

Functional Analysis of the Human Antibody Response to Meningococcal Factor H Binding Protein

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ABSTRACT Two licensed serogroup B meningococcal vaccines contain factor H binding protein (FHbp). The antigen specifically binds human FH, which downregulates complement. In wild-type mice whose mouse FH does not bind to FHbp vaccines, the serum anti-FHbp antibody response inhibited binding of human FH to FHbp. The inhibition was important for eliciting broad anti-FHbp serum bactericidal activity. In human FH transgenic mice and some nonhuman primates, FHbp was able to form a complex with FH and FHbp vaccination elicited anti-FHbp antibodies that did not inhibit FH binding. To investigate the human anti-FHbp repertoire, we cloned immunoglobulin heavy- and light-chain-variable-region genes of individual B cells from three adults immunized with FHbp vaccines and generated 10 sequence-distinct native anti-FHbp antibody fragments (Fabs). All 10 Fabs bound to live meningococci; only 1 slightly inhibited binding of human FH, while 4 enhanced FH binding. Affinity-purified anti-FHbp antibody from serum of a fourth immunized adult also enhanced binding of human FH to live meningococcal bacteria. Despite the bound FH, the affinity-purified serum anti-FHbp antibodies elicited human complement-mediated bactericidal activity that was amplified by the alternative pathway. The lack of FH inhibition by the human anti-FHbp Fabs and serum antibodies suggests that binding of human FH to the vaccine antigen skews the anti-FHbp antibody repertoire to epitopes outside the FH-binding site. Mutant FHbp vaccines with decreased FH binding may represent a means to redirect the human antibody repertoire to epitopes within the FH binding site, which can inhibit FH binding and, potentially, increase safety and protective activity.

IMPORTANCE Two meningococcal vaccines contain factor H binding protein (FHbp). Immunized mice whose mouse factor H (FH) does not bind to FHbp develop serum anti-FHbp antibodies that block binding of human FH to the bacteria. With less bound FH, the bacteria become more susceptible to complement killing. To investigate human responses, we isolated 10 recombinant anti-FHbp antibody fragments (Fabs) from immune cells of three immunized adults. One slightly inhibited binding of FH to the bacteria, and four enhanced FH binding. Purified serum anti-FHbp antibodies from a fourth immunized adult also enhanced FH binding. Although bound FH would be expected to block the alternative pathway, the human anti-FHbp antibodies retained bactericidal activity and the ability to activate the alternative pathway. Mutant FHbp vaccines with decreased binding to human FH may redirect the human antibody repertoire to epitopes within the FH binding site that inhibit FH binding, which are expected to increase protective activity.

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Meningococci cause meningitis and sepsis worldwide. The strains can be subdivided into serogroups based on antigenically and chemically distinctive capsular polysaccharides. Conjugate vaccines that target the polysaccharide capsule are available against serogroups A, C, W, and Y (1). Because the group B capsular polysaccharide is an autoantigen (2), two recently licensed serogroup B vaccines target protein antigens (3, 4). Both of these vaccines contain factor H-binding protein (FHbp), which is a surface-exposed lipoprotein that binds human complement factor H (FH) (5). A key property of FHbp is its ability to recruit human FH to the bacterial surface, which downregulates the complement alternative pathway (5). This mechanism is important for

the ability of the organism to evade human complement-mediated serum bactericidal activity and invade the host (5, 6).

To date, more than 800 natural amino acid sequence variants of FHbp have been identified and are accessible in the public database at <http://pubmlst.org/neisseria/fHbp>. Based on amino acid sequence relatedness, FHbp can be classified into two subfamilies, A and B (7), or three variant groups, 1, 2, and 3 (8). In general, serum anti-FHbp bactericidal activity is specific against strains expressing FHbp sequence variants from the homologous antigenic variant group or subfamily as the vaccine antigen.

FHbp immunization of humans (9–11) and mice (3, 7, 8, 12) elicits serum anti-FHbp bactericidal antibodies. However, bind-

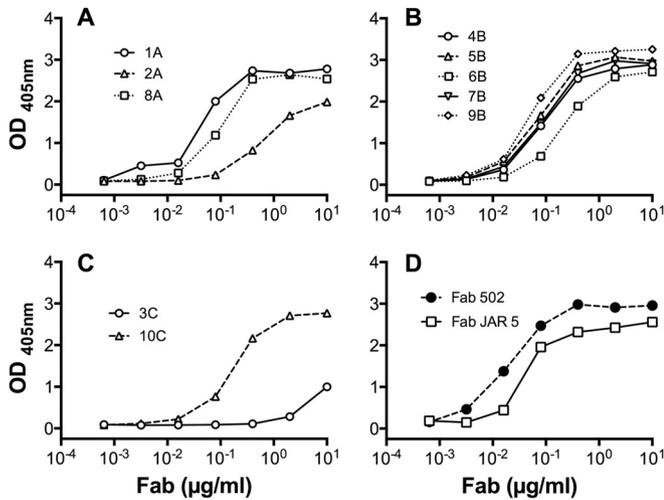


FIG 1 Binding of anti-FHbp Fabs to recombinant FHbp ID 1 as measured by ELISA. (A) Fabs prepared from subject A. (B) Fabs prepared from subject B. (C) Fabs prepared from subject C. (D) Control human-mouse chimeric anti-FHbp Fabs, 502 and JAR 5, which were derived from murine MAb 502 (19) and JAR 5 (20), respectively.

ing of FH to FHbp is specific for human FH (13) and FH from some nonhuman primates (13, 14). When humans and nonhuman primates are immunized, the vaccine antigen can form a complex with FH, which does not occur in wild-type mice, whose mouse FH does not bind to FHbp. In immunized human FH transgenic mice (15–17) and infant rhesus macaques (18), binding of FH to the vaccine antigen skews the serum anti-FHbp antibody repertoire to epitopes outside the FH binding site. These antibodies have lower complement-mediated bactericidal activity than anti-FHbp antibodies that inhibit human FH binding.

To investigate the anti-FHbp repertoire of immunized humans, we cloned the heavy- and light-chain immunoglobulin variable-region genes (VH and VL genes, respectively) from individual peripheral blood plasmablast cells from three humans immunized with vaccines containing FHbp. The natively paired VH and VL genes were expressed in *Escherichia coli*, and the resulting recombinant antibody fragments (Fabs) were characterized for antigen binding and the ability to inhibit binding of human FH to live bacteria. We also investigated the ability of affinity-purified serum anti-FHbp antibodies from a fourth immunized adult to inhibit binding of human FH and elicit complement-mediated bactericidal activity.

