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Fine Antigenic Specificity and Cooperative Bactericidal Activity of Monoclonal Antibodies Directed at the Meningococcal Vaccine Candidate Factor H-Binding Protein▽

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No broadly protective vaccine is available for the prevention of group B meningococcal disease. One promising candidate is factor H-binding protein (fHbp), which is present in all strains but often sparsely expressed. We prepared seven murine immunoglobulin G monoclonal antibodies (MAbs) against fHbp from antigenic variant group 2 (v.2) or v.3 (~40% of group B strains). Although none of the MAbs individually elicited bactericidal activity with human complement, all had activity in different combinations. We used MAb reactivity with strains expressing fHbp polymorphisms and site-specific mutagenesis to identify residues that are important for epitopes recognized by six of the v.2 or v.3 MAbs and by two v.1 MAbs that were previously characterized. Residues affecting v.2 or v.3 epitopes resided between amino acids 174 and 216, which formed an eight-stranded beta-barrel in the C domain, while residues affecting the v.1 epitopes included amino acids 121 and 122 of the B domain. Pairs of MAbs were bactericidal when their respective epitopes involved residues separated by 16 to 20 Å and when at least one of the MAbs inhibited the binding of fH, a downregulatory complement protein. In contrast, there was no cooperative bactericidal activity when the distance between residues was ≥27 Å or ≤14 Å, which correlated with the inhibition of the binding of one MAb by the other MAb. Thus, a model for anti-fH MAb bactericidal activity against strains expressing low levels of fHbp requires the binding of two MAbs directed at nonoverlapping epitopes, which activates the classical complement pathway as well as inhibits fH binding. The latter increases the susceptibility of the organism to complement-mediated bacteriolysis.

Neisseria meningitidis is an encapsulated gram-negative bacterium that causes meningitis and sepsis. In recent years, approximately 40 to 50% of cases of disease in the United States, and an even higher proportion in Europe, have been caused by group B strains (24, 25). Although polysaccharide–protein-conjugated vaccines are available for the prevention of diseases caused by strains with group A, C, W-135, or Y capsules, no broadly protective vaccine is available against group B strains, in part because the group B capsular polysaccharide is an autoantigen (9, 10, 19) and is a poor immunogen (8). Outer membrane vesicle vaccines, which are free of capsular polysaccharide, have been used successfully to control group B epidemics (15). However, serum bactericidal antibodies elicited by outer membrane vesicle vaccines tend to be strain specific, being directed largely against PorA (21, 23), which is antigenically variable.

New vaccine antigens for the prevention of group B meningococcal disease have been identified by genomic and proteomic studies and offer the possibility of eliciting broadly protective antibodies (5, 7, 12, 20). Among the most promising of these candidates is a surface-exposed lipoprotein, designated factor H-binding protein (fHbp), which earlier was referred to as genome-derived neisserial antigen 1870 (17) or lipoprotein 2086 (11, 31). This antigen is part of two promising meningococcal recombinant protein vaccines being developed for the prevention of group B disease. The protein binds fH, an important negative regulatory molecule in the human complement cascade (16, 22). The antigen is unique as a vaccine candidate, since it elicits serum antibodies that both directly activate classical complement pathway bacteriolysis (28) and also block the binding of fH (16). If fH is not bound on the bacterial surface, the organism becomes more susceptible to bacteriolysis mediated by the alternative complement pathway (16, 22, 28).

The gene encoding fHbp is present in all meningococcal strains that have been examined to date (2, 3, 17). However, fHbp exists in at least three variant groups based on amino acid sequence identity and antibody cross-reactivity. In general, antiserum prepared against fHbp in the variant 1 (v.1) group is bactericidal against other *N. meningitidis* strains expressing fHbp in the v.1 group but not against strains expressing fHbp in the v.2 or v.3 group, and vice versa (1, 3, 17). For fHbp v.1, amino acid residues 101 to 255 of the mature protein have been reported to encompass the region of the molecule that is critical for eliciting bactericidal antibodies (13). However, no information is available on the epitopes expressed by fHbp in the v.2 or v.3 group that are recognized by bactericidal antibodies. In one study, strains expressing v.2 or v.3 fHbp accounted for nearly half of isolates causing group B meningococcal disease in some regions of the United States (3).

In the present study, we prepared and characterized a panel of anti-fHbp monoclonal antibodies (MAbs) from mice immunized with v.2 or v.3 recombinant fHbp (rfHbp) proteins. None

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of the MAbs individually elicited bactericidal activity, but all of them were bactericidal in different combinations. We used the alignments of fHbp sequences from *N. meningitidis* strains differing in their reactivity with the respective MAbs to predict the potential amino acid residues involved in the MAb epitopes. We then used site-specific mutagenesis of fHbp genes to confirm that changes in the amino acid residues of the respective recombinant proteins affected epitope expression. We also performed similar studies of two other previously described anti-fHbp MAbs specific for proteins in the v.1 group (29). The results identified specific regions of the fHbp molecule that were important for eliciting bactericidal antibodies against strains expressing fHbp from different variant groups.

MATERIALS AND METHODS

MAb preparation. Anti-fHbp MAbs JAR 3, 4, and 5 (27) and MAb 502 (13) (a gift of Marzia Giuliani, Novartis Vaccines, Siena, Italy) were prepared from mice immunized with fHbp in the v.1 group. Anti-fHbp MAbs JAR 10, 11, and 13 were generated from a mouse immunized with fHbp in the v.2 group (a gene from strain 2996) (3). We generated four new hybridoma cell lines from the spleens of CD-1 mice immunized with rfHbp in the v.3 group (a gene from strain M1239) using methods previously described (29). The MAbs were precipitated from cell culture supernatants with 50% saturated $(NH_4)_2SO_4$ and dialyzed against phosphate-buffered saline (PBS; Roche).

Bactericidal activity. Complement-mediated bactericidal activity was measured as described elsewhere using washed, log-phase bacteria grown in Mueller-Hinton broth supplemented with 0.25% glucose and 0.02 mM CMP–N-acetylneuramic acid to an optical density at 620 nm of 0.6 (26). The buffer was Dulbecco's PBS (Mediatech, Inc.) containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, $6H_2O$, and 1% (wt/vol) bovine serum albumin (BSA; Sigma). The complement source was human serum from a healthy adult with no detectable intrinsic bactericidal activity. The bactericidal activity of the MAb was defined by the concentration that gave a 50% decrease in the number of CFU (BC_{50}) after 60 min of incubation at 37°C compared to the number of CFU at time zero in the negative control reactions.

