Molecular characterization of two sub-family specific monoclonal antibodies to meningococcal Factor H binding protein

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Abstract

Factor H binding protein (FHbp) is a component of two licensed vaccines for prevention of sepsis and meningitis caused by serogroup B meningococci. FHbp binds human Factor H (FH), which contributes to evasion of host immunity and FHbp sequence variants can be classified into two sub-families. Antibodies against FHbp elicit complement-mediated killing and can inhibit recruitment of FH to the bacterial surface. We report epitope mapping studies of two murine IgG mAbs, designated JAR 31 and JAR 36, isolated from a mouse immunized with FHbp in sub-family A, which is present in ~30–40% of invasive isolates. In the present study, we tested the reactivity of mAbs JAR 31 and JAR 36 with seven natural FHbp sequence variants from different phylogenetic groups. We screened bacteriophage-displayed peptide libraries to identify amino acid residues contributing to the JAR 36 epitope. Based on the reactivities of mAbs JAR 31
and JAR 36 with the seven FHbp variants, and the frequent occurrences of aspartate (D) and lysine (K) residues in the JAR 36-bound phage peptides, we selected six residues in the carboxyl-terminal region of FHbp for replacement with alanine (A). The D201A and K203A substitutions respectively eliminated and decreased binding of mAbs JAR 31 and JAR 36 to FHbp. These substitutions did not affect binding of the control mAb JAR 33 or of human FH. JAR 31 or JAR 36 mediated cooperative complement-mediated bactericidal activity with other anti-FHbp mAbs. The identification of two amino acid residues involved in the epitopes recognized by these anti-FHbp mAbs may contribute to a more complete understanding of the spatial requirements for cooperative anti-FHbp mAb bactericidal activity.

Keywords: Biochemistry, Immunology, Microbiology, Molecular biology

1. Introduction

Factor H (FH) is an important down-regulatory protein of the alternative complement pathway and binding of FH to microbial pathogens contributes to evasion of host-mediated immunity [1, 2]. FH-binding protein (FHbp) is a surface-exposed lipoprotein that recruits human FH to the surface of meningococci [3]. Recombinant FHbp is part of two licensed vaccines, which are referred to as MenB-FHbp (Trumenba®) and MenB-4C (Bexsero®), for prevention of meningococcal disease caused by serogroup B strains [4].

Antibodies to FHbp activate complement-mediated bactericidal activity [5, 6, 7] and also inhibit binding of FH to the bacteria [3], which increases susceptibility of the organisms to complement-mediated bacteriolysis [8, 9]. The development of broadly protective protein-based meningococcal vaccines requires overcoming two limitations of the FHbp antigen. First, FHbp exists in two antigenic sub-families with no cross-protective activity between sub-family A (which can be subdivided into variant groups 2 and 3 [5]) and sub-family B (variant group 1) [6]. Therefore, two or more FHbp variants might be necessary to elicit protective immunity against the majority of strains. Second, FHbp has low expression in many strains, which limits susceptibility to anti-FHbp bacteriolysis [10, 11]; thus, other protein antigens might be needed to confer broad protection against meningococci.

In previous studies, we prepared 13 murine anti-FHbp mAbs against representative FHbp sequence variants from the three major phylogenetic variant groups [7, 12, 13]. Against strains with low to moderate FHbp expression, no individual mAb had complement-mediated bactericidal activity but certain pairwise combinations of anti-FHbp mAbs were able to activate complement-mediated bacteriolysis (i.e., cooperative or synergistic anti-FHbp mAb bactericidal activity [8, 12, 14, 15]). To
define the regions of FHbp recognized by the different mAbs in previous studies, we used multiple sequence alignments of reactive and non-reactive FHbp sequence variants [12], a random mutant FHbp library expressed on the surface of yeast [13, 16] or peptide phage display [14], each coupled with site-specific mutagenesis, to identify amino acid residues that affected mAb binding. Collectively, the data suggested that the distances between epitopes recognized by different combinations of mAbs were important for the ability of two IgG molecules to engage C1q, activate the classical complement pathway and elicit cooperative bactericidal activity [12]. Recently, crystal structures of two FHbp-Fab complexes were used to elucidate the structural basis for cooperative mAb bactericidal activity [15, 17].