RESULTS

Binding of recombinant human Fabs to FHbp. We selected 10 anti-FHbp Fabs for characterization based on their having distinct immunoglobulin gene rearrangements (see Table S1 in the supplemental material). Thus, each Fab expressed a distinct paratope, which was derived from an unrelated B cell. By enzyme-linked immunosorbent assay (ELISA), each of the 10 anti-FHbp Fabs from the three immunized humans showed concentration-dependent binding to recombinant FHbp (ID 1), which is the variant group 1 antigen in the vaccines (Fig. 1A, B, and C for subjects A, B, and C, respectively). Most of the human Fabs had concentration-dependent binding similar to the binding of two control chimeric human-mouse anti-FHbp Fabs, derived from

TABLE 1 Summary of cross-reactivity of the human anti-FHbp Fabs^a

Fab no. ^b	Cross-reactivity result for FHbp with indicated ID ^c							
	Variant group 1				Variant group 2		Variant group 3	
	1	4	13	74	55	22	77	28
1A	+ ^d	–	–	–	–	–	–	–
2A	+	–	–	–	–	–	–	–
3C	+	–	–	–	–	–	–	–
4B	+	+	–	–	–	–	–	–
5B	+	+	–	–	–	–	–	–
6B	+	+	–	+	–	–	–	–
7B	+	+	+	+	+	–	–	–
8A	+	+	+	+	+	–	–	–
9B	+	+	+	+	+	+	+	+
10C	+	+	+	+	+	+	+	+

^a Tested by inhibition ELISA using soluble FHbp variants as inhibitors (see Materials and Methods).

^b Suffixes A, B, and C correspond to the three different human subjects.

^c FHbp ID number from the public database at <http://pubmlst.org/neisseria/fhbp> and variant group number as described by Masignani et al. (8).

^d +, at least 40% inhibition at the highest inhibitor concentration tested (50 µg/ml); –, less than 40% inhibition at the highest inhibitor concentration tested.

monoclonal antibody (MAb) 502 and MAb JAR 5 (Fig. 1D). The control Fabs were encoded by V region gene segments from murine anti-FHbp MAbs 502 (19) and JAR 5 (20).

Cross-reactivity of human anti-FHbp Fabs. To assess the FHbp cross-reactivity of binding by the human anti-FHbp Fabs, we measured the ability of soluble FHbp amino acid sequence variants from variant groups 1, 2, and 3 to inhibit binding of the Fabs to the nominal FHbp vaccine antigen (ID 1) by ELISA (Table 1). Representative data for four human Fabs and five different FHbp amino acid sequence variants are shown (Fig. 2). Overall, three of the Fabs were inhibited only by FHbp ID 1 (see, for example, Fig. 2A). Five additional Fabs were inhibited by FHbp ID 1 and by one to four additional FHbp sequence variants in variant

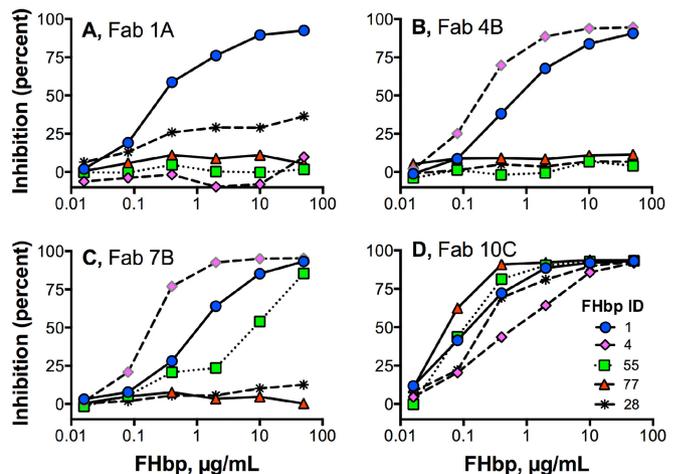


FIG 2 Cross-reactivity of human anti-FHbp Fabs with divergent FHbp sequence variants. Reactivity was determined by the ability of soluble FHbp sequence variants to inhibit binding of Fabs to immobilized FHbp ID 1 by ELISA. Representative data are shown for four Fabs and five FHbp sequence variants. The key for all four panels is shown in panel D. FHbp ID 1, 4, and 55 are in variant group 1; ID 77 is in variant group 2; ID 28 is in variant group 3. The cross-reactivity data for all 10 Fabs and 8 FHbp sequence variants are summarized in Table 1.

TABLE 2 Summary of kinetic data on binding of human Fabs to FHbp ID 1 as measured by surface plasmon resonance^a

Fab no. ^b	$k_a \times 10^{-6}$ (M ⁻¹ s ⁻¹)	$k_d \times 10^3$ (s ⁻¹)	$K_D \times 10^9$ (M)	R_{max} (RU)	χ^2 (RU ²)	GenBank accession no.	
						Light chain	Heavy chain
1A	2.25	0.1	0.44	45	0.02	KP770107	KP770108
2A	0.53	61.0	115	31	0.38	KP770109	KP770110
3C	0.03	3.2	130	11	0.28	KP770111	KP770112
4B	0.72	0.6	0.86	119	0.88	KR017714	KR017716
5B	0.39	4.1	10.6	141	2.1	KP770113	KP770114
6B	0.50	0.4	0.82	98	0.20	KP770115	KP770116
7B	0.18	4.1	22.9	114	0.87	KR017715	KR017717
8A	2.30	2.0	1.04	57	0.02	KP770117	KP770118
9B	1.85	0.04	0.019	58	0.55	KP770119	KP770120
10C	0.93	0.5	0.68	132	0.73	KP770121	KP770122
JAR 5	8.84	0.5	0.054	84	0.14	JF715927	JF715926
Fab 502	1.09	5.3	4.8	105	1.1	EU835941	EU835942

^a k_a , association rate constant; k_d , dissociation rate constant; K_D , equilibrium dissociation constant; R_{max} , maximal binding response units (RU); χ^2 , chi-square value indicating quality of fit of data to a 1:1 binding model. χ^2 was <3% of R_{max} .

^b Each of the Fab numbers except JAR 5 and Fab 502 includes a letter (A, B, or C) which corresponds to one of the three subjects. JAR 5 and Fab 502 are recombinant chimeric human-mouse Fabs derived from murine anti-FHbp MAb JAR 5 (20) and MAb 502 (19), respectively.

group 1. For example, Fab 4B was inhibited by FHbp ID 1 and ID 4, and Fab 7B was inhibited by all five FHbp sequence variants tested in variant group 1 (Fig. 2B and C; note that data are shown only for inhibition by FHbp ID 1, 4, and 55 in variant group 1). Two Fabs (9B and 10C), which were from donors B and C, respectively, were broadly cross-reactive; each was inhibited by all of the FHbp sequence variants tested in variant groups 1, 2, and 3 (see, for example, Fig. 2D).

Affinity of Fabs for FHbp. We measured the binding affinity of the human Fabs to immobilized FHbp ID 1 by surface plasmon resonance (SPR). The 10 human Fabs had a >6,000-fold range in affinity, with equilibrium dissociation constant (K_D) values from 0.019 to 130 nM (Table 2). The two human Fabs that showed the lowest binding to FHbp by ELISA, Fabs 2A and 3C (Fig. 1A and C), had the lowest affinities as measured by SPR ($K_D = 115$ and 130 nM, respectively). In comparison, the control chimeric human-mouse Fab JAR 5 had relatively high affinity ($K_D = 0.05$ nM) and Fab 502 had moderate affinity ($K_D = 4.8$ nM).

