Enhanced Bacteremia in Human Factor H Transgenic Rats Infected by *Neisseria meningitidis*

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Neisseria meningitidis binds the complement downregulating protein, factor H (fH), which enables the organism to evade host defenses. Two fH ligands, fHbp and NspA, are known to bind specifically to human fH. We developed a human fH transgenic infant rat model to investigate the effect of human fH on meningococcal bacteremia. At 18 h after intraperitoneal challenge with 560 CFU of group B strain H44/76, all 19 human fH-positive rats had positive blood cultures compared to 0 of 7 human fH-negative control littermates ($P < 0.0001$). Human fH-positive infant rats also developed bacteremia after challenge with isogenic mutants of H44/76 in which genes encoding fHbp and NspA (Δ fHbp Δ NspA mutant) or the lipooligosaccharide sialyltransferase (Δ lst mutant) had been inactivated. A fully encapsulated Δ fHbp Δ NspA Δ lst mutant unable to sialylate lipooligosaccharide or bind human fH via the known fH ligands did not cause bacteremia, which argued against global susceptibility to bacteremia resulting from random integration of the transgene into the rat genome. *In vitro*, the wild-type and Δ fHbp Δ NspA mutant strains were killed by as little as 20% wild-type infant rat serum. The addition of 3 μ g of human fH/ml permitted survival of the wild-type strain in up to 60% infant rat serum, whereas ≥ 33 μ g of human fH/ml was required to rescue the Δ fHbp Δ NspA mutant. The ability of meningococci lacking expression of fHbp and NspA to cause invasive disease in human fH transgenic rats and to survive in wild-type infant rat serum supplemented with human fH indicates an additional human fH-dependent mechanism of evasion of innate immunity.

The Gram-negative bacterium *Neisseria meningitidis* causes epidemic meningitis and sepsis in sub-Saharan Africa and also is an important cause of invasive disease in industrialized countries. In 2006, two studies demonstrated that *N. meningitidis* bound the complement-downregulating molecule, factor H (fH) (21, 27). Binding of fH to the bacterial surface decreased deposition of C3b and increased the survival of the organism in human serum (21). To date, two meningococcal fH-binding ligands have been identified: factor H binding protein (fHbp) (21) and neisserial surface protein A (NspA) (19). Both ligands are specific for binding human fH only (10, 19).

The hallmark of meningococcal disease is invasion and proliferation of the bacteria in the bloodstream. For more than a decade, the infant rat model has been used to investigate meningococcal bacteremia (26, 32, 33) and to measure the ability of antibodies to confer passive protective activity against meningococcal bacteremia (12, 30, 35). For reasons that are unclear, not all meningococcal strains that cause invasive disease in humans cause bacteremia in the rat model. We describe here the development of a human fH transgenic rat line that permitted investigation of bacteremia caused by a meningococcal strain that is invasive in humans but that is rapidly cleared from the bloodstream in wild-type rats. We used this model to investigate the role of binding of human fH to fHbp and NspA on meningococcal bacteremia.

MATERIALS AND METHODS

Generation of human fH transgenic rats. Wistar rat embryos (Charles River, Wilmington, MA) were microinjected with an ~6-kb transgenic cassette, which contained a cytomegalovirus enhancer, chicken β -actin promoter, the full-length cDNA encoding human fH, and a rabbit β -globin poly(A) sequence that had been isolated and purified from plasmid pCAGGS-human fH (3). Microinjected embryos were implanted into pseudopregnant adult Wistar rats at the University of Massachusetts Transgenic Core facility. Blood sampling of the resulting F₀ founder gen-

eration was performed at 4 weeks of age, and serum samples from individual rats were screened by Western blotting with polyclonal goat antibody to purified human fH (24). The goat anti-human fH antibody used to detect the presence of human fH had been shown previously to react strongly with fH from humans and other primates but minimally with fH from rats. The presence of the human fH cDNA in genomic DNA isolated from the tails of animals was confirmed by PCR. The positive animals were then mated with wild-type Wistar rats, which resulted in F₁ generation rats. Subsequent generations were produced by sibling-to-sibling crosses of rats that were positive for the expression of human fH by Western blotting or enzyme-linked immunosorbent assay (ELISA) (see below). The rats in the present study were offspring of F₂- to F₄-generation sibling-sibling crosses. The protocols for developing the human fH transgenic rats and for performing the meningococcal challenge experiments described below were approved by the Institution Animal Care and Use Committees of the University of Massachusetts and Children's Hospital Oakland Research Institute, respectively.