Because of the central role of FHbp in meningococcal pathogenesis [3, 8, 18, 19, 20], and the inclusion of this antigen in the development of two licensed vaccines [21, 22], it is important to increase our understanding of the mechanisms by which anti-FHbp antibodies elicit cooperative complement-mediated bactericidal activity. The purpose of the present study was to identify FHbp amino acid residues affecting binding by two anti-FHbp mAbs, designated JAR 31 and JAR 36, which are two anti-FHbp mAbs whose epitopes remained undefined. The epitopes recognized by these mAbs were of interest because in previous studies the two mAbs cross-reacted with all FHbp amino acid sequence variants tested from FHbp sub-family A [11, 12, 13] and JAR 36 was previously reported to elicit cooperative complement-mediated bacteriolysis when tested with other anti-FHbp mAbs [12].

2. Materials and methods

2.1. Peptide selection

Peptides that bound to mAb JAR 36 were selected by panning five phage libraries constructed in the two-gene phagemid vector pC89 [23]. Details on the construction of the libraries, designated as pVIII-9aa, pVIII-12aa, pVIII-9aa.Cys, pVIII-Cys.Cys and pVIII-15aa, have been already described in detail elsewhere [23].

Specific phage clones were isolated from the libraries by immuno-affinity selection, using two different techniques to minimize the risk of selecting non-specific clones. In the biopanning approach, 1 μg/ml of JAR 36 mAb was incubated overnight at 4 °C with 10^10 transducing units of each library, in a total volume of 20 μl of PBS. The mixture was incubated with 0.8 μg of biotinylated goat anti-mouse IgG antibody (Fc specific, Sigma, St Louis, MO, USA), which had been pre-adsorbed overnight at 4 °C with 3 × 10^10 UV-inactivated M13 KO7 phage particles to prevent non-specific binding. This mixture was added to a streptavidin-coated 6 cm Falcon 1007 Petri dish and incubated for 10 min at room temperature. After 10 washes with 1 ml of washing solution, bound phages were eluted with 800 μl 0.1 N HCl, adjusted to pH 2.2 with glycine and 10 mg/ml BSA. The solution was neutralized using 60 μl
of 2 M Tris–HCl, pH 9.6 and the phage were amplified by infecting *Escherichia coli* TG1 cells. The second and third rounds of selection were done in the same way, except 10 ng/ml and 1 ng/ml of the mAb were used, respectively.

In the Dynabeads approach, the JAR 36 mAb (1 µg/ml) was incubated with magnetic beads conjugated with protein G (50 µg protein G-Dynabeads®, Dynal, Norway) for 1 h at room temperature under agitation. The beads were washed 3 times with washing solution (PBS, 0.5% Tween-20), and approximately $10^{10}$ ampicillin-transducing units of library preparation ($\sim 10^{11}$ phage particles) in a volume of 100 µl, were added to 900 µl of blocking solution (PBS, 5% non-fat dry milk, 0.05% Tween-20) and agitated for 3–4 h at room temperature. After 10 washes with 1 ml of washing solution, bound phages were eluted with 500 µl 0.1 N HCl, adjusted to pH 2.2 with glycine and 10 mg/ml BSA. The solution was neutralized and the phages were amplified by infecting *E. coli* TG1 cells. The second and third rounds of panning were performed as described above, but using $10^{10}$ ampicillin-transducing units obtained from the first and the second round of amplified phage pools, respectively.

For each library, either individually or in combination, three rounds of selection were performed using the JAR 36 mAb as bait. After each selection cycle, the reactivity of enriched pools was tested by phage ELISA as described below, using the pC89 phage vector (not encoding a foreign peptide) as a negative control, in order to detect presence of JAR 36-specific clones in the selected mixtures containing foreign peptides.

The observed frequency is defined as the ratio between the number of times an amino acid is observed versus the total number of amino acids in sequenced peptide clone inserts; the expected frequency is the ratio between the number of different codons in the library for each amino acid and the total number of different codons that are present in the inserts in each phage library. For the construction of pVIII-9aa and pVIII-9aa. Cys libraries all 64 codons were used; for the pVIII-Cys.Cys and pVIII-15aa libraries only 32 codons were used (NN C/G); and for the pVIII-12aa library the most frequently used codon for each of the 20 amino acids, according to a codon usage table for highly expressed genes in *E. coli* [24], were used. Positive phage clones were identified through immunoscreening as described previously [25].