ELISA. To measure the binding of the MAbs to bacterial cells, *N. meningitidis* strains were grown for 3 h in Mueller-Hinton broth at 37°C in 5% CO_2. The culture was heated for 1 h at 65°C, and the heat-killed bacteria were washed once in PBS and resuspended in PBS to an optical density at 620 nm of 0.6. The suspension (100 μl) was added to each well of a 96-well microtiter plate (Immulon 2B; Thermo Electron Corp.) and allowed to dry. The plates were blocked with blocking buffer (PBS containing 0.1% Tween-20 [PBST; Sigma] and 1% BSA). All antibodies were diluted in blocking buffer. The primary antibodies were anti-fHbp MAbs (5 μg/ml; incubated for 1 h at room temperature), and the secondary antibody was rabbit anti-mouse immunoglobulin G (IgG)-alkaline phosphatase (1:5,000; incubated for 1 h at room temperature; Zymed). Alkaline phosphatase substrate (Sigma) was added, and the absorbance at 405 nm was measured after 30 min. A strain was considered positive for binding if the absorbance value was >10-fold greater than that for the background. Values for negative reactions generally were <3-fold greater than the value for the background. The binding of the MAbs to rfHbp was measured using an enzyme-linked immunosorbent assay (ELISA) procedure similar to that described above for binding to bacteria, except that the wells of the microtiter plate were coated with 1 μg/ml of rfHbp v.1, v.2, or v.3, purified as described previously (1), and diluted in PBS.

Inhibition of binding of fH. The ability of an anti-fHbp MAb to inhibit the binding of fH to fHbp was measured by ELISA. The wells of a microtiter plate were coated with rfHbp as described above. Dilutions containing 0.016 to 50 μg/ml of the MAb were added to the wells together with 50 μg/ml purified fH (Complement Technology, Inc.). The plates were incubated overnight at 4°C. Bound fH was detected with goat polyclonal anti-fH (Bethyl Laboratories) (1:1,000) followed by mouse anti-goat IgG-alkaline phosphatase conjugate (Santa Cruz Biotech) (1:2,000). Both of the latter steps were performed at room temperature for 2 h. After being washed, substrate was added and developed as described above for the antibody-binding ELISA.

Prediction of residues involved in epitopes. The DNA sequencing of the fHbp genes from a panel of 104 isolates identified 38 unique encoded protein sequences. Subsets of these sequences were aligned with ClustalW (6) to assess whether specific amino acid residues corresponded with the MAb reactivity patterns as measured by ELISA with bacterial cells. For the amino acid alignments, the numbering of the residues is based on that of the mature protein (i.e., lacking the signal sequence) of fHbp from strain MC58. In reality, the lengths of the mature v.2 protein (encoded by a gene from strain 8047) and v.3 fHbp (encoded by a gene from strain M1239) differ by −1 and +7 amino acid residues, respectively, from that of fHbp encoded by the gene from strain MC58. Thus, when we refer to a lysine residue at position 180, the residue is actually at position 179 of the v.2 protein or at position 187 in the v.3 protein.

Cloning and site-specific mutagenesis. The gene encoding fHbp was amplified from various meningococcal strains by PCR using primers described previously (17). The PCR products were cloned into a T/A plasmid (pGEM-T-Easy; Promega) and subcloned into the NdeI and XhoI sites of pET21b (Novagen) to encode a C-terminal hexahistidine (His_6) tag. The plasmid encoded the full-length fHbp except for the amino-terminal 26 amino acids, which contained the signal sequence. Site-specific mutagenesis was performed using the QuikChange II kit (Stratagene), using 10 ng of plasmid template and the manufacturer's protocols. Wild-type and mutant fHbp expression plasmids were confirmed by DNA sequence determination.

fHbp expression. Expression plasmids were transformed into *Escherichia coli* BL21(DE3) (Novagen). Cultures (2 ml) were grown in Luria-Bertani medium containing 100 μg/ml ampicillin, and fHbp expression was induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside for 2 to 3 h at 37°C. Cells were collected by centrifugation in an Eppendorf 5415D microcentrifuge at 16,100 × *g* and resuspended in 1× LDS sample buffer (Invitrogen) containing 25 mM 2-mercaptoethanol (Bio-Rad).

Western blotting. Bacterial lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4 to 12% NuPAGE polyacrylamide gels and morpholinepropanesulfonic acid sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer (Invitrogen). The proteins were transferred to polyvinyl difluoride membranes (Immobilon-P; Millipore). The membranes were blocked using PBST containing 2% nonfat dry milk (Carnation; Nestle). The membranes were washed and incubated with the different anti-fHbp MAbs (1 to 5 μg/ml) or, as a control for protein expression by the different clones, 0.02 μg/ml of Penta-His MAb (Qiagen). The membranes were washed in PBST and incubated with a 1:10,000 dilution of a rabbit anti-mouse IgG-horseradish peroxidase conjugate (Zymed) and washed again. The membranes were developed with a chemiluminescent substrate (ECL^+; GE Healthcare) and visualized on a Storm 840 imager (Molecular Dynamics).

Nucleotide sequence accession numbers. The DNA sequences for fHbp from strains M3153, GB988 (also referred to as M01-0240988), 03S-0408, and RM1090 have been deposited in GenBank (accession numbers EU337062, EU337063, EU828659, and EU310268, respectively).

RESULTS

MAbs prepared against v.2 or v.3 fHbp. We investigated concentration-dependent binding by performing ELISAs of three MAbs from a mouse immunized with recombinant fHbp v.2 and four MAbs from a mouse immunized with fHbp v.3 (Fig. 1). The MAbs prepared against the v.2 protein were designated JAR 10, 11, and 13 and were IgG1, IgG2a, and IgG2a, respectively. The reactivities of these MAbs against different *N. meningitidis* strains (3) and recombinant chimeric fHbp vaccines (1) were previously described, but the characteristics of their binding to recombinant proteins and functional activity were not characterized. Four newly prepared MAbs against the v.3 protein were designated JAR 32, 33, 35, and 36. Two of these, JAR 32 and 33, were IgG2a, and the other two, JAR 35 and 36, were IgG2b. As determined by ELISA, all seven MAbs showed concentration-dependent binding to the respective recombinant proteins that were used for the immunization of the mice (Fig. 1). In addition, JAR 13 cross-reacted with the v.3 protein (Fig. 1B), and JAR 36 cross-reacted with the v.2 protein (Fig. 1A). By flow cytometry, each of the MAbs showed similar respective patterns of binding and cross-reactivity to live, encapsulated bacteria that expressed fHbp v.2 (8047) or v.3 (M1239) (data not shown).