Measurement of human fH. Measurement of human fH concentrations in rat sera were performed using a recombinant fHbp capture ELISA, which was specific for human fH and performed as previously described (3). Bound human fH was detected using polyclonal sheep antibody to human fH (Complement Technologies Inc., Tyler, TX), followed by alkaline-phosphatase-conjugated donkey anti-sheep IgG (Sigma, St. Louis, MO). The fH capture assay had a lower limit of detec-

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TABLE 1 Mutants of *N. meningitidis* group B strain H44/76

Strain	Genotype ^a	Phenotype
Wild type	Wild type	B:15:P1.7,16, ST-32, and fHbp ID 1 ^b
ΔfHbp mutant	<i>fHbp::Erm</i>	fHbp not expressed
ΔfHbp ΔNspA mutant	<i>fHbp::Erm; nspA::Spc</i>	fHbp and NspA not expressed
Δ <i>lst</i> mutant	<i>lst::Kan</i>	α-2,3-Sialyltransferase (<i>lst</i>) interrupted, prevents terminal LOS sialylation
ΔfHbp ΔNspA Δ <i>lst</i> mutant	<i>fHbp::Erm; nspA::Spc; lst::Kan</i>	fHbp, NspA, and α-2,3-sialyltransferase not expressed

^a Erm, erythromycin resistance; Spc, spectinomycin resistance; Kan, kanamycin resistance.

^b Capsular group, PorB serotype, and PorA variable region type, respectively. ST, multilocus sequence type. The fHbp identification numbers are the same as those designated on the website (<http://pubmlst.org/neisseria/>).

tion of 12 μg of human fH/ml. Human serum samples used as positive controls were obtained from participants of a protocol approved by the Institutional Review Board of Children's Hospital and Research Center Oakland.

Measurement of rat fH. Rat fH was measured in serum samples by an anti-fH capture ELISA specific for rodent fH. In brief, wells of a microtiter plate were incubated at 4°C overnight with 25 μg of a mouse anti-mouse fH monoclonal antibody (MAB)/ml that cross-reacted with rat fH (MAB 1A2; Hycult Biotech, Netherlands). After washing and blocking the samples with 5% milk in phosphate-buffered saline (PBS), dilutions of pooled sera from wild-type ($n = 3$ animals) or human fH transgenic ($n = 3$ animals) rats were added to the wells, followed by incubation overnight at 4°C. Bound rat fH was detected with a rabbit polyclonal antiserum to mouse fH that cross-reacted with rat fH (provided by Wenchao Song, University of Pennsylvania School of Medicine), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). As a control for the specificity of detecting rodent fH, dilutions of a human serum or purified human fH were negative in the assay (see Results).

Measurement of complement activity. The pooled sera from the wild-type or human fH-positive transgenic rats were assayed for total hemolytic complement activity by measuring the absorbance (that is, the optical density at 415 nm) of oxyhemoglobin released from hemolyzed commercially prepared sensitized sheep erythrocytes (EZ Complement Cells; Diamedix, Miami, FL) after 1 h of incubation with test sera as the complement source. As a control, a commercially prepared human serum sample was used as a comparator (Diamedix). We also assessed alternative complement pathway activation by the wild-type or human fH-positive transgenic serum pools by measuring C3b deposition on zymosan in the presence or absence of Mg²⁺ and EGTA (1, 2). In brief, 15% (vol/vol) of each serum pool was incubated at room temperature for 15 min with 0.1 mg of zymosan in each of three buffers: (i) PBS buffer containing Ca²⁺ and Mg²⁺ (Dulbecco PBS; MediaTech, Manassas, VA) without chelator (both classical and alternative complement pathways active), (ii) PBS without Ca²⁺ but supplemented with 10 mM EGTA plus 10 mM MgCl₂ (final concentration) to test alternative complement activation only, and as a negative control, (iii) PBS without Ca²⁺ or Mg²⁺ supplemented with 10 mM EDTA to inactivate both the classical and the alternative complement pathways. After the zymosan particles were washed, C3b deposition was detected by flow cytometry using a mouse MAb to rat C3 (MAB 12E2; Abcam, Cambridge, MA), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen).

***Neisseria meningitidis* strains.** Group B strain H44/76-SL (B:15:P1.7,16; sequence type 32 [ST-32]) (4), referred to hereafter as H44/76, was used as the test strain. This strain is a relatively high expresser of fHbp (36) and a relatively low expresser of NspA (19, 22). This strain was chosen for the present study because in previous unpublished experiments by the authors it failed to cause sustained bacteremia in wild-type infant rats challenged intraperitoneally (i.p.) with inocula as high as 10⁵ CFU/rat. Other investigators have reported bacteremia in wild-type infant rats after i.p. challenge with 10⁶ CFU/rat coadministered with diluted brain heart infusion broth (31). To investigate the role of bacterial expression of fHbp and NspA on bacteremia, we used isogenic mutants of strain H44/76 in which genes encoding either fHbp (H44/76 ΔfHbp) or both fHbp and