### 2.2. Direct binding ELISA

Binding of JAR 36 mAb to peptides displayed by the phage library selected clones was confirmed by phage ELISA. Ninety-six well plates were coated with mAb JAR 36 (100 µl per well, 0.2 µg/ml in 50 mM NaHCO₃, 0.02% (w/v) Na₃N, pH 9.6) and incubated overnight at 4 °C. The plates were washed 8 times with TBST (50 mM Tris–HCl, 150 mM NaCl, pH 7.5, 0.05% (v/v) Tween-
20). One hundred μl per well of phage supernatant were added and the plates were incubated for 2 h at 37 °C. After washing, 100 μl of a horseradish peroxidase-conjugated anti-M13 mAb (Amersham Biosciences, Buckinghamshire, UK, 1:5,000) were added and incubated for 1 h at 37 °C. Antibody binding was detected by adding 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) and reading the OD at 450 nm after 45 min.

2.3. Construction of site-specific mutants

Site-specific mutants were chosen by targeting charged residues (D or K) that were over-represented in phage peptide inserts (Table 1), were present in the last 70 residues of the FHbp ID 28 sequence and that were predicted to be surface-exposed based on the crystal structure of FHbp in a complex with a fragment of human FH [26]. The sequences of the mutagenic oligonucleotides were: K199A_fwd, GCAGCAGATGAAAATCACCAG; K199A_rev, GAGTTCCGGCGGCCGGCA; D201A_fwd, GCTGAAAAATCA-CACGCGC; D201A_rev, TGCTTTGAGTTCGCGG; K203A_fwd, GCATCACACGCGCTC; K203A_rev, TTCACTGTATTAGTTAGGTCGC; D211A_fwd, GCCACGCACGCTACGCCAGC; D211A_rev, GCCCAAAATGACGGCGT; K241A_fwd, GCAGTTCACGAAATC; K241A_rev, CACGGTTGCCGAGCCG; K245A_fwd, CAGTTCACGAAATCGCC; K245A_rev, CTTCCTATCTACGGTGC; mutated nucleotides in the forward oligonucleotide sequences are underlined. The oligonucleotides were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) prior to PCR amplification. Mutants were constructed using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific, Waltham, MA) using the manufacturer’s protocols. The mutagenesis reactions were transformed into chemically competent E. coli DH5α (Invitrogen, Carlsbad, CA) and independent mutant clones were verified by DNA sequencing (Davis Sequencing, Davis, CA).

2.4. Purification of recombinant FHbp

The wild-type FHbp ID 28 and site-specific mutant proteins were expressed from the T7 promotor using the E. coli plasmid pET21b (Novagen, Madison, WI) as described previously [5, 27]. The recombinant proteins were purified by immobilized metal ion chromatography using Ni-NTA agarose (Qiagen, Valencia, CA) as described previously. Purified FHbps were dialyzed against PBS, sterilized by filtration (Millex 0.22 μm; Millipore, Billerica, MA), and stored at 4 °C prior to use. The protein concentrations were determined by UV absorbance (Nanodrop 1000, Wilmington, DE) based on the extinction coefficient calculated from the amino acid sequence [28].
2.5. Binding of anti-FHbp mAbs and human FH to FHbp

Binding of anti-FHbp mAbs to purified recombinant FHbp was measured by ELISA. Purified FHbp (2 μg/ml) was adsorbed to the wells of a microtiter plate overnight, which subsequently were blocked with PBS containing 0.1% (v/v) Tween-20 (Sigma-Aldrich, St. Louis, MO) and 1% (w/v) BSA. Anti-FHbp mAbs were added at concentrations ranging from 0.008 to 25 μg/ml and incubated for 1 h at 37 °C. Bound antibody was detected (1 h at room temperature) with goat anti-mouse IgG conjugated with alkaline phosphatase (AP) (Invitrogen, Carlsbad, CA). After 30 min of incubation with p-nitrophenyl phosphate (1 mg/ml; Sigma) at room temperature, the optical density at 405 nm was measured.

To measure binding of human FH to FHbp, an ELISA was employed with the same coating and blocking steps as above. Human FH (Complement Technology, Inc., Tyler, TX) was added at concentrations from 0.008 to 25 μg/ml and incubated for