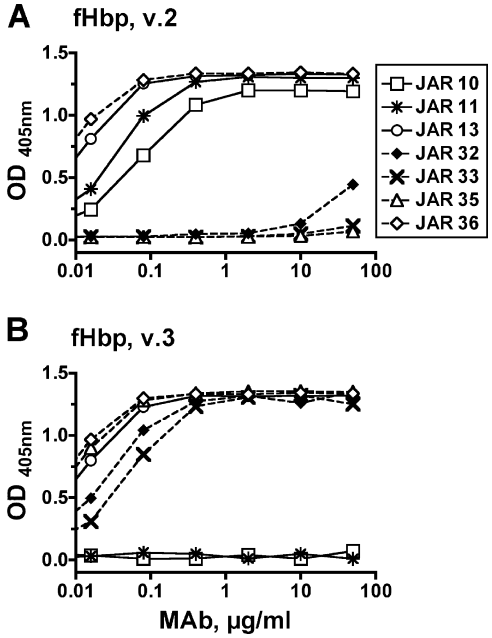

FIG. 1. Concentration-dependent binding of anti-fHbp MAbs to recombinant fHbp v.2 or v.3 as measured by ELISA. JAR 10, 11, and 13 (solid lines) were from a mouse immunized with a recombinant v.2 protein. JAR 32, 33, 35, and 36 (dashed lines) were from a mouse immunized with a recombinant v.3 protein. (A) Binding to fHbp v.2 (encoded by a gene from strain 8047). (B) Binding to fHbp v.3 (encoded by a gene from strain M1239). OD, optical density.

Although JAR 32, 33, and 35, which were from a mouse immunized with fHbp v.3, did not cross-react with the control recombinant v.2 protein used in this study (expressed from the gene from strain 8047), these three MAbs cross-reacted with fHbp expressed by other strains with subvariants of fHbp in the v.2 group (for example, strain RM1090 [2]). These results were consistent with the reported cross-reactivity between polyclonal antisera prepared against v.2 and v.3 proteins (17). JAR 10, which was from a mouse immunized with fHbp in the v.2 group, also cross-reacted with subvariants of fHbp in the v.1 group (for example, strain NZ98/254 [3]).

None of the MAbs individually was bactericidal with human complement (Table 1). However, each of the MAbs was bactericidal when combined with a second anti-fHbp MAb. The respective minimal bactericidal MAb concentrations (BC_{50}) for each of the pairs are summarized in Table 1, which also shows the pair-wise activity of three additional previously described MAbs, JAR 3, 4, and 5, which were from a mouse immunized with fHbp v.1 (28, 29). For each of the individual MAbs, the minimum antibody concentration when combined with a second MAb was as low as 1 to 5 μg/ml (i.e., 0.5 to 2.5 μg/ml of each MAb). Note that JAR 4, which cross-reacted with fHbp in the v.1 and v.2 groups, had cooperative bactericidal activity with other MAbs that were specific for fHbp in the v.1 or v.2 group (i.e., with JAR 3 or JAR 5 against strain NZ98/254, which expressed fHbp in the v.1 group, or with JAR 11 or JAR 13 against strain 8047, which expressed fHbp in the v.2 group).

Inhibition of binding of fH. We previously reported that anti-fHbp MAbs JAR 3 and 5, but not JAR 4, inhibited the binding of human fH to the surface of live bacterial cells of *N. meningitidis* as measured by flow cytometry (16). For the present study, we used an ELISA to measure the ability of each of the MAbs to inhibit the binding of fH to recombinant fHbp from the v.1, v.2, or v.3 group. The anti-fHbp MAbs JAR 3 and 5 gave nearly complete inhibition of fH binding to fHbp v.1, whereas JAR 4 gave no inhibition (Fig. 2A). Three of the

TABLE 1. Complement-mediated cooperative bactericidal activity of anti-fHbp MAbs[a]

JAR MAb (Ig isotype)	BC_{50} (μg/ml) for JAR[b]:									
	3[c]	4	5	10	11	13	32	33	35	36
Strain NZ98/254, fHbp v.1										
JAR 3 (G3)	>50	1	>50	>50						
JAR 4 (G2a)	1	>50	4	>50						
JAR 5 (G2b)	>50	4	>50	>50						
JAR 10 (G1)	>50	>50	>50	>50						
Strain 8047[d], fHbp v.2										
JAR 4 (G2a)		>50		>50	5	4				>50
JAR 10 (G1)		>50		>50	5	>50				>50
JAR 11 (G2a)		5		5	>50	>50				1
JAR 13 (G2a)		4		>50	>50	>50				1
JAR 36 (G2b)		>50		>50	1	1				>50
Strain M1239, fHbp v.3										
JAR 13 (G2a)						>50	>50	>50	>50	1
JAR 32 (G2a)						>50	>50	1	>50	>50
JAR 33 (G2a)						>50	1	>50	5	>50
JAR 35 (G2b)						>50	>50	5	>50	>50
JAR 36 (G2b)						1	>50	>50	>50	>50

[a] JAR 3, 4, and 5 were from a mouse immunized with rfHbp v.1. JAR 10, 11, and 13 were from a mouse immunized with fHbp v.2. JAR 32, 33, 35, and 36 were from a mouse immunized with rfHbp v.3.

[b] Total concentration of two MAbs that resulted in 50% survival of bacteria after 60 min of incubation with human complement. Data shown are for MAb pairs for which the respective strain expressed epitopes recognized by both MAbs.

[c] Data for the MAb pair JAR 3-JAR 4 were published previously (28).

[d] Strain 8047 expressed fHbp that differed by one amino acid from that of strain 2996, which was the source of the gene for preparing the recombinant fHbp v.2 vaccine. Strain 8047 was used as the test organism, since strain 2996 expressed low amounts of fHbp.