NspA (H44/76 ΔfHbp ΔNspA) were inactivated (19). As a comparator, we also investigated bacteremia caused by a mutant strain (H44/76 Δ*lst*), which binds fH through fHbp and NspA but lacks the *lst* gene that encodes for an α-2,3-sialyltransferase. This enzyme is required for terminal sialylation of neisserial lipo-oligosaccharide (LOS) that expresses lacto-*N*-neotetraose (L3,7,9 immunotypes) or P^K-like (L1 immunotype) LOS species (33). To decrease complement resistance of the ΔfHbp ΔNspA mutant further, we also tested a triple-knockout mutant whose terminal LOS sialylation was inactivated and fH binding to fHbp and NspA were also knocked out (H44/76 ΔfHbp ΔNspA Δ*lst*, Table 1).

Infant rat bacteremia model. *N. meningitidis* were grown to early log phase in Mueller-Hinton broth (BD, Franklin Lakes, NJ) supplemented with 0.02 mM cytidine-5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA; Sigma) and 0.25% glucose. The bacteria were washed once in PBS containing 1% (wt/vol) bovine serum albumin (PBS-BSA; Equitech-Bio, Kerrville, TX), centrifuged, and resuspended in PBS-BSA. As described in Results, in different experiments, rats aged 5 to 7 days or 8 to 10 days were challenged i.p. with 100 μl of a suspension containing ~5 × 10² to 5 × 10³ CFU of bacteria. Quantitative blood cultures were obtained at 6 or 18 h, which were performed as previously described (34, 35).

In vitro survival of bacteria in rat serum. We determined the survival of *N. meningitidis* incubated in different concentrations of a serum pool taken from 8- to 9-day-old wild-type Wistar rats (Charles River). Bacteria were grown as described above for the animal challenge model, and washed and resuspended in PBS-BSA containing Ca²⁺ and Mg²⁺ (Dulbecco PBS, MediaTech, Manassas, VA). Pooled rat serum (final concentration of 20, 40, or 60%) was added to wells of a microtiter plate (Nunc-clon Δ Surface; Thermo Fisher Scientific, Rochester, NY). Purified human fH (Complement Technologies, Inc.) was added to the wells at a final concentration of 3, 10, 33, or 100 μg/ml. Approximately 400 CFU of bacteria were added to each well, and the microtiter plates were incubated for 60 min at 37°C in 5% CO₂ with agitation using a Clay Adams Nutator mixer (BD Biosciences, Bedford, MA). At time zero and 60 min, 10-μl aliquots from each well were dripped onto chocolate agar plates. Colony counts were ascertained after incubation overnight, and the percent survival was determined by comparing CFU at T_{60} compared to that at T_0 .

Binding of human fH to *N. meningitidis*. Binding of human fH to live bacterial cells was measured by flow cytometry, which was performed as previously described (9). The control MABs included anti-PorA P1.7 (product number 01/514; National Institute for Biological Standards and Control, Potters Bar, United Kingdom), anti-fHbp JAR 3 (37), and anti-NspA MAB 14C7 (22).

Statistical analyses. Statistical calculations were performed using Prism for Mac version 5.0a (GraphPad Software, La Jolla, CA). All probability values reported are two tailed. Comparisons of the proportions of rats that developed bacteremia at different time points or with different mutant strains were performed by using the Fisher exact test. Comparisons of the geometric mean CFU of bacteria per ml of blood in multiple groups of rats were performed by analysis of variance (ANOVA) on the log₁₀-transformed CFU/ml values. Pairwise comparisons between two groups used a Student *t* test. The lower limit of detection for bacteremia in blood cultures was 10 CFU/ml; for calculations of geometric means, sterile blood cultures were assigned a value of half of the lower limit (i.e., <10

FIG 1 Construction of human fH transgenic rat line. (A) PCR confirmation of the presence of the human fH cDNA in genomic DNA isolated from rat tails. Lane 1, amplification product (232 bp) from genomic DNA extracted from a human factor H transgenic rat; lane 2, amplification product from genomic DNA extracted from a wild-type rat; and lane 3, amplification product from a plasmid containing human factor H cDNA. (B) Serum human factor H concentrations measured in infant rats, aged 3 to 4 days by a capture ELISA. Open circles, human fH-positive rats; gray-filled circles, human fH-negative rats ($<12 \mu\text{g}$ of human fH/ml); open triangles, fH concentrations in control sera from healthy adult humans. The mean human fH concentration in the positive rat sera was lower than that in sera of healthy human adults ($P < 0.0001$). (C) Serum rat fH measured using a capture ELISA specific for rodent fH. Open circles, solid line, pooled sera from three human fH-positive transgenic rats. Crosses with dashed line, pooled sera from three wild-type rats. Open boxes, dotted line, pooled sera from three human adults.

