of FH6,7/HuFc and C3 fragments deposited on bacteria was measured by flow cytometry as described previously (17, 20). All reactions were carried out in Hanks balanced salt solution (HBSS) containing 2 mM CaCl$_2$ and MgCl$_2$ and 0.1% bovine

serum albumin (HBSS^{++}/BSA) in a final volume of 50 µl. Antibodies were used at a dilution of 1:200. Data were collected on a Becton Dickinson (BD) FACSCalibur system and analyzed using FlowJo (Tree Star, Inc.). Binding is shown as histogram tracings and quantified as median fluorescence.

Serum bactericidal assay. Susceptibility of meningococci to killing by FH6,7/Fc in the presence of human complement (20% [vol/vol]) was determined as described previously (22). Survival was calculated as the number of viable colonies at 30 min relative to the number at time zero.

Infant rat protection assay. *N. meningitidis* 4243 was grown (37°C in 5% CO$_2$) to early log phase in Mueller-Hinton broth (BD, Franklin Lakes, NJ) supplemented with 0.02 mM CMP-*N*-acetylneuraminic acid (CMP-NANA; Sigma) and 0.25% glucose. The bacteria were pelleted, washed, and suspended in phosphate-buffered saline (PBS)-BSA. Infant Wistar rats, 5 to 6 days old, were treated intraperitoneally (IP) with PBS alone ($n = 20$), 150 µg of FH6,7/Fc ($n = 15$), 45 µg of FH6,7/Fc ($n = 15$), 45 µg of FH18–20/Fc ($n = 15$), 10 µg of anti-group C capsule MAb ($n = 10$), or 10 µg of anti-PorA MAb 1.2 ($n = 10$). Two hours later, the animals were
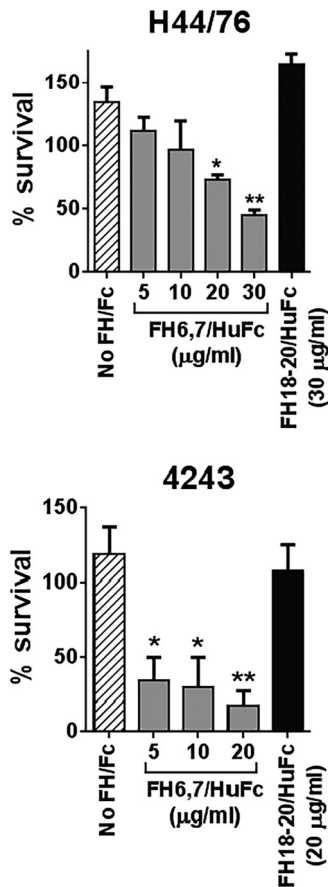
H44/76

4243

FIG 3 FH6,7/HuFc enhances complement-dependent killing of *N. meningitidis*. Bacteria were incubated with 20% complement (IgG/IgM-depleted serum) alone or with complement plus increasing concentration of FH6,7/HuFc, and percent survival at 30 min (relative to counts at 0 min) was measured in a serum bactericidal assay. The means and SEM from 3 independent experiments are shown. *, $P < 0.05$, and **, $P < 0.01$ (ANOVA), compared to the value for bacteria plus complement alone (0 μg/ml FH6,7/HuFc).

challenged IP with 100 μl of a suspension containing 730 CFU of bacteria. Quantitative blood cultures were obtained between 8 h and 9 h after the bacterial challenge as previously described (23). Data are presented as CFU per 100 μl of blood.

Statistical analyses. Comparisons across multiple groups were carried out using one-way analysis of variance (ANOVA) with Tukey's posttest. Comparisons of the geometric mean CFU counts of bacteria per ml of blood between the groups of animals that received FH6,7/Fc and each of the control groups were done using the Mann-Whitney test. The lower limit of detection for bacteremia in blood cultures was 1 CFU/100 μl; for calculations of geometric means, sterile blood cultures were assigned a value of half of the lower limit (i.e., <1 CFU/100 μl was assigned a value of 0.5 CFU/ml).