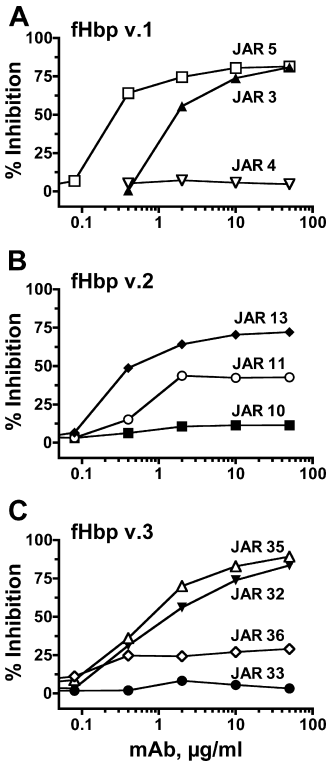

FIG. 2. Inhibition of the binding of human fH to recombinant fHbp by anti-fHbp MAbs as measured by ELISA. (A) Inhibition of binding to fHbp v.1. (B) Inhibition of binding to fHbp v.2. (C) Inhibition of binding of fH to fHbp v.3. The recombinant v.1 protein is from the gene of strain MC58. Respective v.2 and v.3 recombinant proteins are those used in experiments shown in Fig. 1.

MAbs isolated from mice immunized with fHbp v.2 or v.3 (JAR 13, 32, and 35), gave >75% inhibition of the binding of fH to rfHbp v.2 or v.3 (Fig. 2B and C, respectively), while four other MAbs gave partial (JAR 11 and 36) or no inhibition (JAR 10 and 33). All of the MAbs that gave >75% inhibition of fH binding by ELISA also gave strong inhibition of the binding of fH to live, encapsulated bacteria as measured by flow cytometry, while the MAbs that gave no inhibition by ELISA also did not inhibit the binding of fH to bacteria (data not shown). MAbs JAR 11 and 36, which showed a partial inhibition of fH binding by ELISA, gave inconsistent inhibition by flow cytometry (data not shown).

Mapping amino acid residues affecting epitope expression. From the amino acid sequence alignments of fHbp from informative strains (Table 2), we identified polymorphisms that were consistent with certain residues contributing to MAb reactivity for six of the seven MAbs prepared against v.2 or v.3 protein. For these six MAbs (JAR 10, 11, 13, 32, 33, and 35), the residues predicted to affect epitope expression were between positions 174 and 216 (inclusive). These residues were in the part of the protein previously designated the C domain (13), which formed an eight-stranded beta-barrel in the solution structure of fHbp (4). Predictions of residues involved in the epitopes recognized by these six MAbs were tested by the site-specific mutagenesis of plasmid-encoded fHbp expressed in *E. coli*. A residue from a reactive protein was changed to another residue naturally present in a nonreactive protein (knockout [KO]). A converse change at the same position in the sequence from a nonreactive protein to the corresponding residue of a reactive protein also was performed to test whether the epitope could be introduced (knock-in [KI]).

JAR 10 and 33. The epitope for MAb JAR 10, which was raised against a v.2 fHbp, was predicted from sequence alignments to involve both a lysine (K) at position 180 and a glutamate (E) at 192 (Table 2). The respective residues in the JAR 10-negative strains were R180 (strains RM1090, M1239, and GB988) or D192 (strains MC58 and GB988). The immunoblotting of recombinant fHbps containing single-amino-acid substitutions showed that JAR 10 binding was greatly decreased in K180R or E192D mutants of v.2 fHbp from strain 8047 (Fig. 3A, lanes 4 and 5). Moreover, the epitope could be introduced by a converse single-amino-acid substitution, R180K, in the nonreactive v.3 fHbp from strain M1239 (Fig. 3A, lane 7), which naturally contained E192 at the second position of the pair (Table 2), or by D192E in the v.1 fHbp

TABLE 2. Alignment of amino acid sequences of selected fHbps with distinct MAb reactivity patterns[a]

Strain	Anti-fHbp JAR MAb reactivity for group:							Amino acid sequence at position[b]:				
	v.1	v.2			v.3			170	180	190	200	210
	3/5	10	11	13	32/35	33	36					
MC58 (v.1)	1	0	0	0	0	0	0	IDFAAKQGNG	KIEHLKSPEL	NVDLAAADIK	PDGKRHAVIS	GSVLYNQAEK
NZ98/254 (v.1)	1	1	0	0	0	0	0	IDFAAKQGHG	**K**IEHLKSPEL	NV**E**LATAYIK	PDEKHHAVIS	GSVLYNQDEK
M3153 (v.2)	0	1	1	0	0	0	1	IDFA**A**KQGHG	**K**IEHLKTPEQ	NV**E**LASAELK	ADEKSHAVIL	GDTRYGGEEK
8047 (v.2)	0	1	1	1	0	0	1	IDFA**A**KQGHG	**K**IEHLKTPEQ	NV**E**LAAAELK	ADEKSHAVIL	GDTRYG**S**EEK
RM1090 (v.2)	0	0	0	0	1	1	1	IDFT**K**KQGYG	**R**IEHLKTPEQ	NV**E**LASAELK	ADEKSHAVIL	GDTRYGGEEK
M1239 (v.3)	0	0	0	1	1	1	1	IDFT**K**KQGYG	**R**IEHLKTLEQ	NV**E**LAAAELK	ADEKSHAVIL	GDTRYG**S**EEK
GB988 (v.3)	0	0	0	1	0	0	1	IDFTNKQGYG	RIEHLKTPEL	NVDLASAELK	ADEKSHAVIL	GDTRYG**S**EEK
								***: *** *	:*****: *	**:**:* :*	.* * ****	*.. *. **

[a] The MAb reactivity is defined by the binding of anti-fHbp MAbs with bacteria in a whole-cell ELISA. A value of 1 indicates reactivity, which is defined as an absorbance value that is >10-fold above the background level.

