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Meningococcal factor H-binding protein vaccines with decreased binding to human complement factor H have enhanced immunogenicity in human factor H transgenic mice

Raffaella Rossi, Dan M. Granoff, and Peter T. Beernink*

Center for Immunobiology and Vaccine Development, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, California, 94609

Abstract

Factor H-binding protein (fHbp) is a component of a meningococcal vaccine recently licensed in Europe for prevention of serogroup B disease, and a second vaccine in clinical development. The protein specifically binds human factor H (fH), which down-regulates complement activation and enhances resistance to bactericidal activity. There are conflicting data from studies in human fH transgenic mice on whether binding of human fH to fHbp vaccines decreases immunogenicity, and whether mutant fHbp vaccines with decreased fH binding have enhanced immunogenicity. fHbp can be classified into two sub-families based on sequence divergence and immunologic cross-reactivity. Previous studies of mutant fHbp vaccines with low fH binding were from sub-family B, which account for approximately 60% of serogroup B case isolates. In the present study, we evaluated the immunogenicity of two mutant sub-family A fHbp vaccines containing single substitutions, T221A or D211A, which resulted in 15- or 30-fold lower affinity for human fH, respectively, than the corresponding control wild-type fHbp vaccine. In transgenic mice with high serum concentrations of human fH, both mutant vaccines elicited significantly higher IgG titers and higher serum bactericidal antibody responses than the control fHbp vaccine that bound human fH. Thus, mutations introduced into a sub-family A fHbp antigen to decrease fH binding can increase protective antibody responses in human fH transgenic mice. Collectively the data suggest that mutant fHbp antigens with decreased fH binding will result in superior vaccines in humans.

Keywords

fHbp; animal model; bactericidal; mutant fHbp vaccine; fH; variant group 2; sub-family A

1. Introduction

Factor H-binding protein (fHbp) is a principal antigen in a meningococcal vaccine recently licensed in Europe that targets serogroup B strains [1]. A second meningococcal vaccine containing two recombinant fHbp sequence variants is in late-stage clinical development [2, 3]. Based on amino acid sequence similarity and antigenic cross-reactivity, fHbp can be sub-classified into three variant groups [4] or two sub-families [5]. Sub-family A includes fHbp

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*Corresponding Author: Peter T. Beernink, 5700 Martin Luther King Jr. Way, Oakland, CA, USA. Tel: 01 510 450-7970; Fax: 01 510 450-7910; pbeernink@chori.org.

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sequences in variant groups 2 and 3, and sub-family B includes fHbp sequences in variant group 1. Vaccines containing fHbp elicit serum antibodies with complement-mediated bactericidal activity [4–6], which in humans is the serologic hallmark of protection against developing meningococcal disease. In general, antibodies elicited by fHbp vaccines confer protection only against strains expressing fHbp sequence variants from the same variant group or sub-family as the vaccine antigen.

Surface-exposed bacterial proteins that bind complement inhibitors such as factor H (fH) are attractive vaccine candidates since the resulting antibodies can bind to the bacterial surface and elicit bactericidal activity mediated by the complement classical pathway. The antibodies also can inhibit the binding of the host complement inhibitor to the bacteria. In the case of fHbp, inhibition of binding of fH to fHbp resulted in increased susceptibility of the bacteria to complement-mediated bactericidal activity [7–9]. However, one potential limitation of vaccine antigens that bind host proteins is that the binding may mask important epitopes and decrease vaccine immunogenicity [10]. Since binding of fH to fHbp is specific for human fH [11], this hypothesis could not be tested in conventional animal models but can be addressed in studies using transgenic mice expressing human fH. To date, three studies investigated the immunogenicity of fHbp vaccines in human fH transgenic mice [12–14]. Two of the studies found enhanced immunogenicity of mutant fHbp vaccines engineered to have decreased binding to human fH, compared to control fHbp vaccines that strongly bound human fH [12, 13], while the third study did not find enhanced immunogenicity of similar mutant vaccines [14]. The reasons for not having found enhanced immunogenicity in the third study are not known but may have result from the use of a different human fH transgenic mouse model with low serum concentrations of fH [15].