CFU/ml was assigned a value of 5 CFU/ml). Nonparametric correlation coefficients were determined by the Spearman test.

RESULTS

Generation of human fH transgenic rats. We generated human fH transgenic rats by microinjection of embryos with a transgene containing human fH cDNA (Fig. 1A). By Western blotting with a polyclonal goat antibody against human fH, the sera from the transgenic rats showed a band at ~ 150 kDa that corresponded to full-length human fH (data not shown). Figure 1B shows the human fH concentrations in individual sera from 3- to 4-day-old littermates that resulted from F_1 sibling-sibling crosses. Sera from wild-type rats (without the transgene) had no detectable human fH ($<12 \mu\text{g}/\text{ml}$) by ELISA (data not shown); sera from transgenic rat littermates with human fH measurements below $12 \mu\text{g}/\text{ml}$ were considered human fH negative (Fig. 1B, gray-filled circles). Human fH concentrations in positive rats (Fig. 1B, open circles) ranged from 116 to $389 \mu\text{g}/\text{ml}$. The mean $\pm$ two standard errors (2SE) in the positive rats was 226 ± 34 , which was lower than in six

FIG 2 Meningococcal bacteremia in human fH transgenic rats (experiment 1). Six- to seven-day-old animals were challenged i.p. with 560 CFU of group B strain H44/76. Blood cultures were obtained from the human-fH positive rats at 6 or 18 h after challenge (individual animals were bled once). For human fH-negative animals, cultures were obtained only at 18 h because of insufficient numbers of animals. Open circles, CFU/ml of human fH-positive animals; gray-filled circles, CFU/ml of human fH-negative animals. Dotted line indicates the lower limit of detection for positive blood cultures (10 CFU/ml). At 6 h, the geometric mean (horizontal solid line) of the CFU/ml of blood was 394, which increased to 1.2×10^5 CFU/ml at 18 h ($P < 0.0001$). At 18 h, none of the human fH-negative animals had positive cultures of blood (0 of 7 versus 19 of 19 human fH-positive animals at 18 h, $P < 0.0001$ [Fisher exact test]).

adult humans whose serum samples were assayed in parallel (open triangles, mean $\pm$ 2SE, 412 ± 72 , $P < 0.0001$). Based on a capture ELISA specific for rodent fH, the endogenous rat fH concentrations were similar in sera from 2-month-old human fH-positive transgenic rats and wild-type rats (Fig. 1C). The respective sera also had similar total hemolytic activity (see Fig. S1A in the supplemental material) and elicited similar C3b deposition on zymosan in the presence of Ca^{2+} and Mg^{2+} (classical and alternative pathway [see Fig. S1B in the supplemental material]) or Mg^{2+} -EGTA (only alternative complement pathway active [see Fig. S1C in the supplemental material]). Thus, in the human fH transgenic rats, expression of human factor H did not appear to decrease rat fH concentrations or compromise serum complement functional activity.

Human fH-positive rats have enhanced meningococcal bacteremia. In experiment 1 (Fig. 2), infant rats aged 6 to 7 days were inoculated i.p. with 560 CFU of group B strain H44/76. Blood cultures were obtained at 6 or 18 h (individual animals were bled once). At 6 h, all of the human fH-positive animals tested had positive blood cultures (geometric mean, 3.9×10^2 CFU/ml). At 18 h, bacteremia had increased to a geometric mean of 1.2×10^5 CFU/ml ($P < 0.0001$). In contrast, none of the seven human fH-negative control animals tested at 18 h had positive blood cultures (0 of 7 versus 19 of 19 fH-positive animals, $P < 0.0001$ [Fisher exact test]). The human fH-negative rats were not tested for bacteremia at 6 h because of insufficient numbers of available animals.

We conducted three additional infant rat challenge experiments. A graphic display of the levels of bacteremia in individual animals in each experiment is shown in Fig. 3. The respective descriptive statistics of the proportions of animals in each group with positive blood cultures and the group geometric means of the CFU/ml are summarized in Table 2.