| Amino acid | Observed | Expected | Relative (95% CI) |
|------------|----------|----------|-----------------|
| Alanine (A) | 0.072 | 0.063 | 1.16 (0.62, 1.94) |
| Arginine (R) | 0.061 | 0.088 | 0.69 (0.35, 1.22) |
| Asparagine (N) | 0.050 | 0.038 | 1.33 (0.61, 2.48) |
| Aspartate (D) | 0.078 | 0.038 | 2.07 (1.15, 3.39) |
| Glutamine (Q) | 0.039 | 0.038 | 1.04 (0.43, 2.11) |
| Glutamate (E) | 0.050 | 0.038 | 1.33 (0.61, 2.48) |
| Glycine (G) | 0.139 | 0.063 | 2.22 (1.47, 3.17) |
| Histidine (H) | 0.006 | 0.038 | 0.15 (0.00, 0.83) |
| Isoleucine (I) | 0.028 | 0.045 | 0.61 (0.20, 1.41) |
| Leucine (L) | 0.078 | 0.088 | 0.89 (0.49, 1.45) |
| Lysine (K) | 0.083 | 0.038 | 2.22 (1.25, 3.57) |
| Methionine (M) | 0.006 | 0.030 | 0.19 (0.00, 1.04) |
| Phenylalanine (F) | 0.028 | 0.038 | 0.74 (0.24, 1.71) |
| Proline (P) | 0.033 | 0.063 | 0.53 (0.19, 1.15) |
| Serine (S) | 0.017 | 0.088 | 0.19 (0.05, 0.55) |
| Threonine (T) | 0.022 | 0.063 | 0.36 (0.10, 0.90) |
| Tryptophan (W) | 0.111 | 0.030 | 3.74 (2.32, 5.61) |
| Tyrosine (Y) | 0.028 | 0.038 | 0.74 (0.24, 1.71) |
| Valine (V) | 0.067 | 0.063 | 1.07 (0.56, 1.82) |

* Cys (C) residues were eliminated from the analysis because they were fixed in a subset of the phage libraries.

* Observed frequency of each amino acid in peptides bound by JAR 36.

* Expected frequency of each amino acid in the five phage libraries used.

* Ratio between observed frequency and expected frequency and 95% confidence interval calculated from the Gaussian distribution.
2 h at room temperature. Bound FH was detected with sheep anti-human FH (Abcam, Cambridge, MA; 1:7,000) and donkey anti-sheep IgG conjugated with AP (Sigma-Aldrich, St. Louis, MO; 1:10,000). Development was performed as described above.

For measuring inhibition of binding of anti-FHbp mAbs to FHbp, JAR 31 was harvested from hybridoma culture supernatant, purified by chromatography using a protein G column (HiTrap Protein G HP 1 ml, GE Life Sciences) and conjugated to AP (EasyLink Alkaline Phosphatase Conjugation Kit, AbCam) according to the manufacturer’s protocol. Equal volumes of AP-conjugated JAR 31 (2 μg/ml) and anti-FHbp mAb inhibitors (starting concentration of 40 μg/ml) were premixed and 100 μl were added to the wells of a microtiter plate that had been sensitized and blocked as described above. After addition of the antibody mixtures, the reactions were incubated for 2 hours at room temperature. The alkaline phosphatase was detected as described above.

2.6. Cooperative complement-mediated bactericidal activity

Human complement-mediated bactericidal activity was measured against serogroup B strains 8047 and M1239 as previously described [29]. The complement source was serum from a healthy adult obtained under a protocol that was approved by the UCSF Benioff Children’s Hospital Oakland Institutional Review Board (IRB). Written informed consent was obtained from the subject. The serum had normal hemolytic complement activity and no detectable serum bactericidal activity against the two strains. The serum was depleted of IgG using a protein G column (HiTrap Protein G, GE Life Sciences, Piscataway, NJ) as previously described [30] to remove non-bactericidal IgG antibodies. The bactericidal concentration (BC50) was calculated as the interpolated mAb concentration that yielded a 50% decrease in colony forming units (cfu/ml) after 60-min incubation compared with cfu/ml in negative control wells at time zero.

3. Results

3.1. Reactivity of JAR 31 and JAR 36 mAbs with different FHbp sequence variants

FHbp can be classified into modular groups [11, 31] based on different combinations of five variable segments, each encoded by genes from one of two lineages (shown in Fig. 1, Panel A as white or gray symbols). FHbp can contain all five segments from the same lineage (e.g. ID 1 and 28) or have individual segments derived from different lineages (e.g. ID 22, 15, 79, 77 and 207; Fig. 1, Panel A). Based on reactivity with FHbp variants in different modular groups, the modular architecture of FHbp can be useful for coarse-level mapping of anti-FHbp mAb epitopes.
JAR 31 and JAR 36 are IgG2b mAbs that were isolated from a mouse immunized with recombinant FHbp identification (ID) number 28 from sub-family A/variant group 3 (FHbp ID numbers are designated in the FHbp database at http://pubmlst.org/neisseria/fHbp/). The JAR 31 and JAR 36 mAbs reacted with recombinant FHbp ID 28, which was the immunogen used to generate the mAbs, by ELISA (Fig. 2, Panels A and B). Both mAbs cross-reacted with FHbp ID 79, ID 22 and 77 (in sub-family A) but not with FHbp ID 1 or 15 (in sub-family B). The JAR 31 and JAR 36 mAbs cross-reacted with a natural chimeric FHbp, ID 207. Although JAR 31 reactivity previously was reported with diverse FHbp variants in terms of positive or negative reactivity [13], we retested some of the same variants in parallel with JAR 36, using concentration-dependent binding to show relative binding affinities.