[b] The alignment contains the regions of the C domain involved in the binding of six of the MAbs prepared against fHbp v.2 or v.3. Residues involved in the epitopes are shown in boldface type: JAR 10 (K180 and E192), JAR 11 (A174), JAR 13 (S216), JAR 32 (K174), JAR 33 (R180 and E192), and JAR 35 (K174). The alignment was performed with ClustalW (6). The amino acid conservation is indicated below the alignment: *, identical; :, conserved; ., semiconserved. The numbering is based on the amino acid sequence of MC58 v.1 fHbp lacking the signal sequence (17). The DNA sequences for fHbp from strains M3153, GB988 (also referred to as M01-0240988), and RM1090 have been deposited in GenBank (accession numbers EU337062, EU337063, and EU310268, respectively). Accession numbers for fHbp genes from the strains described in previous studies are EU310268, AY548375, DQ523569, and NC_003112.

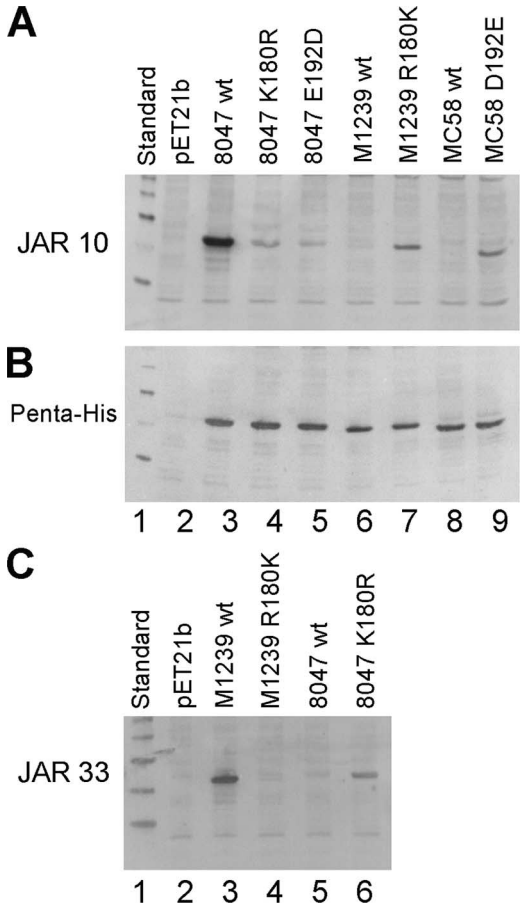

FIG. 3. Western blot showing residues that are important for the binding of anti-fHbp MAbs JAR 10 and JAR 33. Residues lysine (K) 180 and glutamate (E) 192 were involved in the JAR 10 epitope, and arginine (R) 180 was essential for the JAR 33 epitope. The numbering was based on the amino acid sequence of MC58 v.1 fHbp lacking the signal sequence (17) (see Materials and Methods). wt, wild type. (A) JAR 10. (B) Penta-His MAb from the same samples as those used for panel A. (C) JAR 33.

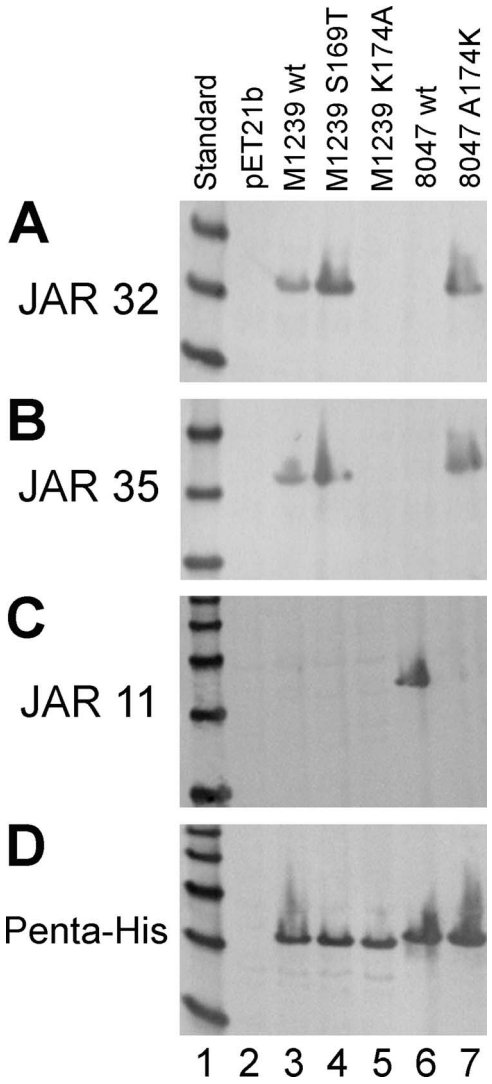

FIG. 4. Western blot showing residues that are important for the binding of anti-fHbp MAbs JAR 11, JAR 32, and JAR 35. Residue lysine (K) 174 was essential for the JAR 32 and JAR 35 epitopes, and an alanine (A) residue at position 174 was involved in the JAR 11 epitope. The numbering is described in the legend to Fig. 3. wt, wild type. (A) JAR 32. (B) JAR 35. (C) JAR 11. (D) Penta-His MAb.

from strain MC58 (Fig. 3A, lane 9), which naturally contained K180 at the first position (Table 2). A control blot probed with a Penta-His MAb specific for the C-terminal His_6 tag showed that similar amounts of the protein were expressed from wild-type and mutant clones (Fig. 3B), which indicated that the mutant proteins were not destabilized globally.

The reactivity of MAb JAR 33, which was raised against a v.3 fHbp, also was associated with residues at positions 180 and 192, which corresponded to those of JAR 10 reactivity with the v.2 protein (Table 2). JAR 33 reactivity could be knocked out by an R180K mutation in fHbp v.3 of strain M1239 (Fig. 3C, lane 4) and introduced in the fHbp of a nonreactive v.2 strain, 8047, by the reciprocal K180R mutation (Fig. 3C, lane 6). A control blot probed with a Penta-His MAb showed that similar amounts of the proteins were expressed from the respective wild-type and mutant clones (Fig. 3B).

JAR 11, 32, and 35. The reactivity of JAR 32 and JAR 35 was associated with the presence of a lysine residue at position 174 (K174) of strains RM1090 and M1239 (Table 2). The immunoblotting of wild-type and KO mutant clones indicated that both JAR 32 and JAR 35 reactivity was abolished in a mutant of M1239 fHbp containing the K174A substitution (Fig. 4A and B, lanes 5). Reactivity could be introduced with the converse substitution, A174K, in the nonreactive fHbp from strain 8047 (Fig. 4A and B, lanes 7). JAR 32 and 35 had different IgG subclasses (IgG2a and IgG2b, respectively). By ELISA, using secondary antibodies specific for each of these subclasses, the addition of JAR 32 gave the concentration-dependent inhibition of the binding of JAR 35 to fHbp v.3 (>90%; data not shown), which was consistent with the two epitopes being overlapping or identical. However, JAR 35 gave only a partial inhibition of the binding of JAR 32 (24%).