RESULTS

Binding of FH6,7/HuFc to *N. meningitidis*. Studies to date have identified three molecules expressed by *N. meningitidis* that can bind to human FH through domains 6 and 7, namely, factor H binding protein (FHbp), NspA and PorB2 (17, 22, 24, 25). We have previously demonstrated binding of a chimeric protein comprising human FH domains 6 and 7 fused to mouse IgG2a Fc to *N. meningitidis* strains H44/76 and 4243 (17). As the first step in the development of FH6,7/Fc as an immunotherapeutic for humans, we replaced mouse IgG2a Fc with human IgG1 Fc and showed that

FH6,7/HuFc bound to *N. meningitidis* H44/76 and 4243 by flow cytometry (Fig. 1A). 4243 bound larger amounts of FH6,7/Fc than H44/76. The strain difference likely reflects the fact that in addition to FHbp and NspA, which can serve as ligands for FH domains 6 and 7 on both strains, PorB from strain 4243, but not PorB from H44/76, also binds FH through domains 6 and 7 (17). Binding of FH18–20/HuFc (negative control) is shown in Fig. S1 in the supplemental material.

The ability of FH6,7/HuFc to bind to bacteria in the presence of increasing concentrations of human FH (3 μg/ml to 100 μg/ml) or human serum (5% to 40%) as a second source of FH was studied next. IgG/IgM-depleted serum was used to permit detection of the human Fc fragment of the chimeric protein without interference by IgG in human serum. Further, the serum was heat inactivated to prevent deposition of the C3 fragment, which in turn could bind to FH domains 6 and 7 (9, 26) and confound results. FH6,7/HuFc bound to bacteria in an uninhibited manner even in the presence of the highest concentrations of FH or serum tested (Fig. 1B). We next asked whether FH6,7/HuFc could block binding of human FH to meningococci. Bacteria were incubated with human FH (20 μg/ml) in the presence of FH6,7/HuFc (concentrations ranging from 5 μg/ml to 40 μg/ml), and FH binding to bacteria was measured by flow cytometry. As shown in Fig. 1C, FH binding to both strains was abrogated even by the lowest concentration (5 μg/ml) of FH6,7/HuFc tested. In contrast, FH18–20/HuFc even at 20 μg/ml did not impede FH binding (see Fig. S2 in the supplemental material). Note that in the absence of the inhibitor, 4243 binds less FH than H44/76, which likely reflects the fact that 4243 is a relatively low expresser of FHbp, as measured by flow cytometry (27). Collectively, these data show that FH6,7/HuFc binds to bacteria even in the presence of high concentrations of FH and can effectively block FH binding to the organism surface.

FH6,7/HuFc enhances C3 and C4 fragment deposition on *N. meningitidis*. To demonstrate the ability of FH6,7/HuFc to activate complement on the bacterial surface, we incubated live bacteria from strains H44/76 and 4243 with complement (human serum depleted of IgG and IgM; final concentration, 20%) alone or with 20% complement in the presence of increasing concentrations (5, 10, and 20 μg/ml) of FH6,7/HuFc and measured C3 fragment deposition on bacteria by flow cytometry (Fig. 2A and B). FH6,7/HuFc increased C3 deposition on both strains in a dose-dependent manner. As expected, FH18–20/HuFc that did not bind to meningococci did not increase C3 deposition on either strain (see Fig. S3 in the supplemental material). Enhanced C4 fragment deposition provided evidence for the ability of FH6,7/HuFc to activate the classical pathway on meningococci (Fig. 2C).

FH6,7/HuFc enhances human complement-dependent killing of *N. meningitidis*. The ability of antibodies against *N. meningitidis* to protect against invasive infection correlates with their ability to mediate complement-dependent killing in a serum bactericidal assay (28). We next studied the ability of FH6,7/HuFc to mediate complement-dependent killing of meningococci in a serum bactericidal assay using 20% human complement as the complement source. FH6,7/HuFc mediated killing of both strains, although H44/76 was more resistant than 4243 (Fig. 3). Consistent with its inability to bind to meningococci and enhance C3 fragment deposition, the control protein, FH18–20/HuFc, did not mediate killing of either strain.