Binding of the Fabs to live bacteria. By flow cytometry, all 10 human anti-FHbp Fabs bound to the surface of live bacteria of serogroup B strain H44/76, which expresses FHbp ID 1. The binding of Fab 1A with high affinity for FHbp and of Fab 3C with low affinity is shown in Fig. 3. At 10 μ g/ml, the higher-affinity Fab gave greater binding than the lower-affinity Fab (Fig. 3A). At 2 μ g/ml, the relative difference in binding between the two Fabs was even greater (Fig. 3B). For comparison, data are shown for binding of 2 μ g/ml of the two control chimeric human-mouse Fabs (Fig. 3C).

The anti-FHbp Fabs enhance FH binding to live meningococci. In previous studies, the ability of mouse anti-FHbp MABs or serum anti-FHbp antibodies to inhibit FH binding to FHbp was important for eliciting broad human complement-mediated anti-FHbp bactericidal activity (12, 21). With less human FH bound to the bacterial surface, there was less complement downregulation, which resulted in greater C3b deposition and greater bactericidal activity (12, 21, 22). We therefore used flow cytometry to measure the ability of the human Fabs to inhibit binding of human FH to

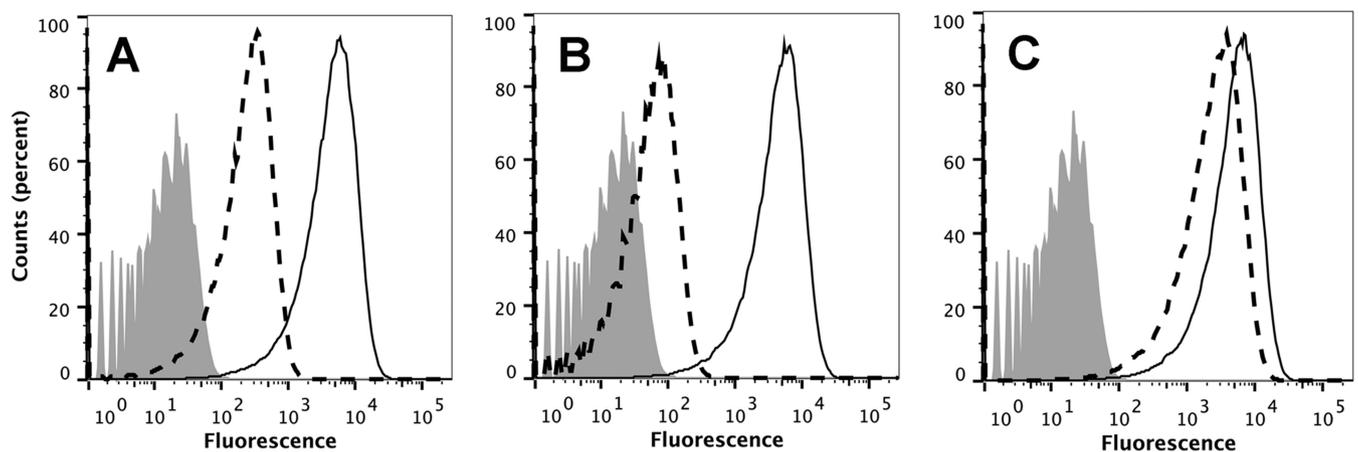


FIG 3 Binding of Fabs to live meningococci as measured by flow cytometry. The test strain was serogroup B strain H44/76 with FHbp ID 1 in variant group 1. (A) Binding of Fabs 1A (solid line) and 3C (dashed line) at 10 μ g/ml. (B) Binding of the same Fabs at 2 μ g/ml. (C) Binding of control chimeric human-mouse Fabs JAR 5 (solid line) and Fab 502 (dashed line) at 2 μ g/ml. Bound Fab was detected with (Fab-specific) biotinylated goat anti-human IgG and streptavidin conjugated to phycoerythrin. Shaded gray histogram, bacteria without FH or Fab.

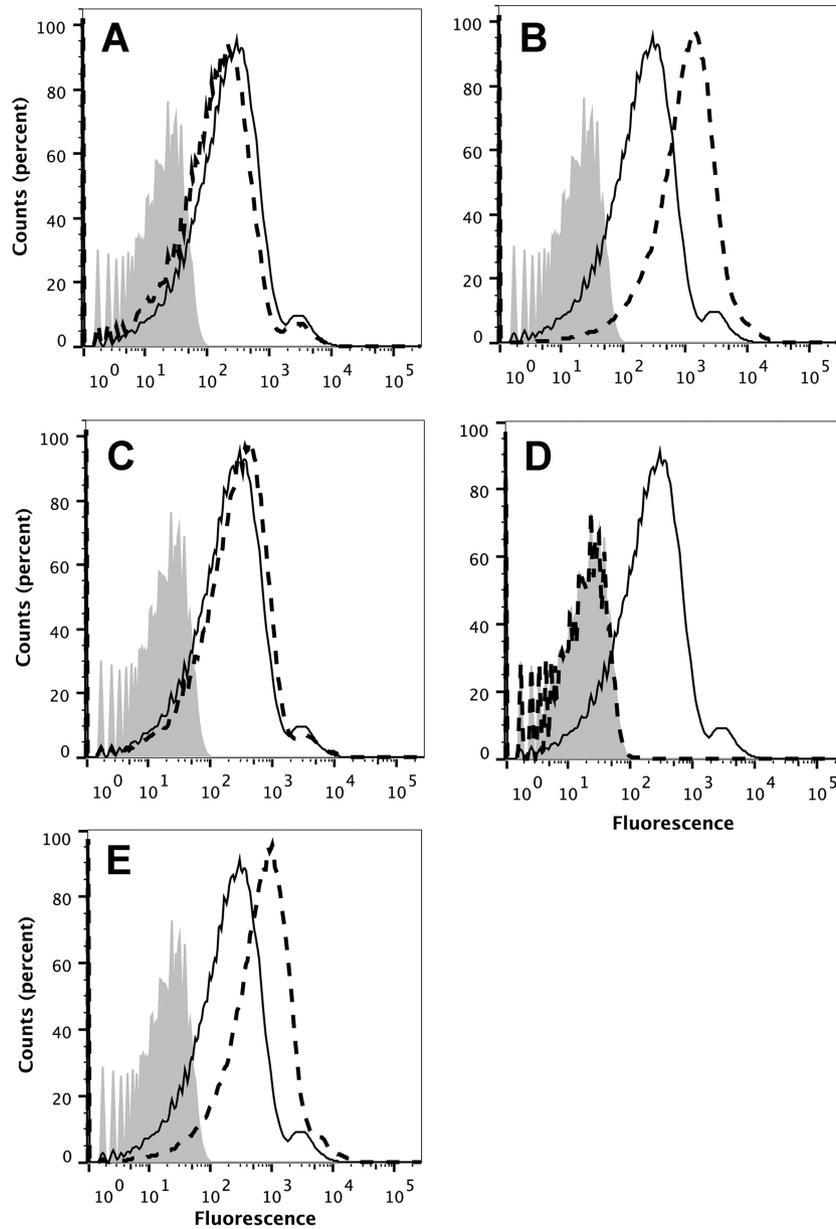


FIG 4 Effect of anti-FHbp Fabs on binding of FH to live meningococci. The test strain was serogroup B strain H44/76. Solid line, FH alone (2 $\mu\text{g/ml}$); dashed line, FH plus Fab (10 $\mu\text{g/ml}$); shaded gray histogram, bacteria without FH or Fab. (A) Slight inhibition of FH binding by human anti-FHbp Fab 5B (see also Fig. 5). (B) Enhancement of FH binding by Fab 2A. (C) Slight enhancement of FH binding by Fab 10C (see also Fig. 5). (D) Inhibition of FH binding by control chimeric human-mouse chimeric Fab JAR 5. (E) Enhancement of FH binding by control chimeric human-mouse Fab 502.