The effects of human fH on bacteremia 6 h after challenge were replicated as part of experiment 3, which was performed in 8- to 10-day-old infant rats: all 17 human fH-positive rats challenged with the wild-type H44/76 strain were bacteremic at 6 h compared

FIG 3 Meningococcal bacteremia in human fH transgenic rats after i.p. challenge of infant rats (experiments 2, 3, and 4). Rats were challenged with a wild-type (WT) strain of H44/76 or isogenic knockout mutants. Blood cultures were obtained at 18 h (experiment 2) or 6 h after the challenges (experiments 3 and 4). The ages of rats at the time of challenge in each experiment and the CFU/rat used for the challenges are summarized in Table 2. Gray-filled circles, CFU/ml of individual human fH-negative rats (experiments 2 and 3 only); open symbols, CFU/ml of individual human fH-positive rats; horizontal solid lines, geometric means of the CFU/ml of the respective groups; horizontal dotted line, limit of detection of bacteremia. For a more detailed description of the statistical analyses of each experiment, see Table 2.

to 1 of 15 human fH-negative animals (the first two rows in experiment 3, Table 2, $P < 0.0001$). In experiments 1 and 3, blood samples were obtained 2 days prior to the bacterial challenge to measure serum human fH concentrations. Among fH-positive animals, the serum concentrations of human fH did not correlate with the level of bacteremia ($r = 0.2$ and 0.4 , $P > 0.2$).

Previous studies identified two human-specific fH ligands in *N. meningitidis*: fHbp (21) and NspA (19). In experiment 2, we challenged groups of human fH-positive transgenic rats with 2,800 CFU of the wild-type H44/76 strain or an isogenic Δ fHbp mutant strain. At 18 h, all of the animals were bacteremic, and there was no significant difference in the geometric mean CFU/ml of blood between the groups given the wild-type or mutant (Table 2). The ability of the Δ fHbp mutant to cause bacteremia in the

human fH transgenic rats was unexpected given the importance of binding of fH to fHbp for survival of strain H44/76 in human serum (21, 36). In experiment 3, therefore, we repeated the challenge experiment in human fH transgenic rats with the H44/76 Δ fHbp mutant and included a second group of rats challenged with an isogenic H44/76 mutant in which both known meningococcal human fH ligands were deleted (Δ fHbp Δ NspA). At 6 h, all 37 rats challenged with either mutant had bacteremia, and there was no significant difference between the geometric means of the CFU/ml of the two groups challenged with mutant strains (experiment 3, Table 2, $P = 0.5$). These geometric means, however, were 2- to 3-fold lower than that of the human fH-positive rats challenged with the H44/76 wild-type strain ($P < 0.05$ by ANOVA). In experiment 4, we confirmed the ability of the Δ fHbp Δ NspA mutant to cause bacteremia in human fH transgenic infant rats (Table 2).

The ability of the Δ fHbp Δ NspA mutant to cause bacteremia in the fH transgenic rat suggested an additional fH-dependent mechanism of survival by strain H44/76. Alternatively, the virulence of this double mutant could be indicative of an unintended disruption of overall protective immunity against bacterial invasion in the transgenic rats caused by random integration of the human fH transgene into the rat genome. To address the latter possibility, we challenged human fH-positive rats with a group B encapsulated *lst* knockout derivative, which was prepared from the H44/76 Δ fHbp Δ NspA mutant strain. In addition to not binding fH to fHbp or NspA, the H44/76 Δ fHbp Δ NspA Δ *lst* mutant was unable to sialylate LOS. We reasoned that the ability of the human fH-positive transgenic rats to resist bacteremia by an encapsulated derivative of the Δ fHbp Δ NspA mutant whose serum resistance may have been attenuated further by the absence of LOS sialylation would serve as a sensitive indicator of the overall robustness of the transgenic rat to resist invasive meningococcal disease. As a control, we also challenged human fH-positive rats with a Δ *lst* mutant of strain H44/76, which was unable to sialylate LOS but still permitted fH binding through fHbp and NspA. Both mutants (the Δ fHbp Δ NspA Δ *lst* mutant and the Δ *lst* mutant) showed growth kinetics in broth culture similar to those of the parent wild-type strain (data not shown). In human fH-positive rats, 15 of 16 animals challenged with the Δ *lst* mutant were bacteremic at 6 h (experiment 4, Table 2). In contrast, all 15 rats challenged with the fully encapsulated Δ fHbp Δ NspA Δ *lst* mutant had sterile blood cultures. These findings argued against a global susceptibility to bacterial invasion caused by random insertion of the human fH transgene into the rat genome.