A replicate blot of the mutant proteins shown (Fig. 4A and B) was probed with JAR 11 and showed that the epitope recognized by this MAb was eliminated by the A174K substitution in fHbp from strain 8047 (Fig. 4C, lane 7). However,

JAR 11 reactivity could not be introduced into the nonreactive v.3 protein of M1239 with the converse substitution (K174A) (Fig. 4C, lane 5). This result suggested that additional residues were important for the JAR 11 epitope. The Penta-His control immunoblot showed that the proteins were expressed at similar levels from wild-type and mutant clones (Fig. 4D).

JAR 13. The JAR 13 epitope was associated with the presence of a serine residue at position 216 (Table 2). The substitution of S216G in the reactive fHbp v.3 protein of strain M1239 eliminated the epitope, while the G216S substitution in the nonreactive fHbp from strain RM1090 introduced the epitope (data not shown).

JAR 3 and 5. As described above, we previously characterized a panel of anti-fHbp MAbs prepared from a mouse immunized with recombinant fHbp in the v.1 group (encoded by a gene from strain MC58) (29). Two of the MAbs, designated JAR 3 and JAR 5, reacted broadly with nearly all subvariants of fHbp v.1. Each MAb inhibited the binding of fH to the surface of encapsulated *N. meningitidis* strains (16), and each was bactericidal against strain NZ98/254 (fHbp v.1) when tested in combination with a second MAb, JAR 4 (Table 1), but not when tested with each other (Table 1).

Based on our previous data, JAR 3 and JAR 5 bound to strains expressing fHbp in the v.1 group, including MC58, 4243, M1390, and NZ98/254, which had a glycine at position 121, but not to strain M6190, which had an arginine at position 121 (29). Another strain, 03S-0408, also did not react with JAR 3 and JAR 5; this strain had residue G121 but contained serine at position 122 rather than lysine, which was present in all of the reactive strains. The data from these natural polymorphisms suggested that this portion of the molecule is important for the expression of the JAR 3 and JAR 5 epitopes. We therefore used site-specific mutagenesis to change the glycine residue at position 121 in the fHbp sequence of strain MC58 to arginine. In a second mutant, we changed the lysine at position 122 to serine. Both substitutions resulted in the loss of JAR 3 and JAR 5 reactivity. The data for the G121R substitution are shown in Fig. 5A and B, lanes 4. A converse change in fHbp from strain M6190, R121G, introduced the JAR 5 epitope (Fig. 5A, lane 6) and, to a lesser extent, the JAR 3 epitope (Fig. 5B, lane 6). The weaker signals for the M6190 mutant R121G protein relative to that of the wild-type protein indicated that additional residues were important for the expression of these epitopes. The Penta-His control MAb showed that the wild-type and mutant proteins were produced in similar quantities (Fig. 5C). A converse change in fHbp from strain 03S-0408, S122K, was not done.

Additional evidence that JAR 3 and JAR 5 recognized overlapping epitopes was derived from competitive inhibition experiments. As reported previously, by ELISA JAR 5 inhibited the binding of JAR 3 to fHbp by >90%, and JAR 3 inhibited the binding of JAR 5 by >90% (28). Thus, JAR 3 and JAR 5 recognized overlapping epitopes, since each of these MAbs inhibited the binding of the other to fHbp.

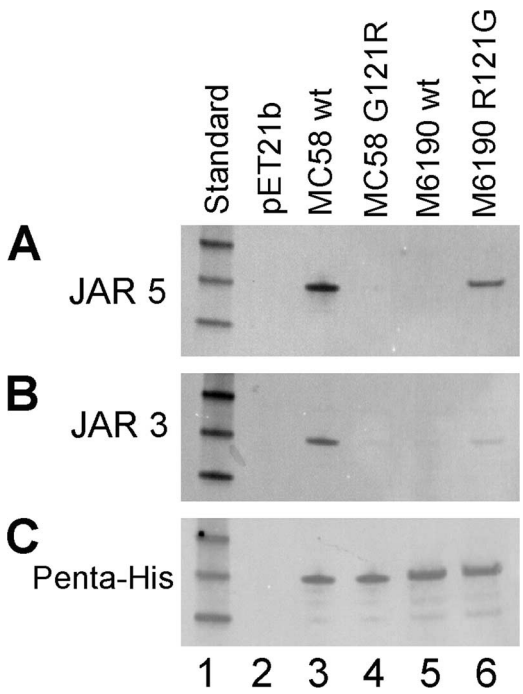

FIG. 5. Western blot showing a residue that is important for the binding of anti-fHbp MAbs JAR 3 and JAR 5. The epitopes for JAR 3 and JAR 5 were eliminated by the replacement of glycine (G) by arginine (R) at position 121 (G121R) in fHbp from strain MC58 and were introduced with substitution R121G in fHbp from strain M6190. The numbering is described in the legend to Fig. 3. (A) JAR 5. (B) JAR 3. (C) Penta-His MAb.

DISCUSSION

In this study, we identified the regions of the fHbp molecule that are important for the binding of six bactericidal MAbs from mice immunized with fHbp v.2 or v.3 and for two additional bactericidal MAbs previously prepared from a mouse immunized with fHbp v.1. Evidence for the locations of the amino acids affecting epitope expression for all but one of the MAbs came both from the loss of binding by KO mutations of naturally reactive proteins and the expression of the epitope by the corresponding KI mutations of wild-type proteins that were negative for binding (Table 3). However, a further definition of the contact residues of the epitopes will require making additional substitutions at neighboring residues in the three-dimensional structure.