The bactericidal activity of FH6,7/HuFc requires the classical pathway of complement. To determine the complement path-
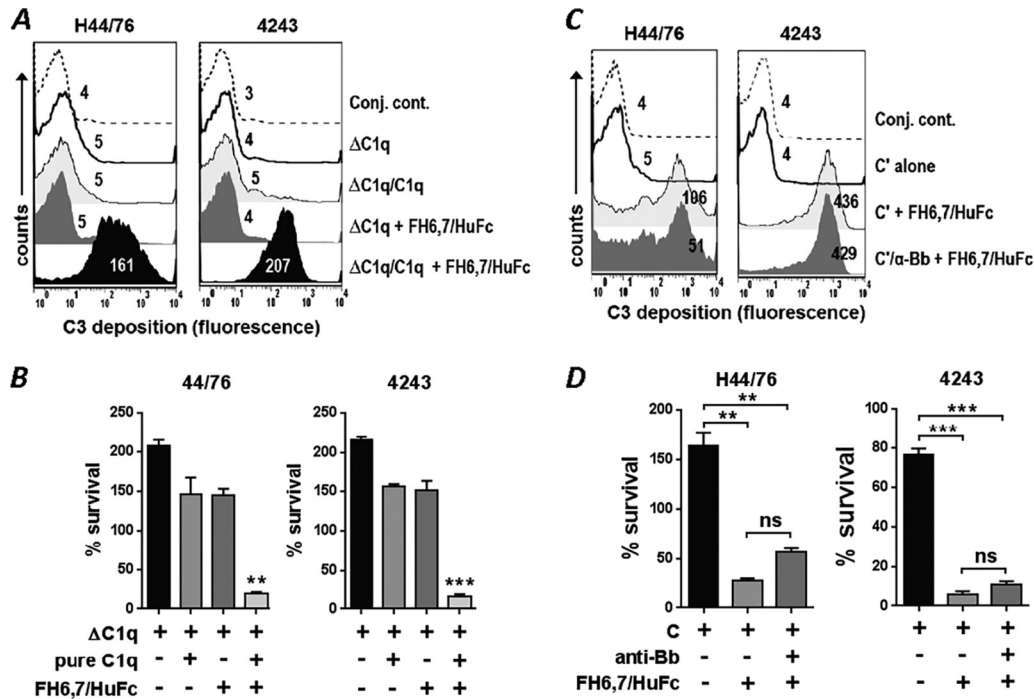
FIG 4 The classical pathway of complement is required for C3 deposition on and killing of meningococci by FH6,7/HuFc. (A) C3 deposition on bacteria is abrogated in the absence of C1q. Strains H44/76 and 4243 were incubated with C1q-depleted serum (ΔC1q) or C1q-depleted serum reconstituted with 70 μg/ml pure C1q (ΔC1q/C1q) either in the absence or in the presence of FH6,7/HuFc (20 μg/ml). C3 deposited on bacteria was measured by flow cytometry. Numbers accompanying each histogram represent the median fluorescence on the entire bacterial population. Results of one representative experiment of two reproducible repeats is shown. (B) The classical pathway is essential for complement-dependent killing of meningococci by FH6,7/HuFc. Serum bactericidal assays were performed with strains H44/76 and 4243 using C1q-depleted serum or C1q-depleted serum reconstituted with C1q, in either the absence or presence of FH6,7/HuFc (30 μg/ml for H44/76 and 20 μg/ml for 4243). Percent survival of bacteria at 30 min relative to 0 min is shown. The means and ranges from two experiments are shown. **, $P < 0.01$, and ***, $P < 0.001$, compared to the other three corresponding groups (ANOVA with Tukey's posttest). (C) Alternative pathway activation plays a limited role in FH6,7/HuFc-mediated C3 deposition on meningococci. H44/76 and 4243 were incubated with complement alone (IgG- and IgM-depleted human serum [C′]), complement plus FH6,7/HuFc, or complement plus anti-factor Bb plus FH6,7/HuFc (20 μg/ml), and the amount of C3 deposited on bacteria was measured by flow cytometry. Results of one representative experiment of two reproducible repeats are shown. Results of the control experiment demonstrating the ability of the anti-Bb MAb to block C3 deposition on yeast glucan particles are shown in Fig. S4 in the supplemental material. (D) The alternative pathway of complement contributes minimally to killing of meningococci by FH6,7/HuFc. Serum bactericidal assays were performed with H44/76 and 4243 using the conditions described for panel C. Data are means and ranges from two experiments. **, $P < 0.01$; ***, $P < 0.001$ (ANOVA with Tukey's posttest).

