Effect of complement Factor H on anti-FHbp serum bactericidal antibody responses of infant rhesus macaques boosted with a licensed meningococcal serogroup B vaccine

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A B S T R A C T

FHbp is a major serogroup B meningococcal vaccine antigen. Binding of complement Factor H (FH) to FHbp is specific for human and some non-human primate FH. In previous studies, FH binding to FHbp vaccines impaired protective anti-FHbp antibody responses. In this study we investigated anti-FHbp antibody responses to a third dose of a licensed serogroup B vaccine (MenB-4C) in infant macaques vaccinated in a previous study with MenB-4C. Six macaques with high binding of FH to FHbp (FH\textsubscript{high}), and six with FH\textsubscript{low} baseline phenotypes, were immunized three months after dose 2. After dose 2, macaques with the FH\textsubscript{low} baseline phenotype had serum anti-FHbp antibodies that enhanced FH binding to FHbp (functionally converting them to a FH\textsubscript{high} phenotype). In this group, activation of the classical complement pathway (C4b deposition) by serum anti-FHbp antibody, and anti-FHbp serum bactericidal titers were lower after dose 3 than after dose 2 (p < 0.02). In macaques with the FH\textsubscript{high} baseline phenotype, the respective anti-FHbp C4b deposition and bactericidal titers were similar after doses 2 and 3. Two macaques developed serum anti-FH autoantibodies after dose 2, which were not detected after dose 3. In conclusion, in macaques with the FH\textsubscript{low} baseline phenotype whose post-dose 2 serum anti-FHbp antibodies had converted them to FH\textsubscript{high}, the anti-FHbp antibody repertoire to dose 3 was skewed to less protective epitopes than after dose 2. Mutant FHbp vaccines that eliminate FH binding may avoid eliciting anti-FHbp antibodies that enhance FH binding, and confer greater protection with less risk of inducing anti-FH autoantibodies than FHbp vaccines that bind FH.

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1. Introduction

Two protein-based meningococcal serogroup B vaccines were licensed in the U.S. in October 2014 and January 2015. Both contain Factor H binding protein (FHbp) [1], which binds complement Factor H (FH). Binding of FH to meningococci down-regulates the alternative complement pathway and enables the bacteria to evade complement [2,3].

Binding of FH to FHbp has been reported to be specific for human and some non-human primate FH [4–6]. In human FH transgenic mice, binding of FH to FHbp vaccines decreased protective anti-FHbp antibody responses [7,8]. In a recent study we investigated the immunogenicity of a licensed four-component meningococcal serogroup B vaccine in infant rhesus macaques in relation to binding of FH to the FHbp vaccine antigen [9]. The vaccine contains FHbp and three other antigens capable of eliciting serum bactericidal activity [10]. In Europe, where currently there is only one licensed serogroup B vaccine, this vaccine, which is manufactured by GSK, is referred to as 4CMenB. However, in the U.S. where there is a second licensed serogroup B vaccine that only contains FHbp (manufactured by Pfizer, referred to as “MenB-FHbp”), the four component vaccine is referred to as MenB-4C [11], which we will utilize herein.

Because of two amino acid polymorphisms in macaque FH domain 6, some macaques have FH that binds strongly to FHbp, while other macaques have FH that binds only weakly [4,6]. In macaques immunized with two doses of MenB-4C, those with the low binding FH phenotype (FH\textsubscript{low}) had higher anti-FHbp serum bactericidal antibody responses than macaques with high binding (FH\textsubscript{high}) phenotypes [9]. Interestingly, in both groups, the vaccine-induced serum anti-FHbp antibodies after dose 2 enhanced binding of FH to FHbp. Thus, after dose 2 the animals with the baseline FH\textsubscript{low} phenotype had functionally converted and had similar FH binding to FHbp as the animals with the FH\textsubscript{high} baseline phenotype. In the
present study, we investigated the anti-FHbp antibody responses of these animals to a third dose of MenB-4C vaccine.

2. Materials and methods

2.1. Immunization of rhesus macaques

The animals were housed at the California National Primate Research Center, Davis, California in accordance with American Association for Accreditation of Laboratory Animal Care Standards. We strictly adhered to the “Guide for the Care and Use of Laboratory Animals” [12]. The study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis. We screened 25 macaques for FH binding and identified 8 with the FH high phenotype (32%) and 17 (68%) with the FH low phenotype. Of these, we selected six animals with the highest FH binding to FHbp (FHhigh phenotype), and six with the lowest FH binding (FHlow) for inclusion in our study. At age 3 months, the 12 animals were immunized IM with the MenB-4C vaccine and were given a second injection one month later. We used the dose recommended for human infants. Six additional macaques (two FHhigh and four FHlow) were followed as non-vaccinated, negative controls [9]. In the present study, we administered a third IM dose of MenB-4C three months after the second dose to the 12 animals immunized in our previous study. Blood samples were obtained 4 weeks after dose 3.