The addition of human fH to wild-type rat serum enhances *in vitro* survival of meningococci. Susceptibility of human fH-positive rats to bacteremia caused by the H44/76 Δ fHbp Δ NspA mutant suggested that a separate human fH-dependent immune evasion mechanism existed in addition to binding of human fH to fHbp and/or NspA. To investigate this possibility, we modeled the *in vivo* experiments by measuring the effect of adding human fH to a serum pool from 8- to 9-day-old wild-type rats on survival of the wild-type H44/76 strain. The results were compared to survival of the isogenic mutant of H44/76 in which genes encoding both fHbp and NspA had been deleted. In the absence of added human fH, both the wild-type and the mutant strains were killed after 60 min of incubation in a serum concentration-dependent manner (Fig. 4, crosses with solid lines). In the case of the wild-type strain (Fig. 4A), the addition of as little as 3 μ g of human fH/ml (closed

TABLE 2 Bacteremia after i.p. challenge of human fH transgenic infant rats

Expt (age of rats)	Serum human fH ^a	H44/76 strain	Challenge dose (CFU/rat)	No. of rats	Bacteremia ^b			
					Time after challenge (h)	No. positive (%)	GM CFU/ml	95% CI
1 (6 to 7 days)	Positive	Wild type	560	18	6	18 (100)	394	178–873
	Negative	Wild type	560	7	18	0 (0) ^A	<10	NA
	Positive	Wild type	560	19		19 (100) ^B	120,000	22,000–680,000
2 (5 to 7 days)	Negative	Wild type	2,800	2	18	0 (0)	<10	NA
	Positive	Wild type	2,800	10		10 (100)	3,614 ^A	217–60,000
	Positive	Δ fHbp mutant	2,800	9		9 (100)	3,311 ^B	129–85,000
3 (8 to 10 days)	Negative	Wild type	5,800	15	6	1 (7)	<10 ^A	<10
	Positive	Wild type	5,800	17		17 (100)	2,065 ^B	893–4,775
	Positive	Δ fHbp mutant	4,900	20		20 (100)	612 ^C	325–1,153
	Positive	Δ fHbp Δ NspA mutant	3,100	17		17 (100)	849 ^D	422–1,710
4 (8 to 9 days)	Positive	Δ fHbp Δ NspA mutant	1,800	16	6	16 (100)	532 ^A	213–1,326
	Positive	Δ fHbp Δ NspA Δ lst mutant	2,000	15		0 (0)	<10	NA
	Positive	Δ lst mutant	3,100	16		15 (94)	105 ^B	41–271

^a Transgenic littermates negative for human fH (<12 μ g/ml) or positive for human fH (79 to 831 μ g/ml) were used.

^b GM, geometric mean; CI, confidence interval. NA, not applicable (all animals in the group had negative blood cultures). *P* values were determined as follows by comparing various values (indicated by superscript capital letters for each experiment). Experiment 1: A versus B, *P* < 0.0001 (Fisher exact test). Experiment 2: A versus B, *P* > 0.9 (Student *t* test). Experiment 3: A, one animal had bacteremia of 80 CFU/ml; B, C, and D, *P* < 0.05 (three-way ANOVA), with pairwise comparisons for B versus C, *P* < 0.02; B versus D, *P* = 0.09; and C versus D, *P* = 0.5 (Student *t* test). Experiment 4: A versus B, *P* = 0.014.

circles with dotted line) enhanced the survival of the organism in up to 60% rat serum, the highest concentration tested. The survival of the Δ fHbp Δ NspA double-knockout mutant also was enhanced by the addition of human fH (Fig. 4B). The dose of human fH required (≥ 33 μ g/ml, gray-filled squares with solid line) was 10-fold higher than that required for survival of the wild-type H44/76 strain. Although the addition of human fH enabled the Δ fHbp Δ NspA mutant to survive in wild-type infant rat serum, by flow cytometry there was no detectable binding of human fH to live bacteria of the Δ fHbp Δ NspA mutant when incubated with 60% wild-type infant rat serum and 100 μ g of human fH/ml (Fig. 5C). As a positive control, the mutant and wild-type strains showed similar binding with an anti-PorA MAbs (Fig. 5A and B). As expected, the mutant showed no binding with MAbs to NspA or fHbp.

FIG 4 Effect of the addition of human fH on survival of H44/76 bacteria after 60 min of incubation with pooled sera from 8- to 9-day-old wild-type rats. (A) Wild-type strain; (B), Δ fHbp Δ NspA mutant. Exogenous human fH concentrations: X's with solid line, 0 μ g/ml; closed circles with dotted line, 3 μ g/ml; open triangles with dashed line, 10 μ g/ml; gray-filled boxes with solid line, 33 μ g/ml; open circles with dashed line, 100 μ g/ml. The data points represent median values from triplicate measurements. Error bars represent the range from three replicate measurements (in cases where two of the three respective results were identical, the bars extend in only one direction, above or below the median value). Similar results were obtained in a separate independent experiment (not shown).