The transgenic mouse immunogenicity studies described above were performed with mutant fHbp vaccines from sub-family B. A broadly protective fHbp vaccine likely will require one fHbp antigen from sub-family B, and a second from sub-family A [5, 6]. However, amino acid substitutions that decrease fH binding in sub-family B do not necessarily decrease fH binding in sub-family A fHbp [16]. We recently described new mutations in fHbp from sub-family A that decreased fH binding [16]. Based on immunogenicity in wild-type mice, in which none of the fHbp vaccines bound to mouse fH, several of the fHbp mutants appeared to be promising vaccine candidates because they elicited similar serum bactericidal antibody responses to the control wild-type subfamily A fHbp vaccine. Thus, the mutations introduced to decrease fH binding did not appear to perturb epitopes required for eliciting serum bactericidal antibodies. In the present study, we investigated the immunogenicity of two of the most promising mutant sub-family A fHbp vaccines in human fH transgenic mice. To maximize the chances of observing an effect of human fH on vaccine immunogenicity, we selected transgenic mice with serum human fH concentrations of 250 µg/ml or higher. These concentrations are similar to the mean serum fH concentrations found in humans (210 to 516 µg/ml [12, 17–19]).

2. Materials and methods

2.1. Recombinant fHbp vaccines

The wild-type sub-family A fHbp sequence used in our studies is ID 22, as designated in the database at <http://pubmlst.org/neisseria/fHbp/>. The numbering of the positions of amino acid residues is based on the mature protein sequence beginning with the lipidated cysteine residue of fHbp ID 1 to facilitate comparison with our previous data [12, 16]. In our previous study of sub-family A mutants the most promising antigens appeared to be D211A and T221A/H223A¹ [16]. We subsequently found that the single amino acid substitution T221A gave an equivalent decrease in fH binding as the double mutant (data not shown). Therefore the T221A and D211A mutant vaccines were investigated in the present study.

Preparation of the D211A mutant was previously described [16]. The desired nucleotide substitutions for the T221A mutant were introduced into the pET21-fHbp ID 22 expression plasmid using the Phusion site-specific mutagenesis kit (Thermo Scientific) and the oligonucleotides: T221A_fwd, gcttaccacctcgcccttttcg; T221A_rev, gcctttttcttcgcccgc; (changes from the wild-type sequences underlined). The recombinant fHbp antigens were purified by Ni²⁺-affinity followed by ion exchange chromatography (5 mL HiTrap Chelating HP and HiTrap SP HP columns, GE Life Sciences) [16]. Purity of the proteins was assessed by SDS-PAGE (NuPAGE; Invitrogen), and retention of important epitopes was determined by binding with anti-fHbp mAbs by ELISA [20]. While a vaccine intended for humans should be prepared without the hexahistidine (His₆) tag, recombinant antigens with His₆-tags are commonly used for demonstrating proof of concept in mice. While a fraction of the serum antibodies elicited by the antigens might be directed at the His₆-tag, these antibodies would not be expected to play a role in serum bactericidal activity, which is measured against bacteria expressing native fHbp antigens that lacked His₆-tags.

2.2. Differential scanning calorimetry

Purified fHbp was dialyzed against PBS overnight at 4 °C. The next day, the absorbance at 280 nm was measured (Nanodrop 1000) and the protein concentration was determined using a molar extinction coefficient of 10430 M⁻¹ cm⁻¹, which was calculated with ProtParam (<http://web.expasy.org/protparam/>) [21]. Thermal stability was measured using a VP-DSC instrument (MicroCal), a protein concentration of 0.5 mg/ml and PBS as the reference solution. The scan rate was 60 °C/h and the passive feedback mode was used. Data were analyzed with Origin 7 software (MicroCal) using a non-2-state unfolding model. Transition midpoint (T_m) values were determined from three independent experiments.