2.2. Serum IgG antibody responses

We used an ELISA to measure serum IgG antibody titers to the four antigens in MenB-4C that elicit serum bactericidal antibodies: FHbp, NadA, NhbA, and PorA P1.4 (present in detergent extracted outer membrane vesicles; OMV) [10,13]. The ELISA was performed as previously described [7], except that bound macaque IgG was detected with alkaline phosphatase-conjugated goat anti-human IgG (Fc specific, Sigma) that cross-reacted with macaque IgG.

2.3. Serum anti-FH autoantibody

Binding of serum IgG antibodies to human FH was measured by ELISA [8] except that macaque IgG anti-FH antibody was detected with goat anti-human IgG (Fc specific) conjugated with alkaline phosphatase (Sigma). In some experiments, we investigated whether the addition of soluble human FH, or previously described recombinant human FH domains 6 and 7 or domains 18 to 20 fused to mouse IgG1 Fc (FH6,7/Fc or FH18-20/Fc, respectively) [14], inhibited serum anti-FH reactivity.

2.4. Serum bactericidal activity

The assay was performed as previously described [9]. The exogenous human complement was serum from a healthy adult that was IgG-depleted using a protein G column (HITrap Protein G; GE Life Sciences) [7]. In some experiments to inhibit alternative complement pathway activation, we added 100 µg/ml of a mouse anti-IgB mAb (A227; Quidel) [15] to the bactericidal reaction as described [16]. Two mouse mAbs, anti-PorA P1.7 (MN14C11.6; [17]) and anti-capsular (SEAM 12 [18]), which elicit bactericidal activity primarily via the classical complement pathway [16,19], were used as negative controls.

2.5. C4b deposition on Neisseria meningitidis

We used flow cytometry to measure deposition of human C4b on the surface of live bacteria [9]. Bound human C4b was detected with a 1:100 dilution of fluorescein isothiocyanate-conjugated anti-human C4b (Meridian Life Science).

2.6. Effect of serum anti-FHbp antibodies on binding of macaque FH to meningococci

The assay was performed with live bacteria and a 1:150 dilution of macaque serum as a source of FH and anti-FHbp antibody [9]. Bound macaque FH was detected with a sheep polyclonal antiserum to human FH (Abcam) followed by washing the bacteria and adding donkey anti-sheep IgG antibody (Sigma) conjugated with AlexaFluor 488.

3. Results

3.1. Lack of serum antibody booster responses to MenB-4C

Six macaques with the FHhigh phenotype and six with the FHlow phenotype as determined in sera obtained before dose 1 (“baseline”) were immunized with MenB-4C three months after dose 2. We compared the respective IgG antibody titers against FHbp, OMV, NadA and NhbA in sera obtained 4 weeks post-dose 2 and post-dose 3 (Fig. 1). For this comparison, we assayed post-dose 2 and 3 sera in parallel. Compared to titers after dose 2, neither group of macaques showed increases in serum IgG titers to any of the antigens after dose 3. Indeed there were small decreases in antibody titers after dose 3 to NadA and NhbA in macaques with the FHhigh phenotype, and to NadA and FHbp in macaques with the FHlow baseline phenotype (p < 0.05 by paired t-test).

There also were no significant increases in serum bactericidal titers after the third dose of vaccine (Fig. 2). For this analysis we tested strains mismatched for three of the four antigens in MenB-4C vaccine that elicit bactericidal antibody [8]. Thus, the bactericidal titers against strains SK016 and 5/99 were specific for antibody to PorA P1.4 and NadA, respectively, and the bactericidal titers to H44/76 were specific for anti-FHbp antibody. FHbp is divided into two sub-families, A and B, based on amino acid sequence similarity [20,21]. For anti-FHbp bactericidal responses we tested wild-type strain H44/76 that expressed sub-family B FHbp ID 1 (that matched the ID 1 antigen in MenB-4C), and a mutant of H44/76 with a different sub-family B FHbp antigen (ID 15) [9]. In some strains, PorB2 or NspA can bind human FH and increase resistance to anti-FHbp bactericidal antibody [22,23]. However, strain H44/76 has PorB3, which shows minimal or no binding with human FH [22], and is a low NspA expresser. Thus, its principal FH ligand is FHbp and, therefore, these alternative FH ligands did not influence anti-FHbp bactericidal activity. For the macaques with the FHlow baseline phenotype, the respective anti-FHbp bactericidal titers were lower after dose 3 than after dose 2 against both H44/76 strains (p < 0.02 by paired t-test). As a result, the anti-FHbp bactericidal titers of the macaques with the FHlow baseline phenotype, which had been higher after dose 2 than in macaques with the FHhigh phenotype (p = 0.02), were no longer different from each other after dose 3 (p > 0.65).

