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Frequency of Factor H-binding Protein Modular Groups and Susceptibility to Cross-reactive Bactericidal Activity in Invasive Meningococcal Isolates

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Abstract

Meningococcal factor H binding protein (fHbp) is a promising vaccine candidate that elicits serum bactericidal antibodies in humans. Based on sequence variability of the entire protein, fHbp has been divided into three variant groups or two sub-families. We recently reported that the fHbp architecture was modular, consisting of five variable segments, each encoded by genes from one of two lineages. Based on combinations of segments from different lineages, all 70 known fHbp sequence variants could be classified into one of six modular groups. In this study, we analyzed sequences of 172 new fHbp variants that were available from public databases. All but three variants could be classified into one of the six previously described modular groups. Among systematically collected invasive group B isolates from the U.S. and Europe, modular group I overall was most common (60%) but group IV (natural chimeras) accounted for 23% of UK isolates and <1% of U.S. isolates ($P < 0.0001$). Mouse antisera to recombinant fHbp from each of the modular groups showed modular group-specific bactericidal activity against strains with low fHbp expression but had broader activity against strains with higher fHbp expression. Thus both modular group and relative expression of fHbp affected strain susceptibility to anti-fHbp bactericidal activity. The results confirmed the modular architecture of fHbp and underscored its importance for the design of broadly protective group B vaccines in different regions.

Keywords

Neisseria meningitidis; vaccine; recombinant protein; bactericidal activity; fHbp expression; bioinformatic

Introduction

The availability of the first meningococcal genomic sequence (strain MC58) [1] allowed the application of high-throughput genomics [2,3], bioinformatics-based antigen predictions

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(reverse vaccinology) [4], and proteomics [5-8] to identify new vaccine targets [9-12]. One of the most promising of these new antigens is factor H-binding protein (fHbp) [13] (previously referred to as GNA1870 [14] or LP2086 [12]). fHbp elicited serum bactericidal antibodies in mice [12,14] and humans [10]. Two vaccines containing recombinant fHbp are in late-stage clinical development (Reviewed in [15]).

Masignani and coworkers classified fHbp into three variant groups based on amino acid sequence diversity and lack of cross-reactive serum bactericidal antibody responses of immunized mice [14]. Using similar analyses, Fletcher and co-workers assigned fHbp variants into two sub-families, designated A and B [12]. Sub-family B corresponded to variant group 1 of Masignani, and sub-family A included variant 2 and 3 groups [16]. Thus far, there has not been a consensus on whether proteins assigned to variant groups 2 and 3 are antigenically or phylogenically distinct [17].

In a recent study, we proposed that the overall fHbp architecture was modular, consisting of combinations of five modular variable segments, each flanked by blocks of two to five invariant amino acid residues [18]. Each of the modular variable segments was derived from one of two lineages, encoded by variant 1 or 3 fHbp genes, respectively. Based on different combinations of the respective segments, all 70 fHbp amino acid sequence variants that had been identified at the time of our publication could be classified into one of six modular groups [18].

Since our report, the publically available fHbp sequence database has been expanded, in large part by a study by Murphy *et al.*, of fHbp nucleotide sequences of 1837 invasive *N. meningitidis* group B isolates [16]. The purpose of the present study was to analyze this expanded dataset to test our hypothesis of the modular architecture of fHbp and to determine the frequencies of the different modular groups among case isolates in different countries. We also report the results of serologic studies that implicated both modular group and level of fHbp expression as affecting strain susceptibility to anti-fHbp complement-mediated bactericidal activity. Collectively the results underscored the relevance of the fHbp modular architecture for classification of the antigen, and for understanding strain-specific and cross-protective anti-fHbp immunity.

Materials and Methods

Source of bioinformatics data and analysis

The dataset included the 70 distinct fHbp amino acid sequence variants previously described [18], and 172 additional distinctive sequences that were subsequently added to the Neisseria.org database (http://neisseria.org/perl/agdbnet/agdbnet.pl?file=nm_fhbp.xml) as of November 2009. In describing the 242 unique proteins (Supplementary Table S1), we employed the protein identification (ID) numbers from the peptide database at the Neisseria.org website.