2.3. Surface plasmon resonance binding studies

Interaction affinities and rate constants between fHbp and human fH were determined using a Biacore X100 instrument (GE Life Sciences). Affinity-purified goat anti-human fH antibody (Complement Technology) was covalently coupled to a CM5 chip by amine coupling (6000 RU). The coupled antibody was used to capture purified human fH (Complement Technology; 500 RU). Multiple-cycle kinetic experiments were performed using five concentrations of each fHbp (3.9 to 62.5 nM for wild-type fHbp, 31.3 to 500 nM for D211A, and 15.6 to 250 nM for T221A). Data were analyzed with Biacore X100 Evaluation software (GE Life Sciences) using a 1:1 binding model. Each fHbp was tested in three to six independent experiments.

2.4. Mouse immunogenicity

Immunogenicity was evaluated in wild-type CD-1 mice and human fH transgenic BALB/c mice [12]. The wild-type mice were 6-week old females; the transgenic mice were 6 to 12-week old males and females. Before beginning immunization, we obtained blood samples and measured serum human fH concentrations using a previously described fHbp capture ELISA [12]. Mice with human fH levels <250 µg/ml were excluded from our study, and the remaining mice were assigned to the different vaccine groups to ensure similar distributions of the serum fH levels. The mean fH concentrations (and ranges) in the vaccine groups were: WT, 444 µg/ml (322–867); D211A, 436 µg/ml (253–782); and T221A, 424 (273–742).

For the immunization experiments, groups of wild-type or transgenic mice received three intraperitoneal injections of the recombinant fHbp vaccines, each containing 10 µg of

¹Previously described in Pajon et al. [16] as T220A/H222A in error

protein adsorbed with 600 µg of aluminum hydroxide as the adjuvant. The wild-type mice were immunized on days 0, 10 and 40 and blood was collected on day 25 by cheek bleeds and on day 55 by terminal cardiac punctures. The transgenic mice were immunized on days 0, 21 and 42 and blood was collected only on day 63. Because of the limited number of transgenic mice available, the number of animals in each vaccine group was smaller than in the CD-1 study, and we did not obtain blood from the transgenic mice post-dose 2 to avoid administering anesthesia and the risk of mortality from the procedure. The experimental protocols strictly followed recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the protocol was approved by the Institutional Animal Care and Use Committee.

2.5. Measurement of serum antibody responses

Because of the relatively large number of wild-type CD-1 mice in each vaccine group, and the limited amount of human complement, we assayed five serum pools (four to five mice per pool) from each vaccine group. For the transgenic BALB/c mice, there were fewer mice in each vaccine group and we assayed individual sera. Serum IgG antibody responses to fHbp were measured by ELISA, which was performed as described previously [16]. The reciprocal anti-fHbp titers in each of the vaccine groups were typically from 10^4 to 10^5 . At these dilutions there was less than ~0.05 µg/ml of human fH, which would not be expected to inhibit binding of anti-fHbp antibody to fHbp [20]. The serum bactericidal titers were measured as previously described [9]. The complement source was 20% IgG-depleted serum [22], which was prepared from blood obtained from a healthy human under a protocol approved by the Institutional Review Board. In the final bactericidal reaction, there is ~100 µg/ml of human fH derived from the complement source. Thus, the effect of the smaller amounts of human fH derived from the different dilutions of transgenic mouse sera on regulation of complement activation would be expected to be negligible.