3.2. Activation of the classical complement pathway by anti-FHbp antibodies

Deposition of human complement C4b on the surface of live N. meningitidis cells is a marker of lectin and/or classical complement pathway activation. In our previous study, the anti-FHbp antibodies in post-dose 2 sera from the macaques with the FHhigh phenotype activated less C4b deposition than the animals with the FHlow phenotype [9]. In the present study we measured C4b deposition by anti-FHbp antibodies in post-dose 3 sera and compared the respective results to post-dose 2 sera (Fig. 3). We assayed the post-dose 2
3 serum anti-FHbp antibodies than post-dose 2. The C4b deposition results for all 12 animals stratified by baseline FH<sup>high</sup> or FH<sup>low</sup> phenotypes are summarized in Fig. 3. Panel B. Among the macaques with baseline FH<sup>high</sup> phenotypes (left panel), there was no significant change in the fluorescence intensities after dose 3, compared to after dose 2. Among macaques with the FH<sup>low</sup> baseline phenotype, there was a significant decline in C4b deposition after dose 3 compared to after dose 2 (p < 0.02, paired t-test). These data suggested that the lower anti-FHbp bactericidal titers after dose 3 than dose 2 in macaques with the FH<sup>low</sup> baseline phenotype were a result of less activation of the classical complement pathway.

3.3. Serum anti-FHbp antibodies elicited by MenB-4C-vaccination enhance binding of FH to FHbp

In human FH transgenic mice or humans, binding of FH to the FHbp antigen redirected the anti-FHbp antibody repertoire to FHbp epitopes outside of the FH binding site, which did...
not inhibit binding of FH to FHbp [8,9,24]. For reasons that remain unknown, the anti-FHbp antibodies enhanced FH binding [8]. In contrast, in immunized wild-type mice whose mouse FH didn’t bind to the FHbp vaccine antigen, the serum anti-FHbp antibodies inhibited FH binding [8]. An unexpected result in our previous macaque study was that the post-dose 2 sera enhanced FH binding irrespective of baseline FH\textsuperscript{High} or FH\textsuperscript{Low} phenotypes. In the present study, we also observed enhanced FH binding by serum anti-FHbp antibodies after dose 3, which was similar in both groups of macaques, and similar to the respective FH enhancement by serum obtained after dose 2 (Fig. 4).

3.4. Alternative pathway amplification of anti-FHbp bactericidal activity despite enhanced FH binding

Because of enhanced FH binding with the post-immunization macaque sera, we expected the alternative complement pathway to be inhibited and for anti-FHbp bactericidal activity to depend largely on the classical complement pathway. To investigate this hypothesis, we determined the effect of blocking the alternative pathway with an anti-Bb mAb on anti-FHbp bactericidal activity. In previous experiments, we showed that this concentration (100 μg/ml) of the anti-Bb mAb completely inhibited C3b deposition on zymosan particles under conditions that blocked the classical and lectin pathways but were permissive for the alternative pathway [24]. In the present study, the addition of the anti-Bb mAb to post-dose 2 or post-dose 3 macaque sera markedly decreased anti-FHbp bactericidal activity (Fig. 5). This result indicated that the alternative pathway remained active despite the bound FH. As expected, the anti-Bb mAb had no effect on bactericidal activity of a control mouse anti-PorA mAb or anticapsular mAb, which, because of the greater abundance of the respective target antigens, did not require alternative pathway amplification for classical pathway bacteriolysis [16].

3.5. Transient serum IgG autoantibodies to FH

In a previous study, two of eleven human FH transgenic mice immunized with MenB-4C developed serum IgM auto-antibodies to human FH [8]. We therefore investigated FH autoantibody activity in serum samples from the immunized macaques. By ELISA, none of the 12 vaccinated animals had IgM anti-FH antibody (data not shown). However, two macaques (designated “2L” with a FH\textsuperscript{low} phenotype, and “18H” with a FH\textsuperscript{high} phenotype) were positive for IgG anti-FH antibody in post-dose 2 immunization sera (Fig. 6, Panel A). Binding was inhibited by the addition of purified human FH to the reaction mixture (Panel B). However, there was no inhibition of anti-FH binding by recombinant human FH domain fragments (6,7 or 18–20, each fused to a murine IgG1 Fc). None of the animals had anti-FH antibodies detected in sera obtained after the third MenB-4C dose (data shown for the two macaques positive for anti-FH antibody after dose 2; Panel C).