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**Fig. 1.** Schematic of the modular architecture of FHbp. A, Seven different FHbp sequence variants, designated by ID numbers, are shown along with their classifications into sub-families, variant groups and modular groups. FHbp is composed of five variable segments, each from one of two genetic lineages [11, 31]. FHbp ID 1 and ID 28 are designated as comprising variable segments from lineage 1 (shaded) and 2 (white), respectively. Each distinctive \( V_E \) segment is designated by two numbers, separated by a decimal point with the first number, 1 or 2, referring to the genetic lineage, and the second number referring to the ID number of the segment as annotated on the website http://pubmlst.org/neisseria/fHbp/. The variants that bind the mAbs JAR 31 and JAR 36 are designated with an asterisk. The schematic figure was adapted from a previous publication [11]. B, Amino acid sequences of the six distinct variable E (\( V_E \)) segments shown in panel A. The sequence begins with position 186 using the numbering of the mature FHbp ID 1 sequence. The residues D and K, which were over-represented in the phage sequences, are shown in bold in the sequence of \( E.2.6 \) and \( E.2.2 \).
Since JAR 31 and JAR 36 reacted with all of the sub-family A sequences tested and with the A/B hybrid ID 207 but not with the two sub-family B sequences tested, it appeared likely that the epitope was contained in the variable E (VE) segment, which comprises residues 186 to 255 (amino acid numbering is based on the sequence of the mature FHbp ID 1; http://pubmlst.org/neisseria/fHbp/). The FHbp variants that were bound by JAR 31 and JAR 36 are denoted with asterisks (Fig. 1, Panel A).

**Fig. 2.** Binding of murine anti-FHbp mAbs to seven purified recombinant FHbp variants by ELISA. A, Concentration-dependent binding of mAb JAR 31. B, Binding of mAb JAR 36. C, Binding of a control mAb, JAR 5, which reacts with the two variants not recognized by JAR 31 or JAR 36. Bound mAbs were detected with goat anti-mouse IgG conjugated to alkaline phosphatase. Binding of JAR 31 and JAR 5 to a larger set of FHbp variants previously was reported as positive or negative [13].
and all have E.2 type V_E segments. The amino acid sequences of the six distinct V_E segments are shown (Fig. 1, Panel B).

As a control, mAb JAR 5 [7], reacted with FHbp ID 1 and 15 in sub-family B but not with any of the sequence variants in sub-family A (Fig. 2, Panel C). The mAb JAR 5 also reacted with the chimeric FHbp ID 207. Binding of JAR 5 is specific for FHbp variants in sub-family B, and the epitope is located in the regions of residues 84—91 and 115—123 [15] in variable segment C (V_C) (Fig. 1, Panel A).

To test whether the JAR 31 and JAR 36 mAbs recognized overlapping epitopes, we used non-labeled mAbs to inhibit binding of alkaline phosphatase-labeled JAR 31 to the nominal FHbp ID 28 antigen by ELISA. Both JAR 31 and JAR 36 yielded 95% inhibition at the highest concentration tested, 20 µg/ml (Fig. 3). JAR 33, which was another mAb raised against FHbp ID 28 that recognizes an epitope involving residues R180 and E192 in the C-terminal domain [12], was used as a negative control and gave <25% inhibition at the highest concentration tested.

3.2. Peptide phage display

Based on data that the two mAbs reacted with the same FHbp sequence variants (Fig. 2), we sought to identify the epitope recognized by one of the mAbs, JAR 36, by peptide phage display. We screened five filamentous phage libraries displaying random peptides of different lengths fused to the N-terminus of the coat protein VIII (pVIII). Forty-five JAR 36-specific positive clones were identified and the sequences of the respective PCR amplified DNA fragments were determined. Among these clones were 15 independent peptide sequences, each of which showed an optical density ≥0.45 by ELISA (Table 2).