We used the coordinates from the nuclear magnetic resonance solution structure of the combined B and C domains of fHbp v.1 (strain MC58) (4) to construct a model showing the locations of the residues involved in the expression of the different epitopes (Fig. 6). The previously described B and C domains (13) are depicted in dark and light gray, respectively. All of the epitopes of MAbs from mice immunized with the v.2 or v.3 proteins were affected by residues spanning amino acids 174 to 216, which were located within an eight-stranded beta-barrel of the C domain (4). In contrast, the expression of the epitopes for JAR 3 and JAR 5, which bound specifically with fHbp v.1 (3, 29), were affected by amino acid substitutions of residues G121 or K122, which were located in the B domain. Note that some other bactericidal MAbs reactive with fHbp v.1 bound to epitopes located in the C domain. These included JAR 10, which was raised against a v.2 protein but which cross-reacted with a subvariant of fHbp v.1 (3). The expression of this epitope required amino acids K180 and E192 in the C

TABLE 3. Summary of Western blotting data for residues involved in MAb epitopes

Immunogen group and MAb[c]	Reactive residue(s)[a]	Nonreactive strain	Nonreactive residue(s)	Evidence[b]
v.1, strain MC58				
JAR 3	G121	M6190	R121	KO, KI
	K122	03S-0408	S122	KO
JAR 5	G121	M6190	R121	KO, KI
	K122	03S-0408	S122	KO
v.2, strain 2996				
JAR 10	K180 and E192	M1239	R180 or D192	KO, KI
JAR 11	A174	M1239	K174	KO
JAR 13	S216	RM1090	G216	KO, KI
v.3, strain M1239				
JAR 32	K174	8047	A174	KO, KI
JAR 33	R180 and E192	8047	K180 or D192	KO, KI
JAR 35	K174	8047	A174	KO, KI

[a] Reactive residue in fHbp from the strain used as the source for immunization.

[b] KO indicates that the KO mutation abolished reactivity in the wild-type protein, and KI indicates that the KI mutation restored reactivity in the nonreactive wild-type protein.

[c] By ELISA, JAR 3 inhibited the binding of JAR 5 and JAR 5 inhibited the binding of JAR 3. JAR 32 inhibited the binding of JAR 35 (but not vice versa), and JAR 13 inhibited the binding of JAR 10 (Fig. 6).

domain. Also, in a previous study, MAb 502, which was specific for fHbp v.1, mapped to R204 in the C domain (13) (Fig. 6B).

Factor H has been reported to bind fHbp from each of the three variant groups (16), which indicated that the critical contact residues must be conserved. With the exception of the MAbs reacting with fHbp epitopes involving the ion pair at residue positions 180 and 192 (Fig. 6), the other MAbs directed at epitopes identified on the B or C domain strongly or partially inhibited the binding of fH (Fig. 2). These results suggested that multiple sites on the fHbp molecule are in contact with fH, or that the binding of the MAbs to fHbp resulted in a conformational change in fHbp that decreased fH binding.

We investigated the interactions of human complement and anti-fHbp MAbs at the surface of three *N. meningitidis* strains expressing fHbp in the v.1, v.2, or v.3 group. The primary endpoint was a complement-dependent decrease of 50% in the number of CFU per milliliter measured after 1 h of incubation of the reaction mixture compared to the respective number of CFU per milliliter present at time zero. This endpoint provides a sensitive and reproducible measure of antibody functional activity and has been used for many decades as a serologic correlate of protection against developing meningococcal disease in humans (14). At antibody concentrations of up to 50 μg/ml, none of the anti-fHbp MAbs individually elicited serum bactericidal activity against the three *N. meningitidis* strains. Note that in our previous study, the binding of anti-fHbp MAbs to the bacterial surface was saturated through a wide range of MAb concentrations (29). Further, binding by an individual MAb did not result in sufficient immune complex to engage C1q and activate classical complement pathway bactericidal activity (18, 30). However, if the amount of immune complex was increased on the bacterial surface by the binding of two anti-fHbp MAbs, or by binding by an individual anti-fHbp to a mutant strain of a strain that naturally expresses a low level of fHbp that was engineered to have increased expression of fHbp, there was an activation of the classical complement pathway and bacteriolysis (28).

The pairs of anti-fHbp MAbs with cooperative bactericidal activity appeared to be specific for binding to certain combi-

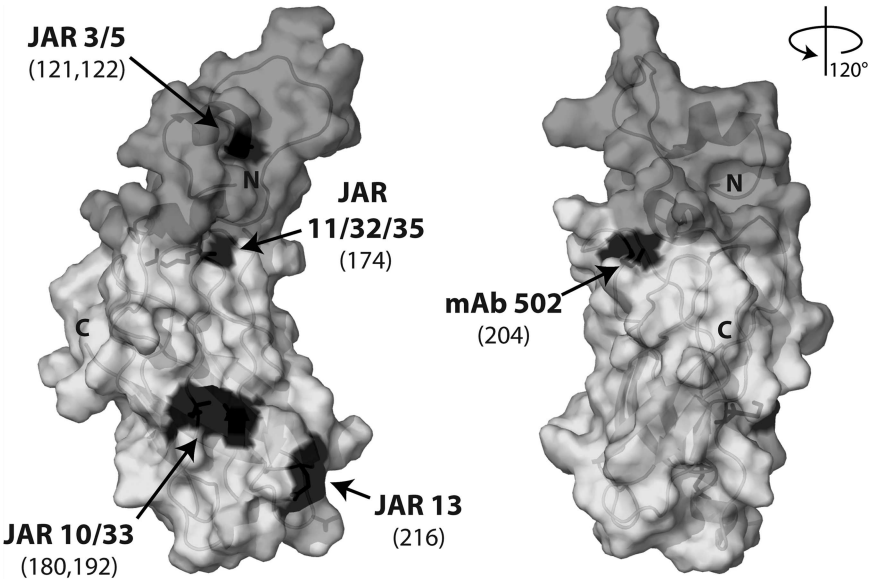

FIG. 6. Model of the locations of amino acid residues affecting the expression of fHbp epitopes. Coordinates were from the solution structure of fHbp v.1 of strain MC58 (4). The B and C domains are depicted in dark and light gray, respectively. The positions of the N and C termini are indicated. (Left) Locations of amino acid residues involved in the MAb epitopes are shown in black, with their respective residue numbers shown in parentheses. (Right) Molecule rotated 120° relative to the image in the left panel. The location of the residue previously reported to be involved in the epitope of MAb 502 is shown (13). Note that not all epitopes are present on fHbp v.1 from strain MC58, but the corresponding amino acid residues involved in the epitopes expressed by fHbp v.2 and v.3 proteins are indicated. The numbering is described in the legend to Fig. 3. The figure was generated using PyMol (http://www.pymol.org).