2.6. *Neisseria meningitidis* test strains

We used three strains for the measurements of bactericidal activity. Expression of fHbp by live bacteria of each of the strains was measured by flow cytometry using anti-fHbp mAb JAR 31 (Figure 1), which was performed as previously described [8]. Serogroup B strains CH597 (also known as 03S-0658 [23]) and CH677 were from patients with invasive meningococcal disease who were hospitalized in the United States in 2002–2003. The PorA variable region (VR) sequence type of CH597 is P1.7-2,13-1; the multilocus sequence type [ST] is 32 (ST clonal complex 32), and the strain is a high expresser of fHbp ID 23, which has one amino acid difference from the wild-type ID 22 fHbp vaccine. The PorA VR type of strain CH677 is P1.5-1,2-2; the ST is 409 (ST clonal complex 41/44), and the strain is a intermediate expresser of fHbp ID 19 (96% amino acid identity with fHbp ID 22). Serogroup W strain CH147 (also known as Nigeria 1/03 [24]) was from a patient with invasive meningococcal disease in 2003 in Nigeria. The PorA VR type is P1.5-1,2-36; the ST is 2881 (ST clonal complex 175), and the strain is a low expresser of fHbp ID 22 (identical to the fHbp ID 22 vaccine).

3. Results

3.1 Characterization of fHbp vaccines

By SDS-PAGE, the respective molecular masses and purities of the wild-type and mutant D211A and T221A fHbp vaccines were similar to each other (Figure 2A). As a more sensitive test of the integrity of the fHbp vaccines, we measured their thermal stability by scanning calorimetry. All three proteins unfolded with two transitions that were centered at 40 and 78 °C (Figure 2B). These transitions are thought to correspond to unfolding of the N-terminal and C-terminal domains, respectively [14, 25]. The thermal stabilities of the wild-

type and mutant proteins were similar, with slight differences in the temperatures for the second transition (Table 1). By ELISA, the anti-fHbp mAb JAR 4, which recognizes a conformational epitope [26], showed similar binding to all three proteins (Figure 2C). By surface plasmon resonance, the wild-type fHbp bound to immobilized human fH with an equilibrium dissociation constant (K_D) of 8 nM (Figure 2D; Table 1). The mutant proteins bound fH with approximately 15 and 30-fold lower affinities (K_D = 125 nM for T221A and 225 nM for D211A). Since the mutants had significantly lower affinity for fH but retained stability and conformational epitopes, the mutant fHbp vaccines were judged to be good candidates for testing immunogenicity in mice.

3.2 Serum IgG anti-fHbp responses

To determine whether the mutations introduced in the fHbp vaccines had a negative effect on immunogenicity in the absence of human fH, we immunized wild-type CD-1 mice whose fH did not bind to the vaccines, and measured IgG anti-fHbp titers in serum pools (4 or 5 sera in each pool); sera were obtained after two or three injections of vaccine. There were no significant differences in the respective titers elicited by the D211A or T221A mutant fHbp vaccines, compared with the wild-type fHbp vaccine (post-second, Figure 3A; post-third dose, Figure 3B; pair-wise comparisons after 2 or 3 doses, $P = 0.10$ by Mann-Whitney test). To determine the effect of human fH on vaccine immunogenicity, we used the same vaccines to immunize human fH transgenic mice. To maximize the statistical power to detect differences among the responses of the vaccine groups, we measured IgG anti-fHbp antibody titers in sera from individual mice. Only sera obtained after the third dose were tested. In the transgenic mice, the IgG anti-fHbp antibody titers were significantly higher in the mice assigned to the D211A or T221A mutant fHbp vaccine groups than control mice immunized with the wild-type fHbp vaccine that bound human fH (1/GMT of 30,000 and 34,000, respectively, for the mutant vaccines, vs. 19,000 for the wild-type fHbp vaccine; $P = 0.04$) (Figure 3C). These results indicated that the mutant fHbp vaccines with decreased binding to human fH had enhanced immunogenicity in the presence of human fH.

3.3. Serum bactericidal antibody responses of wild-type mice

We next evaluated the bactericidal activity of serum pools obtained from the immunized wild-type mice. There were no significant differences between the respective bactericidal antibody titers elicited by the mutant fHbp vaccines and the wild-type fHbp control vaccine when measured against all three test strains (Figure 4; all pair-wise comparisons, $P = 0.18$). Note that the titers after two doses were not measured against one of the strains (CH677) because of insufficient volumes of sera. Thus, in wild-type mice whose fH did not bind to any of the vaccines, the amino acid substitutions introduced in the mutant fHbp vaccines to decrease fH binding did not appear to perturb epitopes required to elicit bactericidal antibodies.