way(s) needed for complement activation on and killing of meningococci by FH6,7/HuFc, bacteria were incubated with C1q-depleted serum (also depleted of IgG and IgM) that lacked classical pathway activity but possessed intact lectin and alternative pathways. As shown in Fig. 4A, there was barely any C3 deposited on both strains when FH6,7/HuFc was added to C1q-depleted serum. C3 deposition was restored when C1q-depleted serum was reconstituted with physiological concentrations of C1q (~70 μg/ml). As expected, in the absence of FH6,7/HuFc, there was barely any C3 deposited on bacteria incubated with C1q-depleted serum or with C1q-depleted serum reconstituted with purified C1q. Accordingly, FH6,7/HuFc did not mediate any killing in the presence of C1q-depleted serum; killing by FH6,7/HuFc was fully restored upon reconstitution with purified C1q (Fig. 4B).

We next blocked the alternative pathway in complement (IgG/IgM-depleted human serum) using a MAb against factor Bb. As a control, we used yeast glucan particles, a potent activator of the alternative pathway (29) to verify that the anti-Bb could block C3 deposition (see Fig. S4 in the supplemental material). Blocking the alternative pathway resulted in a small decrease in the median fluorescence of C3 deposition on strain H44/76 (Fig. 4C, left) but

had no effect on C3 deposition on 4243 (Fig. 4C, right). Accordingly, serum bactericidal assays showed a small increase in survival of H44/76 when the alternative pathway was blocked (Fig. 4D, left). Killing of 4243 proceeded in an unimpeded manner even when the alternative pathway was blocked (Fig. 4D, right).

Collectively, the data above yield the following conclusions: (i) C3 deposition and killing require activation of the classical pathway through Fc (evidenced by minimal C3 deposition and killing mediated by sera lacking IgG/IgM with or without C1q), (ii) the alternative and lectin pathways (in the absence of the classical pathway) activate complement very poorly on meningococci (shown by the ineffectiveness of IgG/IgM-depleted serum, with or without C1q, where the alternative and lectin pathways are intact in depositing C3 or killing bacteria), and (iii) blockade of FH binding by FH6,7/HuFc in the absence of classical pathway activation is insufficient for complement activation on and killing of wild-type meningococci (evidenced by the ineffectiveness of FH6,7/HuFc in depositing C3 and mediating killing in C1q/IgG/IgM-depleted serum).