**Fig. 4.** Effect of post-immunization sera on binding of macaque FH to live bacteria. All sera were tested at a dilution of 1:150 with serogroup B strain H44/76. (A) and (B) Sera obtained before vaccination (gray line) and after dose 2 of vaccine (solid line) or dose 3 (dashed line); (A) Representative macaque with FH\textsuperscript{High} baseline phenotype (B) Representative macaque with FH\textsuperscript{Low} phenotype. By dose 2, the animal with the FH\textsuperscript{High} baseline phenotype has high FH binding, which did not increase further after dose 3. (C) Mean median fluorescence intensities (MFIs) ± SD of 6 macaques with FH\textsuperscript{High} and 6 macaques with FH\textsuperscript{Low} to FHbp.
4. Discussion

FHbp is relatively sparsely exposed [25,26], and there may be insufficient bound anti-FHbp antibody on the meningococcal surface to activate bacteriolysis via the classical complement pathway without alternative pathway amplification [1,16,25]. In FHbp-immunized wild-type mice, serum anti-FHbp antibodies inhibited binding of FH to FHbp [8]. With less bound FH, there was less down-regulation of the alternative pathway, and greater anti-FHbp bactericidal activity than by anti-FHbp antibodies that did not inhibit FH binding [8,16,19].

At the time vaccine trials with the new serogroup B vaccines were started in humans, the function of FHbp in binding FH [1], and the specificity for binding only to human and some non-human primate FH [4–6], were not known. These findings raised questions about the possible effects of binding of a host protein to a vaccine antigen on immunogenicity and safety. To investigate these questions, we performed immunogenicity studies in a human FH transgenic mouse model. Our most important findings were that human FH lowered serum anti-FHbp bactericidal responses of transgenic mice compared to wild-type mice whose mouse FH did not bind to FHbp [7,8]. Further, in human FH transgenic mice, mutant FHbp antigens with decreased FH binding elicited higher serum bactericidal antibody responses than FHbp vaccines that bound human FH [7,27–29]. Finally, two human FH transgenic mice immunized with MenB–4C developed serum IgM autoantibodies to human FH [8], which raised safety concerns. These findings led us to the current investigations of the effect of FH binding on FHbp immunogenicity in infant macaques [9].

Our initial macaque study investigated the responses of infant macaques to two doses of MenB–4C. Macaques with the FHlow phenotype had higher serum anti-FHbp bactericidal responses than macaques with the FHhigh phenotype [9]. These results were consistent with earlier results in MenB–4C-immunized wild-type and human FH transgenic mice [8]. However, the anti-FHbp antibody responses of the wildtype mice, but not the transgenic mice, inhibited FH binding to FHbp, while the anti-FHbp antibodies in both groups of macaques did not inhibit FH binding. Although it is possible that the lack of FH inhibition by the anti-FHbp antibodies of the macaques with the low baseline FH binding phenotype reflected intrinsic differences between mouse and macaque immunoglobulin gene diversity, we think another explanation is more likely. In particular, mouse FH does not bind FHbp whereas even in the macaques with the baseline low FH binding phenotype
there was some FH binding to the FHbp vaccine (albeit of lower affinity than human FH, or macaque FH from animals with the high FH binding phenotype) [6]. Our hypothesis is that in FHbp-immunized wild-type mice, because the FHbp antigen does not bind to mouse FH, the anti-FHbp antibodies are directed at FHbp epitopes in the FH binding site, which inhibit binding of FH to FHbp [7,8]. In contrast, even in the macaques with low-FH binding baseline phenotype, there was sufficient low affinity FH binding to the vaccine to skew the anti-FHbp antibody repertoire to FHbp epitopes outside of the FH binding site, which did not inhibit binding of FH to FHbp.

In the present study, the macaques with the FH high binding baseline phenotype were analogous to human infants whose FH binds strongly to FHbp. In Europe, MenB-4C is licensed for three doses in infants immunized beginning at ages 2 to 5 months. However, in a study of infants given three doses at 2, 4 and 6 months of age, the post-dose 3 anti-FHbp serum bactericidal titers were similar to those after dose 2 (i.e., no clear booster responses) [30]. Thus, the lack of booster responses to dose 3 in the macaques with the FH high-binding baseline phenotype were similar to those of vaccinated human infants.