3.4. Serum bactericidal antibody responses of human fH transgenic mice

To test the hypothesis that mutant fHbp vaccines with decreased fH binding have enhanced protective antibody responses in the presence of human fH, we measured serum bactericidal titers of post-third dose sera from individual transgenic mice. The D211A mutant fHbp vaccine elicited significantly higher serum bactericidal titers against all three strains tested when compared to the control fHbp vaccine that strongly bound human fH. The differences were four-fold higher against strain CH147 (1/GMT of 26 vs. 6; $P = 0.001$; Figure 5A), two-fold higher against strain CH597 (1068 vs. 587; $P = 0.03$; Figure 5B) and eleven-fold against strain CH677 (185 vs. 16; $P = 0.0001$; Figure 5C). The T221A mutant fHbp vaccine elicited higher titers against two of the three test strains. The differences were two-fold higher against strain CH597 (1/GMT of 919 vs. 587 for the control fHbp vaccine that bound fH;

P=0.08, Figure 5B) and seven-fold higher against strain CH677 (112 vs. 16; P=0.001; Figure 5C).

4. Discussion

As described in the Introduction, in two studies mutant fHbp sub-family B vaccines with decreased fH binding elicited higher serum bactericidal antibody responses in human fH transgenic mice than the respective control vaccines that bound fH [12, 13]. In contrast, a third study did not find evidence of increased immunogenicity of several mutant recombinant fHbp vaccines [14]. Each of these studies investigated mutants of sub-family B fHbp ID 1 with substitutions at arginine-41 (alanine or serine). Recently we reviewed these studies, and concluded that the most likely explanation for the lack of enhanced immunogenicity of the mutant vaccines in the third study was the use of a transgenic mouse line with low serum concentrations of human-mouse chimeric fH [27].

Vaccines containing fHbp generally elicit protective antibody responses only against strains expressing fHbp from the same sub-family or variant group as the vaccine [4–6]. Although the R41S substitution decreased fH binding in multiple fHbp sequence variants tested from sub-family B, substitutions at this position did not decrease fH binding in fHbp variants from sub-family A [16]. In the same study, we identified alternative amino acid substitutions that decreased fH binding to sub-family A fHbp, while appearing to preserve epitopes needed for eliciting serum bactericidal antibodies in wild-type mice [16]. In the present study, we have extended the previous studies to investigate the immunogenicity of two of the most promising sub-family A mutant fHbp vaccines with decreased fH binding.

We performed the immunogenicity studies in the two mouse strains for different reasons. Binding of fH to fHbp is specific for human fH, and mouse fH does not bind to the vaccine [11]. The immunogenicity study in wild-type CD-1 mice was done to determine whether the amino acid substitutions, which were introduced in the mutant fHbp vaccines to decrease human fH binding, negatively affected epitopes important for eliciting serum bactericidal activity in an animal model in which mouse fH did not bind to any of the vaccine antigens. The purpose of the study in transgenic BALB/c mice was to compare the immunogenicity of a wild-type fHbp vaccine that bound human fH, to that of mutant fHbp vaccines with decreased binding of human fH. For this study, we selected mice with serum human fH concentrations >250 µg/ml based on our previous data that showed significantly higher responses to a mutant sub-family B fHbp with decreased binding of fH only in transgenic mice with serum human fH concentrations >250 µg/ml [12]. The results are clinically relevant since many humans have serum fH concentrations >250 µg/ml [12]. Our results showed that in the presence of human fH, the mutant fHbp vaccines with decreased fH binding elicited higher serum bactericidal antibody responses. The D211A mutant fHbp vaccine appeared to elicit higher and more consistent serum bactericidal titers in human transgenic mice than the T221A mutant vaccine (Figure 5). Since both fHbp mutants had similar immunogenicity to each other in wild-type mice, the higher immunogenicity of the D211A mutant vaccine in transgenic mice might have reflected the lower affinity of this mutant for binding human fH (30-fold lower than the control fHbp vaccine, as compared with 15-fold lower fH binding for the T221A mutant (Figure 2D; Table 1). For the transgenic mouse immunogenicity experiment, we investigated both males and females because the number of available transgenic mice was limited. In assigning animals to each treatment group we attempted to distribute the small number of males equally among the vaccine groups. Further, we conducted sub-analyses of the responses of the female mice only and found significantly higher bactericidal titers elicited by each of the mutant vaccines (D211A and T221A) than the control wild-type vaccine. The data in the females thus paralleled the findings reported for both males and females when analyzed together.