the surface of live bacteria from serogroup B strain H44/76. Of the 10 Fabs, only 1 (Fab 5B) showed slight (~50%) inhibition of human FH binding (Fig. 4A). Four Fabs showed 3- to 6-fold enhancement of human FH binding; representative data for Fab 2A are shown (Fig. 4B). The remaining five Fabs showed less-than-2-fold enhancement of human FH binding (representative data for Fab 10C are shown; Fig. 4C). As controls, the chimeric human-mouse JAR 5 Fab showed strong inhibition of human FH binding (Fig. 4D) and the 502 Fab enhanced human FH binding (Fig. 4E).

The results of testing inhibition or enhancement of FH binding to the bacteria as measured by flow cytometry were reproducible; the respective results from two independent assays are shown

(Fig. 5). The results are expressed as the ratio of the median fluorescence intensity measured when the bacteria were incubated with the Fab and human FH to that measured when the bacteria were incubated with human FH alone. In this assay, the control chimeric human-mouse JAR 5 Fab gave nearly 100% inhibition (ratio of 0.02) and the control human-mouse 502 Fab gave enhancement (ratio of 3.4). The goodness of fit of a linear equation for the data for all 10 human Fabs in the two assays showed an r^2 value of 0.97 ($P < 0.001$).

Serum anti-FHbp antibodies elicited by vaccination also enhance binding of FH. We did not have serum samples from the three immunized adults whose B cells were used to isolate the

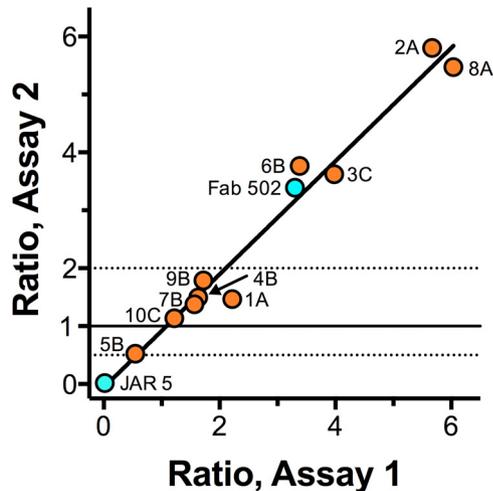


FIG 5 Effect of Fab on FH binding to the bacteria measured in two independent flow cytometric assays. Data are shown as the ratios of the median fluorescence for bound FH in the presence of Fab to the median fluorescence for bound FH without Fab. Ratios of ≥ 2 represent significant enhancement; ratios of ≤ 0.5 represent significant inhibition. Control chimeric human-mouse Fabs 502 and JAR 5 (aqua) showed enhancement and inhibition, respectively. Of 10 human Fabs (orange) tested, 1 Fab, 5B, showed slight inhibition, 5 showed no significant inhibition or enhancement, and 4 showed 3- to 6-fold enhancement of FH binding. There was at least one Fab that enhanced FH binding from each of the three immunized human subjects tested.

anti-FHbp Fabs. To investigate whether serum antibodies from a vaccinated human enhance binding of human FH to FHbp, we investigated serum from an adult immunized with the licensed Novartis serogroup B vaccine (4CMenB) 6 years after receiving two doses as part of a clinical trial. Two weeks before the booster dose, the serum IgG anti-FHbp titer was 1:800, which increased to 1:25,000 3 weeks after the booster (Fig. 6A). Compared with the prebooster serum, there was an increase in binding of human FH to live bacteria of wild-type serogroup B strain H44/76 with the

serum obtained after vaccination (Fig. 6B). To confirm whether the enhanced FH binding was the result of the presence of serum anti-FHbp antibodies, as opposed to antibodies elicited by other antigens in the 4CMenB vaccine, we purified anti-FHbp antibodies from the post-dose 3 serum using an FHbp affinity column. According to ELISA results, the serum anti-outer membrane vesicle (OMV) and anti-neisserial heparin binding antigen (NHba) titers of the adsorbed serum were not affected by adsorption with FHbp, and the affinity-purified anti-FHbp antibody contained no detectable antibodies to OMV or NHba (data not shown). With a 1:600 dilution of the adsorbed serum as a source of antibodies to 4CMenB antigens other than FHbp and endogenous human FH, there was low-level binding of FH to the bacteria (solid line, Fig. 6C). Following the addition of a 1:600 dilution ($\sim 0.1 \mu\text{g/ml}$) of the affinity-purified anti-FHbp antibodies to the 1:600 dilution of the adsorbed serum, there was enhanced binding of FH to the meningococcal surface (dotted line).

Anti-FHbp bactericidal activity. We tested bactericidal activity of the affinity-purified anti-FHbp antibodies against a wild-type serogroup B strain, H44/76, with naturally high FHbp expression and a previously described mutant of H44/76 with lower FHbp expression (23) (Fig. 7). The mutant had about 10-fold-lower binding with a mixture of two anti-FHbp MABs than the wild-type strain (Fig. 7A) but had binding similar to that seen with a control anti-PorA P1.7 MAB (Fig. 7B). Despite the enhanced FH binding, the affinity-purified anti-FHbp antibodies were bactericidal with human complement, particularly against the wild-type strain with higher FHbp expression (Fig. 7C). The wild-type and mutant strains were equally susceptible to the complement-mediated bactericidal activity of a control anti-PorA MAB (Fig. 7D).

With individual chimeric-human anti-FHbp MABs, blocking of binding of human FH to the bacterial surface was critical for eliciting complement-mediated bactericidal activity, which required alternative pathway amplification (22). The high bactericidal activity of the affinity-purified human anti-FHbp antibodies