FH6,7/HuFc protects infant rats against meningococcal bacteremia. We next asked whether FH6,7/HuFc could facilitate
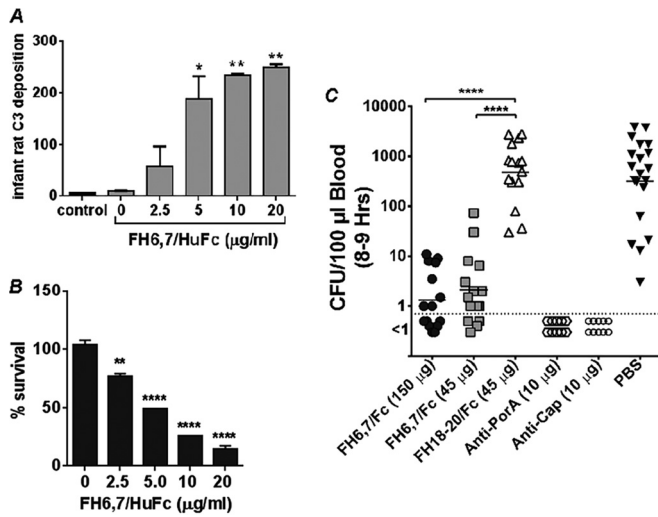
FIG 5 FH6,7/HuFc activates rat complement on *N. meningitidis* and protects infant rats against meningococcal bacteremia. (A) FH6,7/HuFc enhances rat C3 deposition on *N. meningitidis*. Strain 4243 was incubated with 20% infant rat complement alone or in the presence of increasing concentrations of FH6,7/HuFc, as indicated. Following 30 min of incubation at 37°C, rat C3 deposited on bacteria was measured by flow cytometry. The median fluorescence was recorded, and each bar represents the mean and range from two separate experiments. *, $P < 0.05$, and **, $P < 0.01$, compared to C3 deposition in the absence of added FH6,7/HuFc (ANOVA). (B) FH6,7/HuFc enhances killing of strain 4243 by infant rat complement. *N. meningitidis* strain 4243 was incubated with infant rat complement (20%), either alone or in the presence of increasing concentrations of FH6,7/HuFc, and survival at 30 min was measured in a serum bactericidal assay. Data are the means and ranges from two independent observations. **, $P < 0.01$; ****, $P < 0.0001$. (C) FH6,7/HuFc decreases meningococcal bacteremia in the infant rat model. Infant rats (5 to 6 days old) were treated with PBS alone ($n = 20$), with one of two doses of FH6,7/HuFc (150 μg/rat or 45 μg/rat; $n = 15$ in each group), or with FH18–20/HuFc (45 μg/rat; $n = 15$), which does not bind to the bacteria. Positive controls included groups of rats given an anti-group C capsule Ab (10 μg/rat; $n = 10$) or anti-PorA MAb P1.2 (10 μg/rat; $n = 10$). Two hours later, the animals were challenged IP with 730 CFU of serogroup C strain 4243. Rats were sacrificed between 8 and 9 h postinfection, and the CFU per 100 μl of blood were quantified. The horizontal dotted line indicates the limit of detection of the assay (1 CFU/100 μl). Statistical comparisons between groups were made with the Mann-Whitney test. ****, $P < 0.0001$.

clearance of *N. meningitidis* in the infant rat model of meningococcal bacteremia. We chose strain 4243 for this experiment because it can readily cause bacteremia in wild-type infant rats (27). As a prelude to the experiments *in vivo*, we asked whether FH6,7/HuFc could enhance activation of infant rat complement on 4243. As shown in Fig. 5A and B, FH6,7/HuFc enhanced deposition of rat C3 and killing of 4243, respectively, in a dose-dependent manner when incubated with infant rat complement.

We next challenged infant rats intraperitoneally with 4243; the rats were administered FH6,7/HuFc at doses of 150 μg ($n = 15$) or 45 μg ($n = 15$) per rat 2 h before the bacterial challenge. Negative controls (uninhibited bacterial proliferation) for this experiment included rats given PBS alone ($n = 20$) and rats given FH18–20/HuFc ($n = 15$), which does not bind to C3, increase C3 deposition, or mediate killing of meningococci (data not shown). Positive controls (protection against bacteremia) included animals given either anti-group C capsular Ab or anti-PorA P1.2 MAb (10 rats in each group). Both doses of FH6,7/Fc significantly decreased the burden of bacteremia compared to burdens in the negative-con-

trol groups given FH18–20/Fc or PBS (Fig. 5C). Collectively, the data show that FH6,7/HuFc can activate complement on meningococci and protect infant rats against bacteremia.

DISCUSSION

In this study, we show that a chimeric molecule comprising human FH domains 6 and 7 fused to human IgG1 Fc can bind to meningococci, enhance complement deposition, mediate direct killing through complement, and protect infant rats against meningococcal bacteremia. These data provide proof of principle for the development of FH/Fc chimeric proteins that fuse different microbial binding domains of FH with Fc as adjunctive immunotherapeutics against microbial infections.