For both mutant fHbp vaccines, the highest serum bactericidal titers were measured against strain CH597, which was also the strain with the highest expression of fHbp, and the lowest titers were against strain CH147, which was the strain with the lowest fHbp expression (Figure 1). Further, the largest differences in serum bactericidal activity between the respective mutant and wild-type fHbp vaccines were against the strains with low (CH147) or intermediate (CH677) fHbp expression. Note that in previous studies using isogenic and wild-type strains, those with low or intermediate fHbp expression were generally more resistant to bactericidal activity elicited by fHbp-based vaccines than strains with high fHbp expression [6, 24, 28]. Further, strains with low or intermediate fHbp expression are relatively frequent among disease-causing serogroup B isolates [6, 28]. Thus the ability of the mutant fHbp vaccines with decreased fH binding to augment the bactericidal antibody titers against strains with low or intermediate fHbp expression would be particularly valuable for enhancing protection against disease. However, we cannot rule out the possibility that strain factors in addition to fHbp expression could have contributed to the differences in susceptibility to bactericidal activity among the strains.

In previous studies, strains with sub-family A fHbp accounted for approximately 40% of disease-causing serogroup B isolates in the U.S. [29], and 25 to 57% in various other countries [30]. One strategy to broaden protection of fHbp vaccines is to combine two or more fHbp antigens from different sub-families [5, 6]. In our previous studies we showed that R41S mutant vaccines from fHbp sub-family B had decreased fH binding and enhanced immunogenicity in human fH transgenic mice. In the present study, we now show that mutant sub-family A fHbp antigens with decreased fH binding also have increased immunogenicity in human fH transgenic mice. Combining mutant antigens from both sub-families thus offers a promising approach for developing a more broadly protective fHbp vaccine for humans. Use of mutant fHbp vaccines with decreased fH binding has the additional potential benefit of avoiding the theoretical risk of eliciting auto-immune responses to neo-antigens that might be formed when human fH binds to wild-type fHbp vaccines.

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Highlights

- Two meningococcal factor H binding protein mutants decreased binding of human factor H
- The mutations did not impair protective antibody responses in mice
- The mutants elicited significantly augmented antibody responses in human factor H transgenic mice
- Mutants with decreased factor H binding may lead to improved vaccines for humans

Figure 1.

Expression of fHbp in bactericidal test strains by flow cytometry. **Panel A.** Binding of anti-capsular mAb JW-W1 (J.A. Welsch and D.M. Granoff, unpublished data) to serogroup W strain CH147 (solid line) or binding of mAb SEAM 12 [31] to serogroup B strains CH677 (dashed line) and CH597 (dotted line). The negative control was strain CH597 with no primary antibody added (shaded histogram). Negative control histograms for the other two strains were similar. **Panel B.** Anti-fHbp mAb JAR 31 binding to same three strains. Line patterns same as in panel A. The median fluorescence values for JAR 31 binding to the three strains were 46 (CH147), 619 (CH677) and 983 (CH597).

Figure 2.