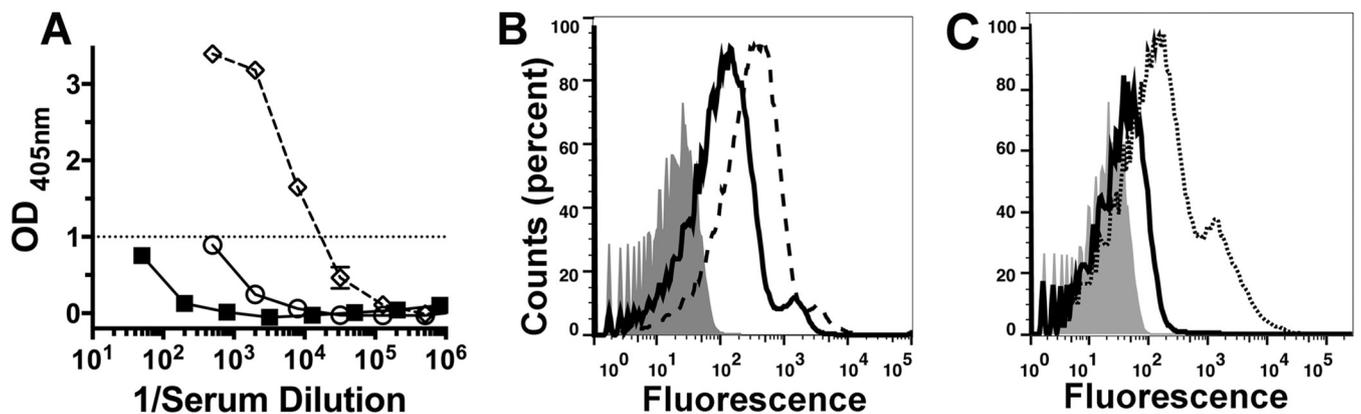


FIG 6 Serum antibodies from an adult human boosted with a serogroup B vaccine containing FHbp enhance FH binding. A healthy adult was immunized with a third dose of a serogroup B vaccine (4CMenB; Novartis Vaccines) which contained FHbp ID 1 in variant group 1. The vaccine was given 6 years after doses 1 and 2. (A) IgG binding to FHbp (ELISA) in serum samples obtained 2 weeks before (open circles) and 3 weeks after (open diamonds) dose 3. Negative-control serum (filled squares) was from a nonvaccinated adult. (B) Binding of serum FH to serogroup B strain H44/76. All sera were heated to inactivate endogenous complement (FH is stable under the conditions used [49]). Gray histogram, bacteria without serum; solid line, 1:600 dilution of serum obtained 2 weeks before dose 3; dashed line, 1:600 dilution of serum obtained 3 weeks after dose 3. (C) Binding of human FH to strain H44/76 incubated with a 1:600 dilution of post-dose 3 serum that had been depleted of anti-FHbp antibodies (solid line) or with a 1:600 dilution of the adsorbed serum mixed with $0.1 \mu\text{g/ml}$ of affinity-purified anti-FHbp antibodies eluted from the FHbp-Sepharose used for antibody depletion (dotted line). The gray histogram represents background binding to bacteria in the absence of FH or serum (secondary antibody alone).

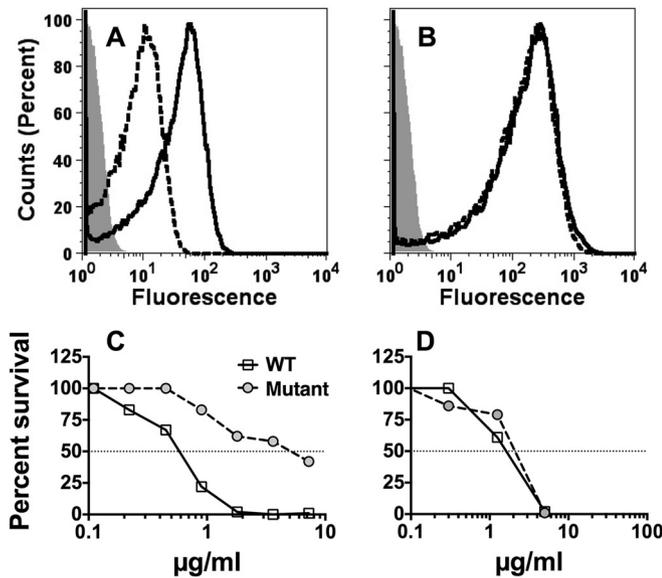


FIG 7 The effect of strain expression of FHbp on complement-mediated bactericidal activity of affinity-purified human anti-FHbp antibodies. Data are from wild-type strain H44/76 with naturally high FHbp expression (solid line) and from a mutant of strain H44/76 with lower FH expression (dashed line). (A and B) Flow cytometric detection of bound antibody. (A) Murine anti-FHbp MAbs (JAR 5 and JAR 4; 50 $\mu\text{g/ml}$). (B) Control anti-PorA P1.7 MAb (5 $\mu\text{g/ml}$). (C and D) Bactericidal activity with human complement. (C) Affinity-purified human anti-FHbp antibody. (D) Control murine anti-PorA P1.7 MAb. Data were replicated in two or three independent experiments.

against the H44/76 wild-type strain, therefore, was surprising in light of the enhanced FH binding induced by these antibodies, which would be expected to block the alternative pathway. To assess the basis for the bactericidal activity, we investigated the relative roles of the different complement pathways.

Deposition of human C4b on the surface of live *Neisseria meningitidis* cells can be used as a marker of lectin and/or classical complement pathway activation (22). The affinity-purified human anti-FHbp antibodies activated C4b deposition on strain H44/76 (Fig. 8A), as did a control mouse polyclonal anti-FHbp antiserum (Fig. 8B). A control chimeric human IgG1 mouse anti-PorA P1.16 MAb (24) gave about 10-fold more C4b deposition than the human or mouse polyclonal anti-FHbp antibodies (Fig. 8C). This result was expected since PorA is more abundant on the bacterial surface than FHbp (25) and since PorA can exist in a trimeric state (26) compared with monomeric FHbp. The resulting anti-PorA classical pathway activity was therefore higher than that elicited by the polyclonal mouse or human anti-FHbp antibodies. As expected, there also was no effect of the addition of an anti-factor B (Bb) MAb on C4b deposition elicited by the anti-PorA MAb (Fig. 8, dashed lines), since the anti-Bb MAb inhibits only the alternative pathway (22).

We also measured deposition of C3b on H44/76 as a marker of activation of any of the three complement pathways individually or in combination. The anti-Bb MAb downregulated deposition of C3b elicited by the control mouse polyclonal anti-FHbp antiserum, which is known to inhibit binding of human FH to the bacteria (Fig. 8E) (12). This result was expected (22), since, with less bound human FH, classical pathway activation by the anti-FHbp antibodies can recruit alternative pathway amplification of

C3b deposition, which would be blocked by the addition of the anti-Bb reagent. However, the anti-Bb MAb also decreased C3b deposition elicited by the affinity-purified human anti-FHbp antibodies (Fig. 8D), which did not inhibit FH binding. This result was unexpected since the bound human FH should have downregulated the alternative pathway and the effect of the addition of anti-Bb MAb on C3b deposition should have been small. These data indicate that the human anti-FHbp antibodies can recruit the alternative pathway despite the presence of bound human FH. As a negative control, we tested the effect of the anti-Bb MAb on C3b deposition elicited by the chimeric human IgG1 mouse anti-PorA P1.16 MAb (Fig. 8F). C3b deposition was not affected by inhibiting the alternative pathway since the anti-PorA MAb does not block FH binding, and with bound FH, the alternative pathway is downregulated and anti-PorA C3b deposition results only from the classical pathway.

We then measured the effect of blocking the alternative pathway on anti-FHbp bactericidal activity. As observed for the C3b deposition, the addition of the anti-Bb MAb markedly decreased the bactericidal activity elicited by the mouse anti-FHbp antiserum or human anti-FHbp antibodies (Fig. 8H and G, respectively) but had no effect on the bactericidal activity of the control chimeric human IgG1 mouse anti-PorA MAb (Fig. 8I). Thus, the bactericidal activity of the affinity-purified human anti-FHbp antibodies, but not the anti-PorA MAb, used the alternative pathway despite the presence of bound human FH.