We used a combination of approaches for analysis of complete or partial protein sequences. Sequences were aligned with MUSCLE (EBI, v3.7, <http://www.ebi.ac.uk/Tools/muscle/index.html>) [19,20] configured for highest accuracy. The accuracy of the alignments was confirmed by visual inspection and using the program JALVIEW [21]. Alignments also were performed on the individual modular variable segments between the blocks of invariant residues previously described [18] (See Results). Networks were generated using SplitsTree, version 4.0 [22], with default parameters. Statistical tests for branch support were performed using the bootstrapping method (1000 replicates).

Cloning, expression and purification of recombinant proteins

Expression plasmids were constructed by PCR amplification of fHbp genes from genomic DNA as described previously [14]. The genes encoded fHbp ID 1 (Modular group I), 28 (group II), 22 (group III), 15 (group IV), 79 (group V) and 77 (group VI). C-terminal hexahistidine-tagged recombinant fHbps were expressed in *Escherichia coli* BL21(DE3) (Novagen, Madison, WI, US), and purified as described elsewhere [23].

Mouse antisera

Groups of five-week-old CD-1 female mice (10 mice per group) were obtained from Charles River (Wilmington, MD, US). The mice were immunized via the intraperitoneal route (IP) with three doses of vaccine given at 3-week intervals. Each 100- μ l dose contained 20 μ g of recombinant protein mixed with Freund's adjuvant (FA) (Sigma-Aldrich, St. Louis, MO, US). (Complete FA for the first dose and incomplete for subsequent doses). Terminal blood samples were obtained three weeks after the last dose. The animal procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of the Children's Hospital Oakland Research Institute.

Bacterial strains

Characteristics of the *N. meningitidis* strains used to measure serum bactericidal activity are summarized in Table 1. Two isolates each were selected from modular groups I to VI. For five of the modular groups, one strain from each pair was a low expresser of fHbp and the other a higher expresser as measured as described below. For modular group V, only strains that expressed intermediate quantities of fHbp were identified and, therefore, used for the assays.

Complement-dependent serum bactericidal antibody activity

The bactericidal assay was performed as described elsewhere, using early log phase bacteria grown for approximately 2 h in Mueller-Hinton broth (BD Biosciences, Franklin Lakes, NJ, US) supplemented with 0.25% glucose (w/v) and 0.02 mM cytidine 5'-monophospho-N-acetylneuraminic acid (Sigma-Aldrich, St. Louis, MO, US) [11]. The complement source was serum from a non-immune healthy adult with normal hemolytic complement activity. The serum was passed over a protein G Sepharose column (HiTrap Protein G HP, GE Healthcare, Piscataway, NJ, US) to remove IgG antibodies [24].

Quantitative Western Blotting of fHbp

N. meningitidis cells were grown in broth cultures to early log phase, heat-killed (56 °C for 1 h), collected by centrifugation, and resuspended in PBS to an optical density of 0.6. Proteins in the heat-killed cells ($1-4 \times 10^7$ CFU) were separated by SDS-PAGE using 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA, US) as specified by the manufacturer, and were transferred to PVDF membranes (Immobilon-FL; Millipore, Billerica, MA, US) using a Western blot module (Invitrogen, Carlsbad CA, US). After blocking overnight at 4 °C in blocking buffer (Li-Cor Biosciences, Lincoln, NE, US), fHbp modular groups I and IV (variant 1 group of Massignani et al [14]) were detected with anti-fHbp JAR 5 or, JAR 31, for modular groups II, III, V or VI (variant groups 2 or 3) [25]. The secondary antibody was goat anti-mouse IgG-IRDye800CW (1:10,000, Rockland Immunochemicals, Gilbertsville, PA, US).

To determine the quantity of bound protein, the membranes were scanned at 800 nm wavelength using an infrared scanner (Li-Cor Odyssey, Lincoln, NE, US), and the integrated intensities of the bands were calculated with software provided by the manufacturer (version 3.0.21). The results of testing purified, recombinant fHbp from each of the six modular groups indicated that the IR signal intensities were proportional to the $\log_{10}$ of the quantities loaded in the range of 0.03 to 2 μ g (representative data for control proteins in modular groups I and

VI are shown in Figure 1). Representative expression data are shown for two strains with fHbp in modular group I, and two strains in modular group VI (Figure 1).