TABLE 4. Bactericidal activity of combinations of MAbs in relation to the respective locations of the epitopes

JAR MAb pair	Strain (variant)	Combination BC_{50}, in μg/ml[a]	Residues in epitopes[b]	Approx distance[c] (Å)	fH inhibition[d]	Ig isotypes
5 and 502[e]	H44/76	<1	G121 and R204	16	++ and ND	G2b and G2a
10 and 11	8047 (v.2)	5	K180/E192 and A174	18–20	− and +	G1 and G2a
33 and 32	M1239 (v.3)	1	R180/E192 and K174	18–20	− and ++	G2a and G2a
33 and 35	M1239 (v.3)	5	R180/E192 and K174	18–20	− and ++	G2a and G2b
3 and 10	NZ98/254 (v.1)	>50	G121 and K180/E192	31–32	++ and −	G3 and G1
5 and 10	NZ98/254 (v.1)	>50	G121 and K180/E192	31–32	++ and −	G2b and G1
13 and 35	M1239 (v.3)	>50	S216 and K174	27	++ and ++	G2a and G2b
13 and 11	8047 (v.2)	>50	S216 and A174	27	++ and +	G2a and G2a
13 and 32	M1239 (v.3)	>50	S216 and K174	27	++ and ++	G2a and G2a
13 and 33	M1239 (v.3)	>50	S216 and R180/E192	9–14	++ and −	G2a and G2a
13 and 10	8047 (v.2)	>50	S216 and K180/E192	9–14	++ and −	G2a and G1
3 and 5	NZ98/254 (v.1)	>50	G121 and G121	0	++ and ++	G3 and G2b
32 and 35	M1239 (v.3)	>50	K174 and K174	0	++ and ++	G2a and G2b

[a] Data are shown only for MAbs that individually were not bactericidal against the test strain (BC_{50} > 50 μg/ml).
[b] For the respective MAbs in the pair. The numbering of the residues is based on the amino acid sequence of MC58 v.1 fHbp lacking the signal sequence (17).
[c] Distances between the pairs of the MAbs were calculated between alpha-carbon positions for the respective residues using PyMol (http://www.pymol.org). JAR 32 and JAR 35 recognize overlapping (or identical) epitopes including K174.
[d] ++, inhibition of fH binding to rfHbp (>70%); +, partial inhibition (25 to 45%); −, no inhibition (<15%) as measured by ELISA (Fig. 2).
[e] The amino acid affecting the expression of the MAb 502 epitope was described by Giuliani et al. (13). The inhibition of fH binding was not determined (ND).

nations of positions on the molecule (Table 4). For example, the binding of one MAb to an epitope involving an ion pair between amino acid residues 180 and 192, in combination with the binding of a second MAb to an epitope involving residue 174, elicited bactericidal activity against test strains expressing fHbp in the v.2 or v.3 group. Bactericidal activity was independent of the specific respective amino acids at these locations in fHbp from the two strains. However, if one MAb bound to one of these locations and a second MAb bound to an epitope involving residue 216 or 121, there was no bactericidal activity. In contrast, MAbs binding to epitopes involving residue 216 or 121 were bactericidal in combination with other MAbs, such as JAR 4 or JAR 36, for which we do not yet have information on the location of the amino acids affecting epitope expression (Table 1). For pairs of MAbs for which we had information on the residues affecting epitope expression, we observed no bactericidal activity if the respective residues were too close (≤14 Å) (Table 4). For all such pairs of MAbs, we observed the competitive inhibition of the binding of one MAb to fHbp by the second MAb as measured by ELISA. For example, in a previous study, JAR 5 inhibited the binding of JAR 3 (28). In the present study, JAR 32 inhibited the binding of JAR 35, and JAR 13 inhibited the binding of JAR 10 (data not shown). For pairs for which the respective residues were too far apart (≥27 Å) (Table 4), we did not observe an ELISA inhibition of MAb binding to fHbp by one member of the pair. Our hypothesis to explain the lack of the bactericidal activity of these MAb pairs is that the distance between the respective epitopes was too great and/or the orientation of the Fc regions of the respective IgG molecules was not optimal for the engagement of C1q and the activation of the classical complement pathway.

We also observed that for all pairs of anti-fHbp MAbs with bactericidal activity, at least one member of the pair inhibited the binding of fH. This was true for all pairs of MAbs for which the residues affecting epitope expression were identified for each member of the pair (Table 4) and for pairs for which we lacked information on residues affecting the epitope expression of one or both MAbs (for example, pairs with JAR 4) (Table 1). However, there were examples of nonbactericidal pairs for which one or both MAbs inhibited the binding of fH (i.e., JAR 11 and 13 or JAR 13 and 32) (Table 1). Thus, the inhibition of the binding of fH may be necessary but is not sufficient for cooperative anti-fHbp MAb bactericidal activity against *N. meningitidis* strains expressing low levels of fHbp.

Collectively, the data suggested a model for anti-fHbp cooperative MAb bacteriolysis of strains with low levels of fHbp expression. The model requires both the formation of a critical amount of immune complex to permit two IgG molecules to engage C1q and activate complement-mediated bactericidal activity (18, 30) and the inhibition of fH binding, which increases the susceptibility of the organism to bacteriolysis by the amplification of the alternative complement pathway. In contrast, for bactericidal activity by antibodies to other minor meningococcal outer membrane protein antigens that do not downregulate complement activation, it was necessary to have antibodies present that targeted more than one antigen to create sufficient immune complex for complement-mediated bactericidal activity (30).

Finally, the presence of the JAR 3 and 5 epitopes in the B domain of fHbp that were capable of interacting with bactericidal antibodies specific for fHbp v.1, and the presence of other epitopes on the C domain capable of interacting with bactericidal antibodies specific for fHbp in the v.2 and v.3 groups, provided the rationale for the construction of chimeric fHbps containing the respective regions. As recently reported (1), knowledge of the locations of amino acid residues affecting these epitopes allowed us to design a vaccine consisting of a single recombinant chimeric protein that elicited serum bactericidal antibody responses against genetically diverse strains expressing fHbp from different variant groups.

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