DISCUSSION

The purpose of our study was to determine the fine antigenic specificity of human anti-FHbp antibodies and the ability of the antibodies to inhibit or enhance FH binding, the importance of which is discussed below. To investigate the human anti-FHbp antibody repertoire, we characterized 10 recombinant human anti-FHbp Fabs derived from individual B cells from three immunized adults. Each of the Fabs had distinct paratopes since they were encoded by different rearrangements of V region immunoglobulin genes. The VH and VL immunoglobulin genes also were mutated as defined by divergence from germ line sequences (see Table S1 in the supplemental material). For each subject, the affinities of the respective Fabs ranged from ~200-fold to 1,200-fold (Table 2). In a polyclonal serum antibody response, the higher-affinity antibodies would be expected to contribute more to the functional antibody response than the lower-affinity antibodies.

Two of the human Fabs, 4B and 5B, which were isolated from the same subject, had the same H-chain and L-chain germ line gene rearrangements except for different diversity (D) regions (see Table S1 in the supplemental material). However, the respective mutated H-chain V regions (VDJ) of the two Fabs differed by 19 amino acids (80% identity), and the mutated L-chain V regions (VJ) differed by 12 amino acid differences (91% identity). Although the two Fabs also had the same pattern of reactivity with different FHbp sequence variants (Table 1), the mutated sequences resulted in Fab 5B having 10-fold-higher affinity for FHbp ID 1 than Fab 4B (Table 2); also, Fab 5B slightly inhibited binding of FH to FHbp whereas Fab 4B showed slight enhancement (Fig. 5). Thus, although the two Fabs had similar respective germ line gene rearrangements, amino acid sequence differences between the mutated antibodies affected the structure of the paratope and antibody function (27–29).

As described in the introduction, binding of FH to FHbp is

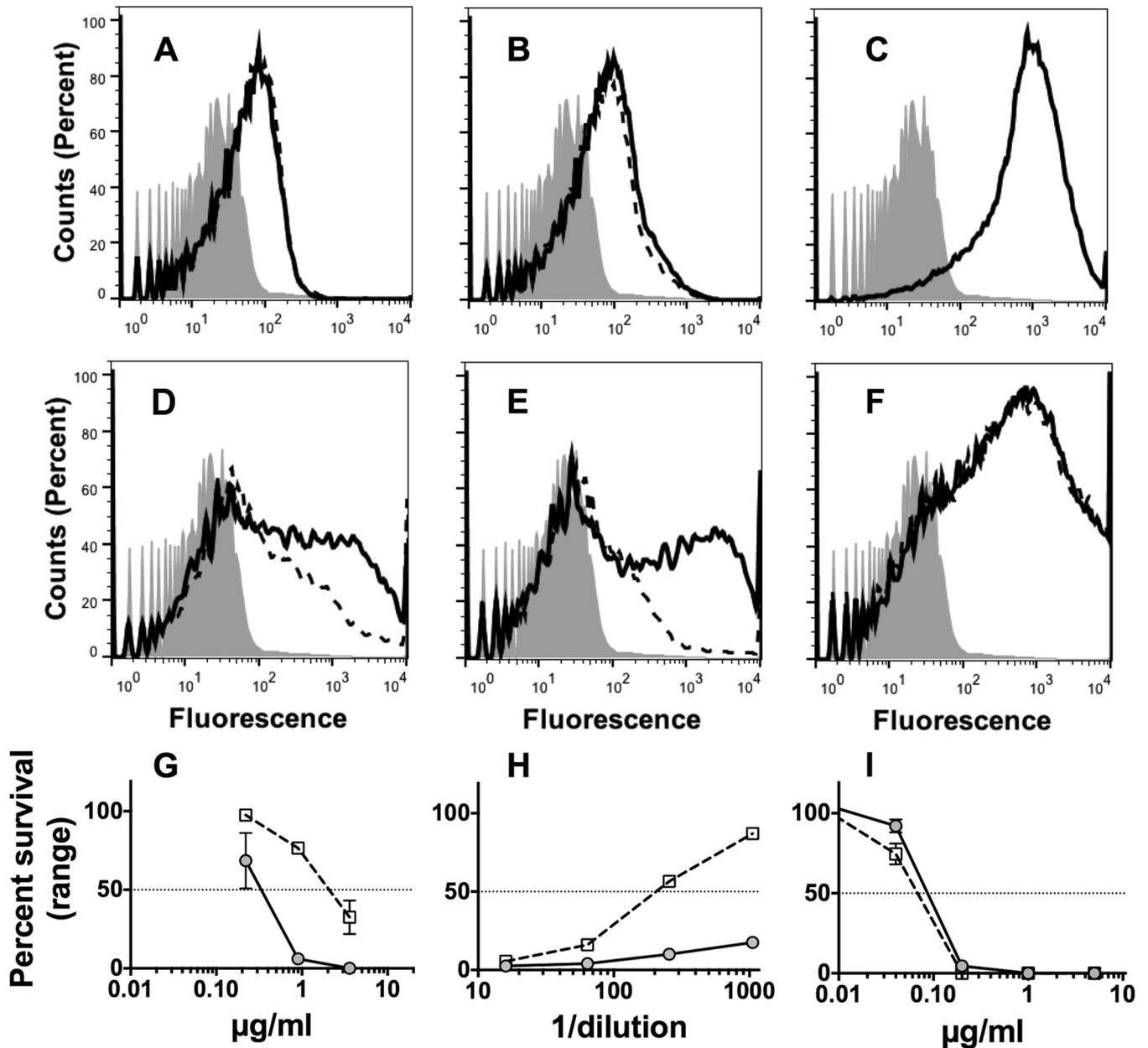


FIG 8 Affinity-purified human anti-FHbp antibodies require alternative pathway amplification for maximum complement-mediated bactericidal activity against the wild-type strain of H44/76. (A to C) Effect of the addition of a murine anti-Bb MAb on C4b deposition measured by flow cytometry. Solid line, no anti-Bb MAb; dashed line, addition of anti-Bb MAb (100 μg/ml); filled histograms, bacteria only without antibody or complement. (A) Affinity-purified human anti-FHbp antibody (1:50 dilution, or 1.16 μg/ml). (B) Mouse polyclonal anti-FHbp serum (1:50 dilution). (C) Chimeric human IgG1-mouse anti-PorA P1.16 MAb (1 μg/ml). (D to F) Effect of the addition of a murine anti-Bb MAb on C3b deposition. Symbols and conditions are the same as those described for panels A, B, and C. Note, in panels C and F, solid and dashed lines are superimposed. (D) Affinity-purified human anti-FHbp antibody. (E) Mouse polyclonal anti-FHbp serum. (F) Chimeric human mouse anti-PorA P1.16 MAb. (G to I) Effect of a murine anti-Bb MAb on bactericidal activity. (G) Affinity-purified human anti-FHbp antibody. (H) Mouse polyclonal anti-FHbp serum. (I) Chimeric human mouse anti-PorA P1.16 MAb. Filled circles with solid line, bactericidal activity with no anti-Bb MAb; open squares with dashed line, bactericidal activity in the presence of anti-Bb MAb (100 μg/ml). Representative data from two to three replicate experiments are shown.