Statistical analysis

Statistical calculations were performed using Prism 5 for Mac OSX, version 5.0a (GraphPad Software, La Jolla, CA, US). The proportion of isolates with fHbp in different modular groups in each country was computed along with the respective 95 percent confidence intervals calculated from the binomial distribution. Differences found in the proportion of isolates in the respective modular groups were compared by Fisher Exact Test (two-tailed) or chi-square analyses.

Results

Phylogenetic analyses of an expanded data set of fHbp protein sequences

Figure 2 depicts a phylogenetic network analysis based on the entire amino acid sequences of the 242 unique proteins. For genes that evolve via horizontal transfer and recombination, this depiction reflects better the relationships of the different sequence variants than conventional tree representations [16]. The network analysis confirmed the two previously described major branches designated as sub-families A and B [12]. Sub-family A contained a total of 104 distinct fHbp variants, which could be subdivided into the previously described Massignani variant 2 (N=51) and 3 groups (N=53) [14]. Sub-family B contained a total of 138 fHbp sequence variants (all in the variant 1 group). In sub-family B we included protein ID 207, which is a natural chimera that had been assigned by Murphy *et al.* to sub-family A (sequence A62 according to the Murphy classification) [16]. As described below, 4 of the 5 modular segments of this protein were derived from sub-family B genes, and several of these segments were known to contain epitopes recognized by anti-fHbp mAbs JAR 3 and JAR 5, which are specific for sub-family B [25,26]. Since the amino acid sequence also had greater identity with protein ID 1 (variant 1/sub-family B) than protein ID 28 (variant 3/sub-family A), we reassigned this protein to the variant 1/sub-family B group.

Invariant amino acids flanking modular segments of natural fHbp chimeras

In our previous study, all 70 fHbp sequence variants had blocks of 2 to 5 invariant residues that flanked five modular variable segments (Figure 3, Panel A) [18]. These invariant residues were conserved in 170 of the 172 newly identified fHbp variants. One exception, protein ID 33, had SHFDF instead of SRFDF between the A and B segments, and the other exception, protein, ID 150, had IEHLE instead of IEHLK between the D and A segments (Figure 3, Panel A).

Diversity of individual modular segments

In our previous study, we had assigned unique identifiers to each of the modular segments with distinctive amino acid sequence variants. The identifier began with a letter, A through E, to represent the modular variable segment, was followed by an α or β , to indicate residues encoded by gene segments derived from modular group I (α) or II (β) lineages, respectively, which was followed by a number for each distinct peptide sequence. The analysis of the individual segments of the new fHbp variants increased the number of unique segments to a total of 242 (185 α and 57 β) distinctive segments (Supplementary Table S2). Using this identification system, each distinctive fHbp variant can be annotated by a combination of the five segments. For example, protein ID 1 is A α 2;B α 1;C α 5;D α 5;E α 8. These annotations were determined for all 242 unique fHbp amino acid sequence variants and are provided in Supplementary Table S1.

We used the program SplitsTree [22] to generate a summary network of each of the segments to display the relatedness of each of the variant sequences. The results represented the potential alternative paths (in terms of amino acids changes) that can be followed when connecting different sequences. The respective split networks for each of the segments were similar. Representative split networks for segments A and E are shown in Figure 4. This network analysis underscored our previous conclusion that each of the respective modular segments was derived from one of two distinct genetic lineages, which we had designated α or β types [18]. The network topology revealed evidence of extensive recombination within the respective α segments (left side of Figure 4), less recombination within the respective β segments (right side of Figure 4), and minimal or no recombination between α and β types.

Classification of fHbp variants by modular groups

All but three of the 172 new fHbp variants could be classified into one of the six previously described modular groups (I to VI, Figure 3, Panel B). The three exceptions (protein ID 207, 67 and 175) had distinctive combinations of modular segments derived from α or β type lineages and were assigned to one of three new modular groups (VII, VIII and IX, respectively).

Of 242 distinct fHbp variants in the expanded database, 125 proteins were in modular group I (Figure 3, Panel B), which contained only α segments, and 20 proteins were in modular group II (entirely β segments). The remaining 97 fHbp sequence variants were natural chimeras of α and β segments and were assigned to modular groups III through IX (together, 40 percent of all variants). Figure 3, Panel B shows the fHbp variant groups as described by Maignani [14] for each of the modular groups. The variant groups were assigned based on the relatedness of the overall amino acid sequences of the respective proteins (Figure 2).