![Fig. 3. Inhibition of binding of mAb JAR 31 to FHbp ID 28 by mAb inhibitors. JAR 31 conjugated to alkaline phosphatase was held at a fixed concentration and the concentrations of the mAb inhibitors, JAR 31, JAR 36 and JAR 33, were varied. Percent inhibition was calculated relative to the optical density of wells containing no mAb inhibitor. Data points are the mean and range from duplicate measurements.](https://doi.org/10.1016/j.heliyon.2018.e00591)
Direct comparison of the peptide sequences isolated from phage display experiments did not allow us to identify a consensus sequence among the JAR 36-reactive peptides. Using an approach that we had shown to be successful in similar cases [32, 33, 34], we predicted that the most abundant amino acids in the bound peptides might be important for the interaction between the immunogen and the mAb. The observed frequency of each amino acid in the mimotopes and the expected frequency of each amino acid in the collective libraries were used to calculate a ratio representing the relative frequency (Table 1). The results indicated higher occurrences of aspartate (D), glycine (G), lysine (K) and tryptophan (W), each with a normalized ratio >2.0 (Table 1). Tryptophan does not occur in any known FHbp sequence [11, 35], and glycine has a hydrogen atom as its side chain. Consequently, we predicted that the electrostatically charged residues D or K likely contribute to the JAR 36 epitope.

### 3.3. Site-specific mutagenesis of D and K residues

Since JAR 31 and JAR 36 reacted with FHbp sequence variants containing variable E (V_E) segments from lineage 2 (Fig. 1, Panel A), we focused our attention on the

### Table 2. Amino acid sequences of the phage-displayed peptides mimicking the JAR 36 epitope.a

| Sequence               | No. of Clones | ELISA OD | SD  |
|------------------------|---------------|----------|-----|
| AKWCAQFCQGYL           | 2             | 3.10     | 0.42|
| AKWCNWLCTWVVG          | 3             | 2.25     | 0.35|
| GKOCAWCEWFA            | 1             | 1.10     | 0.14|
| GKGCTRGGCDVD           | 2             | 1.00     | 0.39|
| WSDKDRNLWGLWYRE        | 4             | 0.78     | 0.05|
| QARCIVEEECKWA          | 3             | 0.68     | 0.16|
| LGWCMDGLCKGV           | 2             | 0.65     | 0.14|
| NKFVSLGLA              | 5             | 0.65     | 0.09|
| QKWFALGAPWYD           | 3             | 0.60     | 0.10|
| WNNWNGKPTRDE           | 1             | 0.57     | 0.04|
| GKCMLVDCNRE           | 1             | 0.57     | 0.04|
| RPGPGIDII              | 13            | 0.57     | 0.16|
| KVCQLWGNNCGE           | 2             | 0.55     | 0.07|
| GCGKWEIDGCAA           | 2             | 0.55     | 0.07|
| VRSKWEVGRPYPDYDV       | 1             | 0.45     | 0.08|

*a positive phage clones ranked by their reactivity with JAR 36.
b deduced amino acid sequences of the peptide inserts displayed through pVIII fusion on the phage library clones positive for JAR 36.
c reactivity of phage clone with mAb JAR 36, determined by ELISA.
d SD value is the standard deviation of the mean (n = 2).
amino acid residues, D and K, that were over-represented in the phage peptide sequences, and that were located between positions 186 and 255 of FHbp ID 28 (Fig. 1, Panel B). This region of FHbp contained three D and six K residues that were conserved in V_E segments from lineage 2 (Fig. 1, Panel B). To decrease further the number of possible residues to test experimentally, we examined the positions of the D and K residues in the structure of FHbp ID 28 (Fig. 4). Seven of the nine D or K residues occurred in two clusters near the FH binding site of the protein, which was consistent with previous observations that JAR 36 inhibits binding of FH to FHbp [12]. Cluster 1 included K199, D201 and K203 on one end of the C-terminal beta-barrel domain, and Cluster 2 included D211, K219, K241 and K245 on the other end of the beta-barrel.