Several medically important microbes bind to human FH to subvert complement (listed in Table S1 in the supplemental material). It is worth noting that many of these pathogens engage FH through regions spanned by domains 6 and 7 or domains 18 through 20. Pathogens may have evolved the ability to recognize patterns in these two regions of FH, which permits them to bind to FH and downregulate complement. Microbes that bind to domains 6 and 7 may also bind to an alternatively spliced variant of FH called factor H-like protein 1 (FHL-1), which comprises FH domains 1 through 7 plus four unique C-terminal amino acids and possesses complement-inhibiting activity (30). Drug resistance remains a major problem with microbes such as *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, and *Candida albicans* (1), all of which are reported to bind FH (see Table S1 in the supplemental material). In some instances, infections caused by these organisms are difficult to eradicate despite appropriate antimicrobial treatment. Novel immunotherapeutics such as the FH/Fc chimeric molecule described here (FH6,7/HuFc) and/or domains 18 to 20 fused to Fc may serve as useful adjuncts to more conventional antimicrobial agents. An advantage of such an adjunctive immunotherapeutic approach is that resistance to FH/HuFc will be accompanied by an inability of the organism to bind to FH; loss of this important virulence mechanism would result in decreased microbial fitness and therefore is less likely to occur.

The bactericidal activity of FH6,7/HuFc requires Fc-initiated classical-pathway activation. These data are consistent with previous work showing that activation of the classical pathway of complement is required for killing of the pathogenic neisseriae (31–34). An important finding in this study was that in the absence of a functional classical pathway, blocking FH binding alone by FH6,7/HuFc (i.e., an uninhibited alternative pathway) was insufficient to mediate C3 deposition or killing of wild-type meningococci. It is worth noting that FH6,7/HuFc was more bactericidal against 4243 than against H44/76. FHbp and NspA are ligands for FH on both H44/76 and 4243; however, in addition to these two FH ligands, 4243 PorB2 also binds to FH through domains 6 and 7 (17), which was reflected by the higher binding of FH6,7/HuFc to 4243 than to strain H44/76 (Fig. 1). PorB2 is one of the most abundantly expressed meningococcal surface proteins (35), and it is reasonable to posit that binding of FH6,7/HuFc to PorB2 will result in efficient complement activation. Accordingly, the higher binding of FH6,7/HuFc to 4243 may have resulted in kinetically overwhelming classical pathway activation that alone was sufficient to kill 4243 (no increase in survival when the alternative pathway was blocked), whereas recruitment of the alternative pathway contributed to a small extent to the killing of H44/76 by

FH6,7/HuFc (29% increase in survival upon adding anti-factor Bb to block the alternative pathway [Fig. 4D, left]).

On a molar basis, specific MAbs directed against PorA and capsule were more effective than FH6,7/HuFc in preventing bacteremia. This is not surprising, because these high-affinity Abs are directed against abundantly expressed surface antigens, and their intact nature (as opposed to that of a chimeric Fc fusion molecule) likely results in very efficient complement activation. However, while specific Abs have a relatively narrow specificity (for example, against specific groups in the case of anti-capsule Abs or specific serosubtypes in the case of anti-PorA Abs), FH/Fc fusion proteins not only have the potential to bind to diverse meningococcal strains but also may be useful to combat a wide array of pathogens (listed in Table S1 in the supplemental material). Thus, such pattern recognition chimeric Fc-bearing molecules may prove useful in treating infections prior to establishing a specific etiologic diagnosis. Further, given the diversity of surface antigens even across strains within a species, raising specific Abs to each strain or subset of strains within a species may not represent a practical therapeutic approach.

In conclusion, this study provides proof of principle of the utility of an FH/Fc fusion protein as an adjunctive anti-infective immunotherapeutic. Studies to assess the efficacy of FH/Fc fusion proteins such as FH6,7/HuFc and FH18–20/HuFc against other microbes—in particular, multidrug-resistant pathogens—are warranted.

ACKNOWLEDGMENTS

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