Effect of LOS sialic acid on survival of mutant H44/76 bacteria in infant rat serum supplemented with human fH. In separate experiments, the Δ lst mutant had less survival in 40 or 60% wild-type infant rat serum supplemented with 100 μ g of human fH/ml than the wild-type H44/76 strain (Fig. 6A). The Δ fHbp Δ NspA Δ lst triple-knockout mutant showed even less survival under these conditions. In contrast, survival of Δ fHbp Δ NspA double mutant in wild-type infant rat serum supplemented with 100 μ g of human fH/ml was indistinguishable from that of the wild-type strain (Fig. 6B).

DISCUSSION

In the present study we developed an animal model to investigate the role of human fH on meningococcal bacteremia in relation to expression of fHbp and NspA. In previously described meningococcal animal models that utilized wild-type mice or rats, bacteremia was limited by lack of human specific interactions of meningococci with host molecules. These molecules included scavenging of iron via human transferrin binding protein (28), crossing the human blood-brain barrier via human CD46 (14, 15), interactions with human T cells or dendritic cells via Opa (18), and evasion of complement activation via binding human fH (21, 27). In wild-type mice, the i.p. coadministration of mucin and hemoglobin along with high meningococcal challenge doses were required for bacteremia (5). Although human CD46 transgenic mice were more susceptible to developing meningococcal meningitis than control mice (15), and human transferrin transgenic mice (38) developed higher levels of meningococcal bacteremia than control mice that did not express the respective transgene, both of these transgenic models required challenges by high numbers of bacteria ($>10^6$ CFU/mouse) to produce disease. These data suggested that, in mice, replacement of a single human-specific mechanism for crossing the blood-brain barrier or inva-

FIG 5 Binding of human fH on the surfaces of live meningococci as measured by flow cytometry. (A and B) Binding of control MABs. (A) H44/76 wild-type strain. Bacteria alone, gray-shaded area; anti-porin P1.7 MAB, thick black line; anti-fHbp MAB JAR 3, dashed black line; anti-NspA MAB 14C7, dark gray line. (B) H44/76 Δ fHbp Δ NspA double-knockout mutant. Symbols are as defined for panel A. (C) Binding of human fH to live bacteria. Wild-type H44/76 strain with 60% infant rat serum and 100 μ g of human fH/ml, dark gray line; Δ fHbp Δ NspA double-knockout mutant with 60% infant rat serum and 100 μ g of human fH/ml, black line; wild-type or Δ fHbp Δ NspA double-knockout mutant without human fH and without infant rat serum; dashed line and shaded gray areas, respectively.

sion of the bloodstream was insufficient to mimic susceptibility of humans to meningococcal disease.

For reasons that are not understood, wild-type infant rats are more susceptible than mice to developing bacteremia caused by some strains of meningococci (12, 13, 22, 35, 37). Not all meningococcal strains that cause invasive disease in humans, however, cause bacteremia in infant rats. For example, group B strains in the ST-32 complex such as MC58 or H44/76 required i.p. challenge doses of $>10^6$ CFU (30–32). In the present study, wild-type infant rats challenged with ~ 560 CFU of group B strain H44/76 cleared the organism from the bloodstream within 18 h after challenge, whereas human fH infant transgenic rats developed high levels of bacteremia (Fig. 2). The addition of as little as 3 μ g of human fH/ml to 60% wild-type infant rat serum also enabled the bacteria to survive *in vitro*. These data suggested that the mechanism of resistance of wild-type infant rats to bacteremia by this strain is related to lack of binding of rat fH. The lack of a significant correlation between serum human factor H concentrations in the human fH-positive rats and magnitude of bacteremia can be ex-

plained by the low concentrations of human fH (i.e., as little as 3 μ g/ml) required for survival of the organism in wild-type infant rat serum, which were exceeded even in the transgenic rats with the lowest serum concentrations of human fH (≥ 79 μ g/ml).

As described in the introduction, two meningococcal ligands for binding of human fH have been identified: fHbp (21) and NspA (19). Of the two, fHbp appeared to be the more important in strain H44/76 because in a previous study binding of fH to the bacterial surface as measured by flow cytometry was markedly decreased in a fHbp knockout mutant of H44/76 (H44/76 Δ fHbp) (7) and was not significantly decreased in an isogenic NspA knockout mutant (H44/76 Δ NspA) (9). An unexpected finding in the present study was the ability of both the H44/76 Δ fHbp mutant and the H44/76 Δ fHbp Δ NspA double-knockout mutant to cause bacteremia in human fH transgenic rats (Table 2 and Fig. 3). Although these mutants were not tested for their ability to cause bacteremia in wild-type infant rats, we can infer that, similar to the wild-type strain, the mutants would have been avirulent in the rat model based on rapid *in vitro* killing by as little as 20% serum from wild-type infant rats (Fig. 4).