Characterization of fHbp vaccines. **A**, SDS-PAGE of the purified proteins. Lane 1, molecular mass standard (Kaleidoscope, BioRad); lane 2, ID 22 wild-type; lane 3, D211A mutant; lane 4, T221A mutant. 2 μg of each protein was loaded, and the proteins were visualized with Simply Blue Safe Stain (Invitrogen). **B**, Thermal stability measured by differential scanning calorimetry. fHbp ID 22 wild-type (WT; solid line); D211A mutant (dashed line); T221A mutant (dotted line). Transition midpoint (T_m) values are given in Table 1. **C**, Binding of mAb JAR 4 to WT ID 22 and its mutants as measured by ELISA. The mean and range of duplicate measurements is shown. **D**, Binding of WT and mutant fHbps to immobilized human fH as determined by surface plasmon resonance. Data are shown for injections of 62.5 nM of each fHbp; the kinetic rate and equilibrium dissociation constants determined from experiments with multiple analyte concentrations are shown in Table 1.

Figure 3.

Serum IgG anti-fHbp antibody responses to mutant fHbp vaccines as measured by ELISA. **A**, Responses of wild-type (WT) mice after two doses. Each symbol represents the titer of a serum pool of 4 to 5 mice. Pair-wise differences between WT and mutant fHbp vaccines were not significant ($P > 0.10$ by Mann-Whitney test). **B**, Responses of WT mice after three doses. Serum pools were tested as in panel A. Pair-wise differences were not significant ($P > 0.10$). **C**, Responses of human fH transgenic mice after three doses. Each symbol represents the titer of an individual mouse. For pairwise comparisons, D211A vs. WT, $P = 0.04$; T221A vs. WT, $P = 0.007$.

Figure 4.

Serum bactericidal antibody responses of wild-type mice to mutant fHbp vaccines. Each symbol represents the titer of a serum pool from 4 to 5 mice. **A**, Post-dose 2 titers measured against serogroup W strain CH147. **B**, Post-dose 3 titers against strain CH147. **C**, Post-dose 2 titers against serogroup B strain CH597. **D**, Post-dose 3 titers against strain CH597. **E**, Post-dose 3 titers against serogroup B strain CH677. None of the pairwise comparisons between the wild-type (WT) and mutant fHbp vaccines was statistically significant ($P = 0.18$). Note that the scale for the Y-axis in panels A and B is different than for panels C through E.

Figure 5.

Serum bactericidal antibody responses of human fH transgenic mice to mutant fHbp vaccines. Each symbol represents serum from an individual mouse given 3 doses of vaccine. **A**, Strain CH147 (D211A vs. wild-type (WT) fHbp vaccine, $P = 0.001$ by Mann-Whitney test; T221A vs. WT $P = 0.41$). **B**, Strain CH597 (D211A vs. WT, $P = 0.03$; T221A vs. WT, $P = 0.08$). **C**, Strain CH677 (D211A vs. WT, $P = 0.0001$; T221A vs. WT, $P = 0.001$). Note that the scale for the Y-axis in panel A is different than for panels B and C.

Table 1
Summary of fHbp thermal unfolding transition temperatures and kinetic constants for interaction between fHbp and human fH.

	Thermal unfolding (DSC) ^a		Rate and affinity constants (SPR) ^b		
	<i>T_m</i> 1 (°C)	<i>T_m</i> 2 (°C)	<i>k_d</i> (s ^{−1})	<i>k_a</i> (M ^{−1} s ^{−1})	<i>K_D</i> (nM)
fHbp variant					
WT	39.9 ± 1.2	77.6 ± 0.5	0.0027 ± 0.0001	(3.29 ± 0.17) × 10 ⁵	8.3 ± 0.6
D211A	39.1 ± 0.7	79.8 ± 0.3	0.0193 ± 0.0015	(8.59 ± 0.71) × 10 ⁴	225.0 ± 6.7
T221A	38.6 ± 0.9	76.8 ± 0.1	0.0285 ± 0.0049	(2.31 ± 0.20) × 10 ⁵	125.0 ± 20.6

^a*T_m*, transition midpoint temperatures for thermal unfolding by differential scanning calorimetry (DSC). Mean and SE determined from 3 independent measurements.

^b Association (*k_a*) and dissociation (*k_d*) rate constants and equilibrium dissociation constants (*K_D*) from surface plasmon resonance experiments (SPR; see Methods). Mean and SE determined from 3 to 6 independent measurements.