specific for human (13) and for FH from some nonhuman primates (13, 14). In previous studies in immunized wild-type mice whose mouse FH does not bind to FHbp vaccines, the serum anti-FHbp antibodies inhibited binding of human FH to FHbp (12, 30). The inhibition of binding of human FH was critical for eliciting broad complement-mediated bactericidal activity. The rea-

son is that FHbp is relatively sparsely exposed on the bacterial surface of many meningococcal strains (6, 20); thus, the ability of IgG anti-FHbp antibodies to engage C1q and activate the classical complement pathway may be insufficient for formation of a functional membrane attack complex (5, 21, 31) without alternative pathway amplification (22). In contrast, serum anti-FHbp anti-

bodies of immunized human FH transgenic mice or rhesus macaques did not block binding of human FH (17, 18). For reasons that remain poorly understood, these anti-FHbp antibodies enhanced FH binding. (Relevant studies are summarized in Table S2 in the supplemental material.)

In the present study, we also observed a lack of inhibition by the human anti-FHbp Fabs isolated from three adults immunized with vaccines containing FHbp. The only exception was 1 Fab (of 10) that showed weak (~50%) inhibition. Further, four of the human anti-FHbp Fabs, and serum anti-FHbp antibodies from a fourth immunized adult, markedly enhanced human FH binding. In our previous studies, individual chimeric human IgG1 mouse anti-FHbp MAbs elicited human complement-mediated bactericidal activity only when the MAb inhibited binding of human FH (22). However, mixtures of anti-FHbp MAbs that individually did not elicit bactericidal activity could have bactericidal activity together (6, 30, 32). In general, the MAb combinations with the greatest bactericidal activity included at least one that blocked FH binding. In future studies, we plan to convert the human Fabs to intact human IgG1 antibodies to investigate their ability to elicit human anti-FHbp bactericidal activity individually and in combination, but this issue was beyond the scope of the present study.

To investigate human anti-FHbp bactericidal activity, we purified anti-FHbp antibodies from serum of a vaccinated adult. As observed with the anti-FHbp Fabs, the anti-FHbp antibodies did not inhibit FH binding but did enhance FH binding to *N. meningitidis*. One hypothesis is that binding of antibodies to epitopes on FHbp results in conformational changes that render FHbp more accessible for FH binding (17). For example, in a previous study, an anti-FHbp MAb (MAb 502) enhanced FH binding to FHbp (33) and, based on results of nuclear magnetic resonance (NMR) studies, binding of this MAb altered the chemical shifts of certain FHbp residues such as Ala206 and Val207 (34), which are located in the FH binding interface (35, 36).

On the basis of the enhanced FH binding, we expected the anti-FHbp bactericidal activity to depend largely on the classical complement pathway since the alternative pathway would be downregulated by the bound FH. However, this prediction proved incorrect in that deposition of C3b and bactericidal activity elicited by the human anti-FHbp antibody were largely dependent on activation of the classical pathway with alternative pathway amplification, as shown by inhibition of C3b deposition and bactericidal activity by the anti-Bb MAb. Since the anti-Bb MAb blocked formation of the C3 convertase C3bBb, the decreased C3b deposition and bactericidal activity suggested either that the bound FH was not fully functional in acceleration of C3bBb decay or that blocking the formation of the C3 convertase had a more potent downregulatory effect than on accelerating decay of the convertase. It also is possible that IgG antibodies bound to the bacterial surface served as sites for deposition of C3b, thus overcoming the downregulatory activities of FH. For example, IgG bound to bacteria can serve as a target for C3b (37) and C3b bound to IgG is resistant to FH inactivation (38). Evidence also suggests that IgG-C3b complexes produce more active C3 convertases than free C3b (39). Understanding the actual basis for the lack of complete downregulation of the alternative pathway despite the bound human FH, however, will require additional study.

The two licensed serogroup B vaccines are efficacious in humans because they can elicit serum bactericidal activity. However, the lack of FH inhibition by the anti-FHbp Fabs in the three sub-

jects given the 4CMenB vaccine and the FH enhancement by four of the Fabs and by the serum anti-FHbp antibodies of a fourth subject informed us that the mechanism by which these human anti-FHbp antibodies confer protection is different from that seen with immunized wild-type mice.

Because of a polymorphism in macaque FH, some animals have FH that binds strongly to FHbp whereas other animals have FH that binds weakly (14). Serum antibodies from 4CMenB-vaccinated macaques with FH that bound weakly to the vaccine elicited greater activation of the classical complement pathway and higher bactericidal activity than were seen with macaques whose FH bound strongly to the FHbp antigen (18). These data, taken together with the results from immunized humans reported here, have important implications for improving FHbp vaccines. We have shown that introduction of single amino acid substitutions in FHbp can decrease FH binding by more than 100-fold (15, 40). In human FH transgenic mice, these mutant vaccines elicited serum anti-FHbp antibodies that blocked FH binding (15, 17), and the anti-FHbp antibodies had higher serum bactericidal activity than the anti-FHbp antibodies elicited by control FHbp vaccines that bound FH (15, 41–43). Thus, although the two licensed serogroup B vaccines elicit serum bactericidal activity in humans, it is likely that the protection elicited can be improved by incorporating mutations that decrease FH binding. The mutant antigens also may be safer, with less risk of eliciting autoantibodies to human FH (observed in two human FH transgenic mice immunized with the Novartis 4CMenB vaccine) (17).

MATERIALS AND METHODS

Production of anti-FHbp Fabs. Three human subjects were immunized in Krakow, Poland, in a study sponsored by Novartis Vaccine, using two doses of multicomponent serogroup B meningococcal vaccines containing recombinant FHbp ID 1 (9, 11). The protocol was approved by the Bioethics Committee of the District Medical Doctors' Chamber in Krakow, and written informed consent was obtained from each of the subjects. Eight days after the second dose, plasmablast cells were isolated from peripheral blood and sorted individually into the wells of a microtiter plate. The plates were frozen and sent on dry ice to the Children's Hospital Oakland Research Institute (Oakland, CA, USA), where VH and VL genes of single cells were amplified separately and then joined by overlap extension PCR (L. Liu and A. H. Lucas, unpublished data). Products from the wells were pooled, cloned into pET22, and expressed in *E. coli*. Bacterial lysates were screened for binding to FHbp ID 1 by ELISA. The V region genes of positive Fabs (optical density at 405 nm [OD₄₀₅] greater than 0.1) were sequenced. Thirteen Fab clones encoded by unique germ line gene rearrangements were expressed and purified by Ni²⁺-affinity chromatography, quantified by capture ELISA, and stored at 4°C. Of the 13 Fabs, 10 were obtained in sufficient quantity for further analysis. The GenBank accession numbers of the immunoglobulin V region genes encoding the 10 Fabs are provided (Table 2). As controls for the functional assays, we prepared two chimeric human-mouse anti-FHbp Fabs from previously described V region genes encoding murine MAbs JAR 5 (20) and 502 (19). The respective mouse V region gene segments were cloned into the same vector described above, using constant regions of the CH1 region from human IgG1 and human kappa L chain.