We were concerned that random insertion of the human fH transgene into the rat genome might have resulted in a global increase in susceptibility to bacterial invasion. Two lines of evidence argued against this possibility. First, the Δ lst mutant, which was unable to sialylate LOS, showed lower bacteremia in the model than the wild-type strain (experiment 4, Table 2 and Fig. 3). Further, deleting *lst* in the background of the Δ fHbp Δ NspA mutant resulted in complete loss of virulence in the human fH transgenic rats. These data suggested that the ability of the wild-type strain and other mutants to cause bacteremia was not a result of complete lack of innate immunity.

The triple Δ fHbp Δ NspA Δ lst mutant also had lower *in vitro* survival than the Δ fHbp Δ NspA mutant in wild-type rat serum supplemented with 100 μ g of human fH/ml (Fig. 6, compare panels A and B). While LOS sialic acid has not been reported to contribute to the amount of human fH that binds directly to H44/76 (21, 27), at higher human fH concentrations LOS sialic acid may contribute to serum resistance by enhancing the affinity of fH for surface-bound C3b (17). C3b and C4b are deposited on *Neisseria* LOS (8, 20) and sialylation may also limit the number of available targets for C3b/C4b.

The ability of the Δ fHbp Δ NspA double-knockout mutant to

FIG 6 Effect of sialylated LOS on survival of *N. meningitidis* in wild-type infant rat serum supplemented with human fH. Rat serum pool was from 8- to 9-day-old animals. Human fH was added to a final concentration of 100 μ g/ml to each dilution of rat serum. (A) Open bars, H44/76 wild-type strain; Gray bars, Δ lst isogenic mutant that lacks a gene required for terminal sialylation of LOS; diagonal hatched bars, Δ fHbp Δ NspA Δ lst triple-knockout mutant that, in addition to not binding fH to fHbp or NspA, is unable to sialylate LOS. (B) Open bars, H44/76 wild-type strain; horizontal hatched bars, Δ fHbp Δ NspA double mutant. The data in panels A and B are from separate experiments, each replicated two or three times. In panels A and B, bars represent median values from triplicate measurements in an individual experiment. Error bars represent ranges.

cause bacteremia in human fH-transgenic rats and to survive in wild-type infant rat serum supplemented with human fH implied the existence of a third human fH-dependent mechanism, which was independent of fH binding to fHbp and/or NspA. In a previous study, binding of human fH to the Δ fHbp Δ NspA double-knockout mutant was indistinguishable from the background reactivity in the absence of human fH (9). This result was confirmed in the present study when we tested binding of human fH to the Δ fHbp Δ NspA mutant in the presence of infant rat serum (Fig. 5C). In both studies, however, the sensitivity of the flow cytometric method used to detect human fH binding likely was too low for detection of small amounts that may have been sufficient for downregulation of complement activation. Note also that in the present study, we found a slightly lower level of bacteremia in the human fH transgenic animals challenged by the H44/76 Δ fHbp Δ NspA mutant compared to that of the wild-type H44/76 strain (Table 2, experiment 3) and a requirement for higher human fH concentrations for survival of the Δ fHbp Δ NspA mutant in 60% wild-type infant rat serum (at least 33 μ g of human fH/ml compared to as little as 3 μ g/ml for the survival of the wild-type H44/76 strain). These results implied that there was lower expression and/or lower stringency of fH binding by the third human fH-dependent mechanism than for binding of fH by fHbp and/or NspA.

Note that the main site of fH synthesis is the liver. fH, however, is also synthesized *de novo* at various sites in the body, including the lung (6), skin fibroblasts (16), kidney (23, 29), spleen (23), thymus (23), endothelial cells (25), and synovial fluid (11), and by primary human cervical epithelial cells (8). The newly developed human fH transgenic rat model expresses fH from a chicken beta-actin promoter, which could result in expression of this protein at sites that do not normally express fH. Although we do not believe that expression of fH at these sites would affect the outcome of bacteremia in the currently described model, it may be a consideration while using this transgenic animal in future studies that involve certain immune privileged sites in the body where fH is normally not expressed.

In summary, the present results indicate that human fH has a profound effect on the ability of the meningococcus to cause bacteremia in infant rats and on survival of the organism in infant rat serum. In the absence of bacterial expression of the two ligands, fHbp and NspA, which are known to bind human fH, the levels of bacteremia and survival in rat serum supplemented with human factor H were lessened but not eliminated. The transgenic model may prove useful for further investigations of the mechanisms whereby fH enhances survival of mutants that lack expression of either of these (or possibly other) ligands. The model also may prove valuable for investigating antibodies that protect against meningococcal bacteremia in humans and that are designed to overcome the complement downregulating effects of factor H binding to the bacterial surface.

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