Fig. 4. Location of residues predicted to affect binding of the JAR 31 and JAR 36 mAbs. The atomic coordinates were from the crystal structure of FHbp ID 28 in a complex with FH domains 6 and 7, which were omitted for clarity (PDB accession number 4AYI) [36]. The figure was generated using PyMol (http://www.pymol.org). A, The protein is oriented with the FH interaction surface at the top. Seven of the nine D or K residues occurred in two clusters, 1 (green) or 2 (blue). The residues K229 and K254 did not cluster with the other D or K residues and are shown in yellow. B, Same coloring as panel A, with view rotated by 90° around the X-axis. Cluster 1 included K199, D201 and K203 (green) and Cluster 2 included D211, K219, K241 and K245 (blue).
We substituted the codons for the three residues in Cluster 1 and three of the four residues in Cluster 2 with codons encoding alanine (A) and expressed and purified the FHbp mutants. JAR 31 and JAR 36 reacted similarly with the wild-type FHbp ID 28 and the Cluster 1 mutant K199A, slightly less with the K203A mutant and not with the D201A mutant (Fig. 5, Panels A and C, respectively for JAR 31 and JAR 36).

Fig. 5. Binding of anti-FHbp mAbs and Factor H to site-specific mutants of FHbp ID 28 by ELISA. A, Binding of mAb JAR 31 to FHbp ID 28 wild-type (WT, solid line) and Cluster 1 mutants K199A, D201A, K203A. B, Binding of control mAb JAR 33 to the WT and three Cluster 1 mutants. C, Binding of mAb JAR 36 to the WT and three Cluster 1 mutants. D, Binding of human Factor H to the WT and three Cluster 1 mutants. The symbols in Panels B—D are the same as in Panel A. E, Binding of mAb JAR 31 to FHbp ID 28 WT and Cluster 2 mutants D211A, K241A and K245A. F, Binding of mAb JAR 33 to FHbp ID 28 WT and three Cluster 2 mutants. The symbols used are shown in panel E. In all six panels, symbols represent the means and error bars represent the standard error (SE) from two to six independent measurements.
As a positive control to demonstrate that the D201 mutant was not structurally impaired, we tested binding of anti-FHbp mAb JAR 33, which reacted similarly with the native ID 28 and all six of the mutants (Fig. 5, Panel B). As a further probe of the conformational integrity of the Cluster 1 mutants, we tested binding of human FH, which interacts with both structural domains of FHbp. FH bound to the WT FHbp and to the D201A and K203A mutants similarly (Panel D). Whereas the K199A mutant bound to all three mAbs similarly to WT FHbp, this mutant had ~10-fold decreased binding of human FH, which also was observed in a previous study using surface plasmon resonance [36]. The decreased binding of FH by the K199A mutant, but preserved binding of the three anti-FHbp mAbs indicates that this substitution specifically affects FH binding.

Alanine substitutions of three residues in Cluster 2 had no effect on binding of JAR 31 or JAR 33 (Fig. 5, Panels E and F, respectively). Although we did not substitute the Cluster 2 residue K219 in the present study, the asparagine (N) substitution, K219N, did not decrease binding of JAR 31 in a related FHbp variant, ID 22, in recent study [37] or in FHbp ID 28 (R. Rossi and P.T. Beernink, unpublished data).

3.4. Cooperative mAb bactericidal activity

Finally, we tested combinations of JAR 31 or JAR 36 with other anti-FHbp mAbs for cooperative bactericidal activity. Either mAb was bactericidal in combination with anti-FHbp mAb JAR 11 or JAR 13 (Table 3), which were raised against FHbp ID

| Anti-FHbp mAb (IgG Subclass) | Reactive Residue(s) | Test Strain (FHbp ID) | Combination BC50, µg/ml |
|-------------------------------|---------------------|------------------------|------------------------|
| JAR 4 (2a)                    | D25, H26, K27       | 8047 (77)              | >50                    |
| JAR 10 (1)                    | K180, E192          | 8047 (77)              | >50                    |
| JAR 11 (2a)                   | A174                | 8047 (77)              | 2                      |
| JAR 13 (2a)                   | S216                | 8047 (77)              | 1                      |
| JAR 13 (2a)                   | S216                | M1239 (28)             | 5                      |
| JAR 32 (2a)                   | K174                | M1239 (28)             | >50                    |
| JAR 33 (2a)                   | R180, E192          | M1239 (28)             | >50                    |
| JAR 35 (2b)                   | K174                | M1239 (28)             | >50                    |

*Reactive residue(s), amino acid residue affecting epitope expression [12, 14].

FHbp ID numbers are from the database at http://pubmlst.org/neisseria/FHbp/ and the corresponding variant groups and modular groups are shown in Fig. 1, Panel A.