Recombinant FHbp. FHbp amino acid sequence variants were expressed as soluble proteins in *E. coli* using T7 expression plasmid pET21b (Novagen) as previously described (8). The recombinant FHbp was purified by Ni²⁺-affinity chromatography using an Akta Purifier liquid chromatography system and HITRAP Chelating HP columns (GE Life Sciences) (5 ml) and buffer solutions recommended by the column manufacturer. Fractions containing FHbp were pooled, dialyzed against phosphate-buffered saline (PBS), and stored at 4°C. The concentrations

of FHbp were determined by UV absorbance using the molar extinction coefficient calculated from the respective amino acid sequences and Prot-Param (44).

Binding of Fabs to FHbp by ELISA. The method was previously described (45) with the exception that bound Fab was detected by (Fab-specific) goat anti-human IgG conjugated to alkaline phosphatase (Sigma) (1:5,000 in PBS-Tween 20 [PBST]–bovine serum albumin [BSA]).

Cross-reactivity of Fabs with FHbp. We used an inhibition ELISA with eight diverse recombinant FHbp amino acid sequence variants to measure Fab cross-reactivity. The sequence variants used as inhibitors included five from variant group 1 (ID 1, 4, 13, 74, and 55), two from variant group 2 (ID 22 and 77), and one from variant group 3 (ID 28). The wells of microtiter plates were coated with FHbp ID 1, blocked, and washed as described above for the direct anti-FHbp binding ELISA. Serial dilutions of each of the individual soluble FHbp inhibitors were added with a constant concentration of the Fab (approximately equal to that needed to yield an optical density of 2.0 in the absence of the inhibitor). The plate was incubated at 4°C overnight. After washing was performed, bound Fab was detected as described above. Percent inhibition by the heterologous FHbp soluble inhibitors was expressed as the percentage of Fab binding in the presence of soluble FHbp compared to Fab binding in the absence of inhibitor.

Fab binding and kinetic analysis by surface plasmon resonance. Binding and kinetic analyses were performed with 200 to 400 response units of purified recombinant FHbp immobilized to a biosensor chip using amine coupling (CM5 chip and amine coupling kit; GE Life Sciences). The running buffer was HBS-EP (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P-20 [GE Life Sciences]); the regeneration buffer was 100 mM glycine (pH 2.0)–3 M NaCl or 10 mM glycine (pH 1.7). For multiple-cycle kinetic experiments, the injection times with the Fabs were 3 min for association, 5 to 10 min for dissociation, and 1 to 2 min for regeneration and the flow rate was 30 μ l/min. Data were collected on a Biacore X100 Plus instrument and analyzed with X100 Evaluation software (GE Life Sciences) using a 1:1 binding model.

Bacterial growth. We used serogroup B strain H44/76 (B:15:P1.7,16; ST-32) that expresses FHbp ID 1 (matched to the FHbp variant group 1 antigen in the vaccines). This strain is mismatched for all of the other antigens in the Novartis 4CMenB vaccine (9, 17). In some experiments, we used a previously described mutant of H44/76 with lower FHbp expression than the wild-type strain (23). The bacteria were grown in regular Franz medium (46) containing 0.02 mM CMP–N-acetylneuraminic acid (CMP-NANA) to the mid-log phase ($OD_{600} = 0.6$) as previously described (21).

Binding of Fabs to the surface of live bacteria and inhibition of FH binding. Binding of Fabs to live meningococci was determined by flow cytometry as previously described (21). Bound Fabs were detected using biotinylated goat anti-human Fab (Rockland Immunochemicals) (1.5 μ g/ml), followed by streptavidin-phycoerythrin (eBioscience) (1:300).

Flow cytometry also was used to measure the ability of the Fabs to inhibit binding of FH to live bacteria (21). The cells were grown and washed as described above, and a mixture of purified human FH (2 μ g/ml final concentration) and Fab (0 or 10 μ g/ml final concentration) was added. The reaction mixtures were incubated for 1 h at room temperature. Bound FH was detected using polyclonal sheep anti-human FH (Abcam) followed by washing and incubation with donkey anti-sheep IgG antibody conjugated with Alexa Fluor 488.

Bactericidal activity. The test sera (see below) were heated for 30 min at 56°C to inactivate complement. The exogenous human complement was human serum depleted of IgG with a protein G column (HITRAP Protein G HP; GE Healthcare) (5 ml) (15).

Classical and alternative complement pathway activity elicited by anti-FHbp antibodies. Flow cytometry was used to measure the deposition of human C3b or C4b on the surfaces of live bacteria of the H44/76 wild-type strain (21). In brief, bacteria were grown and harvested as de-

scribed above and resuspended in Dulbecco's PBS containing 1% BSA to a density of 10^8 CFU/ml. The bacteria were incubated with human complement (5% IgG-depleted human serum) and different concentrations of antibodies. After 10 to 15 min of incubation at room temperature, bound human C3b or C4b was detected with 1:100 dilutions of fluorescein isothiocyanate-conjugated anti-human C3c or C4b (Abcam). In some experiments, to inhibit alternative complement pathway activation, we added 100 μ g/ml of a mouse anti-Bb MAb (Quidel Corp.) to the reaction mixture as previously described (22). Zymosan is a potent activator of the alternative pathway (47). By flow cytometry, this concentration of the anti-Bb MAb completely inhibited C3b deposition on zymosan particles when incubated with 15% IgG-depleted human serum (complement) under conditions that blocked the classical pathway but were permissive for the alternative pathway (chelation of Ca^{2+} but not Mg^{2+} by the addition of 10 mM EGTA and 10 mM $MgCl_2$) (data not shown).

Human sera. A microbiologist had been vaccinated 6 years earlier with two doses of an investigational serogroup B vaccine containing FHbp ID 1 as part of a European clinical trial sponsored by Novartis Vaccines. The subject received a third dose in Europe, where the Novartis 4CMenB vaccine is licensed. Serum samples obtained 2 weeks before and 3 weeks after the booster dose were assayed for IgG antibody titers for FHbp and their ability to inhibit or enhance FH binding to live bacteria. Informed written consent was obtained from the subject, and the studies were approved by the UCSF Benioff Children's Hospital Oakland Institutional Review Board.

Purification of anti-FHbp antibodies. Purified recombinant FHbp ID 1 R41S protein was coupled to activated cyanogen bromide Sepharose as previously described (48). The human serum sample (500 μ l diluted 1:2 in PBS) was incubated with the immunoadsorbent for 1 h at room temperature. Efficiency of serum adsorption of anti-FHbp antibody (>98%) was determined by ELISA. Specificity of the serum antibody adsorption was confirmed by lack of adsorption of antibodies to OMV and to NHba, two other antigens in the 4CMenB vaccine (data not shown). There was no detectable binding by the affinity-purified antibody fraction measured by ELISA against two negative-control antigens, OMV and NHba.

Fab sequences. The GenBank accession numbers for the human Fabs are listed in Table 2. The V region sequences for the control Fabs 502 and JAR 5 were reported previously (19, 20).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00842-15/-/DCSupplemental>.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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