An important question in the present study is why the macaques with the baseline FHlow phenotype showed lower serum anti-FHbp bactericidal titers after dose 3 than after dose 2 (Fig. 2). As noted above, after dose 2, serum anti-FHbp antibodies in the animals with the baseline FHlow phenotype enhanced binding of FH to FHbp [9], which functionally converted them to a FHhigh phenotype. Thus, at the time of dose 3 the FHbp vaccine antigen could form a complex with FH, mask important FHbp epitopes, and skew the serum anti-FHbp antibody repertoire to epitopes giving less protective activity than after dose 2. Thus, the importance of the lower anti-FHbp bacterial antibody responses after dose 3 than after dose 2 in the FHlow baseline group is that they underscore the effect of binding of FH to the vaccine antigen on anti-FHbp repertoire and functional antibody activity.

Note also that the enhanced binding of FH to the bacterial surface by the serum anti-FHbp antibodies in the macaques would be expected to inhibit the alternative complement pathway. However, despite bound FH, the macaque serum anti-FHbp antibodies utilized the alternative complement pathway for bacteriolysis as shown by decreased bactericidal activity in the presence of an anti-Bb mAb that functionally blocked the alternative complement pathway (Fig. 5) [16]. In a recent study, we observed similar results with affinity-purified human anti-FHbp antibodies; despite enhancing binding of FH to the bacteria, anti-FHbp bactericidal activity was decreased by an anti-Bb mAb [24]. Collectively, these results indicate that macaque or human anti-FHbp antibody enhancement of FH binding to the bacteria is not fully functional in down-regulating the alternative pathway. Conceivably, the anti-FHbp antibodies interfere with FH cofactor and/or decay acceleration activities. Further studies are needed to understand the basis for enhanced FH binding by anti-FHbp antibodies and the reason why the bound FH is not more effective in down-regulating the alternative pathway.

A theoretical risk of immunizing with a vaccine antigen that binds to a host antigen is developing autoantibodies to the host antigen. In the present study, two MenB-4C-vaccinated macaques developed serum IgG autoantibodies to FH, which was consistent with previous data in human FH transgenic mice [8]. In both studies the anti-FH reactivity was specific in that the majority of the activity was inhibited by the addition of full-length human FH. The clinical importance of the serum autoantibodies to FH is unknown. In the mice, the isotype was limited to IgM, and in the macaques the IgG serum anti-FH antibodies were present only after dose 2 and not after dose 3. An important limitation of the present study is that we lacked serum samples from the macaques taken immediately...
before dose 3. Therefore we do not know whether the serum anti-FH antibodies present one month after dose 2 were still present two months later at the time of dose 3 and declined after vaccination, or had declined to undetectable levels before dose 3.

Serum IgG anti-FH autoantibodies are found in patients with two rare diseases, atypical hemolytic uremic syndrome [31–34] and C3 glomerulopathies [35]. In patients with anti-FH autoantibodies, these diseases may not manifest clinically for many years. Therefore, the occurrence of these diseases in relation to FHbp vaccination may be difficult to ascertain. To date, we are not aware of studies of development of serum anti-FH autoantibodies in humans immunized with MenB-4C. The experimental “signals” of autoantibody activity to FH in two animal models of FHbp immunization suggest the potential for FHbp vaccines to elicit anti-FH autoantibodies in humans, and underscore the need to investigate the presence of these antibodies in sera from immunized humans.

Finally, a promising strategy to decrease the potential for FHbp vaccines to elicit anti-FH autoantibodies and to increase vaccine immunogenicity is to develop mutant FHbp vaccines with decreased FH binding. While we have investigated such mutant FHbp antigens in human FH transgenic mice [7,27–29,36], future studies will be needed to establish the safety and immunogenicity of these antigens in non-human primates and humans.

Conflict of interest statement

Dan M. Granoff, Serena Giuntini and Peter T. Beernink declare no conflicts of interest.

Acknowledgements

This work was supported by grants R01 AI046464 (D.M.G.), R01 AI099125 (P.T.B.) and R01 AI14701 (D.M.G. and P.T.B.) from the National Institute of Allergy and Infectious Diseases, NIH and by a Pilot Program grant (to D.M.G. and P.T.B.) from grant PS1 OD011107 from the Office of Research Infrastructure Programs to the California National Primate Research Center. The work was performed in a facility funded by the Research Facilities Improvement Program grant C06 RR016226 from the National Center for Research Resources, NIH. We are grateful to Dr. Sanjay Ram, University of Massachusetts School of Medicine, Worcester MA for critical review of the data, and to Koen Van Rompay and Paul-Michael Sosa, California National Primate Research Center, for supervising immunization of the macaques and blood collection, and to Patricia Zuno-Mitchell for performing the serum bactericidal assays.

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