Frequency of fHbp modular groups among isolates causing disease in different countries

The analysis described above provided information on the extent of fHbp modular group diversity but did not address the frequency of different fHbp modular groups among isolates causing disease. For this analysis, we used the fHbp sequence data reported by Murphy *et al.* from systematically collected group B isolates in the U.S. and Europe. The isolates were from cases in the United States between 2001 and 2005 (N=432), and from the United Kingdom (N=536), France (N=244), Norway (N=23) and the Czech Republic (N=27) for the years 2001 to 2006. We supplemented the U.S. data with fHbp sequences we had recently determined for 143 additional isolates that had been systematically collected at multiple sites in the U.S. as part of a previously reported study [27].

Among the total of 1405 systematically collected group B isolates, modular group I was found in 59.7%, group II in 1.7%, group III in 8.1%, group IV in 10.6%, group V in 6.1%, and group VI in 13.6%. The new modular groups, VII, VIII and IX, were each found in $\leq 0.1\%$. The respective distributions of the fHbp modular groups in the different countries as stratified by the variant group classification of Maignani [14] are shown in Figure 5. For this analysis, we excluded the data from Norway and Czech Republic, since the number of isolates in each of these collections was too small for precise estimates of the respective frequencies of the modular groups. The respective percentages of the modular groups in the two U.S. collections are shown separately.

Isolates in variant 1 group of Maignani consisted of modular groups I, IV and VII. Modular group I strains, which have entirely α -type segments, predominated in all three countries (54 to 64 percent of all isolates). In the UK, however, 23% of all isolates were modular group IV, which are natural chimeras of α - and β -type segments (Figure 3, Panel B), as compared with $<1\%$ in the two U.S. collections, and 3 percent of isolates from France ($P<0.001$ by chi square).

Since there was only one isolate in modular group VII (France), the frequency of this modular group is not shown in Figure 5.

Isolates in variant 2 group included modular groups III and VI, which are all natural chimeras of α - and β -type segments. Modular groups III or VI were present in approximately equal proportions of isolates in the two U.S. collections, while modular group VI predominated among variant 2 isolates from the UK and France.

Isolates with variant 3 group included modular group II (entirely β -type segments) and modular groups V, VIII and IX, which are chimeras. In France and the UK, modular group V accounted for the majority of the isolates with variant 3 fHbp, while in the two U.S. collections there were approximately equal numbers of modular group II or V proteins. Modular groups VIII and IX accounted for <0.1% of the isolates. Because of the low percentages, these groups are not shown on Figure 5.

Strain susceptibility to anti-fHbp serum bactericidal activity in relation to modular group and fHbp expression

The results of previous studies suggested that antibodies to fHbp in the variant 1 group were bactericidal primarily against strains with fHbp variant 1 but had little or no activity against strains with fHbp in the variant 2 or 3 groups, and vice versa [14,23]. These studies included relatively few strains and didn't consider the possible role of modular group or level of fHbp expression on susceptibility of a strain to anti-fHbp bactericidal activity.

Figure 6 depicts human complement bactericidal titers of serum pools from mice immunized with recombinant fHbp vaccines representative of modular groups I-VI. The heights of the bars represent the respective median titers of each of the six antisera (3 to 4 pools per modular group) when tested against the specific test strain. For example, the upper left panel shows the data for strain H44/76, which is a high expresser of fHbp in modular group I (relative expression is designated by +++; actual values are shown in Table 1). The median bactericidal titer of the homologous anti-fHbp modular group I antiserum (black bar) was ~1:6000. The respective titers of the five heterologous anti-fHbp modular group antisera (white bars) against strain H44/76 were 1 to 2 log₁₀ lower, ranging from <1:10 (antisera to modular group II) to ~1:200 (antisera to modular group IV). The corresponding median titers of the anti-modular group II and IV antisera when tested against control strains with homologous fHbp modular groups II (strain SK104 in the Variant 3 panel) or IV (NM452 in the Variant 1 panel) were >1:2000. Thus, these and the other heterologous antisera had high antibody activity when measured against control strains with the respective homologous fHbp modular groups.