None of the mAbs individually was bactericidal (BC50 > 50 µg/ml). JAR 4, JAR 10, JAR 32, JAR 33 and JAR 35 elicited cooperative bactericidal activity (BC50 < 50 µg/ml) with other anti-FHbp mAbs not shown [12]. Bactericidal mAb combinations involving JAR 36 were determined previously [12].
Neither mAb elicited cooperative bactericidal activity with JAR 4 or JAR 10 against strain 8047 (sub-family A, variant group 2), or with JAR 32, 33 or 35 against strain M1239 (sub-family A, variant group 3). Although similar data previously were reported for pairwise combinations with JAR 36 [12], we tested JAR 36 in parallel with JAR 31 in order to have directly comparable data. The absence or presence of cooperative bactericidal activity between JAR 31 or JAR 36 and other anti-FHbp mAbs provided the opportunity to examine further the mechanistic basis for the cooperative bactericidal activity.

4. Discussion

Knowledge of the locations of the epitopes recognized by anti-FHbp mAbs has been used for the rational design of recombinant FHbp molecules capable of eliciting broad serum bactericidal antibody responses across strains with FHbp in sub-families A or B [27, 38]. The amino acid residues affecting the epitope(s) of anti-FHbp mAbs JAR 31 and JAR 36 had not been defined, in part because these mAbs reacted with all of the FHbp sequences tested from sub-family A. These two mAbs have been useful in previous studies to distinguish FHbp variants [12, 27], and to provide relative or absolute quantification of FHbp expression by strains [11, 39].

In the present study, we used a combination of peptide phage display approaches, which had been successful in identifying the epitope of another cross-reactive anti-FHbp mAb, JAR 4 [14]. With JAR 36, however, we did not identify a consensus peptide sequence from the phage display studies that also was present in the FHbp sequence. Among the peptides bound by JAR 36, however, we identified several amino acid residues, D and K, that occurred more frequently than by chance. Using this information, along with FHbp sequence alignments and protein structural information, we predicted D or K residues potentially contributing to the epitope recognized by JAR 36. The D201A substitution eliminated binding of JAR 31 and JAR 36 to the mutant recombinant FHbp and the K203A substitution decreased binding of JAR 31 and JAR 36 slightly but reproducibly.

Residues previously reported to be important for anti-FHbp mAb recognition of FHbp in sub-family A were K174, R180, E192 and S216 [12]. The residues D201 and K203 that affect binding of JAR 31 and JAR 36 to sub-family A FHbp variants also are situated in this region. D201 also is present in JAR 31 and JAR 36 non-reactive sequences from sub-family B. Therefore, the presence of D201 is necessary but not sufficient for binding and neighboring residues present in non-binding sub-family B FHbp variants may affect the conformation of the epitopes recognized by JAR 31 and JAR 36. The preceding residue, 200, is A in sub-family A sequences, which bind JAR 31, and proline (P) in non-binding sub-family
B sequences; however, substitution of A200 with P also did not affect reactivity with JAR 31 (authors’ unpublished data).

JAR 31 and JAR 36 elicited cooperative bactericidal activity with JAR 11 and JAR 13. The latter two mAbs recognize epitopes containing A174, which is 20–28 Å from D201, and S213, which is 27–31 Å away. JAR 31 and JAR 36 were not bactericidal with JAR 4, which recognizes D25-K27 (26–35 Å), or with JAR 10, which recognizes R180/E192 (22–30 Å) [12]. JAR 31 and JAR 36 also were not bactericidal with JAR 32 or 35, which recognize a residue at the same position as JAR 11, or with JAR 33, which recognizes residues at the same positions as JAR 10 [12]. Taken together, the distances between epitopes recognized by cooperative versus non-cooperative pairs of mAbs do not appear to be substantially different. Therefore, some other factor, such as the orientation of the bound mAbs likely plays a role in cooperative mAb function.

For one cooperative pair of anti-FHbp mAbs, 12C1 and JAR 5, crystal structures of their respective Fab fragments bound to FHbp have been determined [15, 17]. A model of the ternary complex between the two Fabs and FHbp show that they bind adjacent, non-overlapping epitopes on FHbp. Another model of the intact mAbs bound to FHbp shows that the respective Fc regions are ~115–130 Å apart and was proposed to be consistent with the approximate dimensions of C1q, which contains 6 globular recognition regions and is ~35 nm in diameter [40]. Further structural studies of anti-FHbp mAbs bound to FHbp will better define the contributions of epitope spacing and orientation to synergistic mAb bactericidal activity.

Declarations

Author contribution statement

Carla Lo Passo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lorenza Zippilli, Antonella Angiolillo, Isabella Costa, Ida Pernice, Roberta Galbo: Performed the experiments; Analyzed and interpreted the data.

Franco Felici, Peter T. Beernink: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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