We observed lower bactericidal titers against low fHbp expressing strains than against high expressing strains (compare respective graphs on the right side of Figure 6 showing data for low expressing strains [designated +/-], with those on the left showing higher expressers). For example the anti-modular group I antiserum had a titer of 1:6000 against H44/76, a high fHbp modular group I expressing strain, but a titer of ~1:100 against strain 03S-0408, a low fHbp modular group I expresser. (For modular group V, both test strains were intermediate expressers, since a pair of high and low fHbp expressing strains were not available).

We also observed a trend for lower cross-reactivity of anti-fHbp bactericidal activity against strains with low expression of fHbp from heterologous modular groups than for high expressing strains (see for example, data for low expressing strains 03S-0408 (modular group I), M01573 (modular group IV), and RM1090 (modular group III), which were killed only by their respective homologous modular group antisera as compared with the broader bactericidal activity against the respective higher fHbp expressing strains (H44/76, NM452, and 03S-0673; Figure 6). Strain NMB (fHbp modular group VI) was completely resistant to anti-fHbp

bactericidal activity (titer <1:10), which apparently was a result of low fHbp expression (Table 1). The amino acid sequence of the vaccine used to prepare the anti-modular group VI antisera differed by a single amino acid from fHbp expressed by NMB, and the resulting antisera were bactericidal against a higher expressing strain (961-5945) with fHbp modular group VI. Further, the resistant strain, NMB, was killed by a control anti-PorA mAb at a concentration of 0.15 µg/ml, which was identical to that of an anti-PorA mAb required for killing of the anti-fHbp susceptible 961-5945 strain.

Discussion

fHbp is a principal antigen in two promising meningococcal group B vaccines under development. The Wyeth vaccine contains two recombinant lipidated fHbps, one from sub-family A and the other from B [12]. The Novartis vaccine contains a recombinant fHbp in sub-family B (referred to as variant 1) fused with a second antigen, GNA2091, along with two other recombinant proteins, GNA2132 fused with GNA1030, and NadA [9], and a detergent-treated outer membrane vesicle vaccine (Reviewed in [15]). The success of the either vaccine strategy will depend, in whole or in part, on the ability of antibodies to fHbp to confer broad protection activity against strains with different levels of fHbp expression and with different amino acid amino acid sequence variants in the respective sub-families or variant groups.

Previous classifications of fHbp into two sub-families or three variant groups were based on the amino acid sequences of the entire protein. We have proposed an alternative classification based on a modular fHbp architecture, which we inferred from amino acid sequences of natural chimeras [18]. In the present study, all but three of the 172 newly described fHbp amino acid sequence variants could be classified into one of the six previously described modular groups. The exceptional variants were new combinations of the five segments and were assigned to new modular groups, VII, VIII and IX. Our network analyses confirmed the segregation of the variants into the six originally described modular groups and the three new modular groups (Figure 2).

Overall, more than 50% of all isolates in the United States, United Kingdom and France had fHbp modular group I, which consisted entirely of α segments. Further, 22% of all isolates had an identical fHbp (ID 1) in modular group I, which was the variant 1 protein in the Novartis group B vaccine described above. There were, however, geographic differences in the frequencies of the other modular groups. For example, modular group IV isolates were rare in the U.S. (<1%) or France (4%) but frequent in the UK (23% of all isolates). The high proportion of isolates with modular group IV in the UK may reflect the recent emergence of group B strains from the ST-269 clonal complex in that country [28].

Modular group IV proteins are part of sub-family B/variant 1 but are natural chimeras with A β segments instead of A α segments (Figure 3, Panel B). The high prevalence of modular group IV isolates in the UK potentially could limit vaccine coverage with a modular group I protein since modular group IV strains appear to be less susceptible to anti-fHbp modular group I bactericidal antibodies (Figure 6, NM452 and M01573).

Because of lack of information on the two antigens contained in the Wyeth bivalent vaccine, or the contribution of antibodies to non-fHbp antigens to protection elicited by the Novartis multicomponent vaccine, it was not possible to predict strain coverage elicited by either vaccine. Our sequence analyses and experimental data (Figure 6), however, allowed us to address a long-standing controversy on the degree of cross-reactive bactericidal activity against isolates with fHbp variant 2 and 3. These proteins are considered separate variants by one group of investigators [14], a single sub-family A with two branches by another group [16], and six modular groups according to our expanded modular classification (Figures 2 and 3) [18].

In general, we found high anti-fHbp cross-reactive bactericidal activity against sub-family A strains with intermediate or high expression of fHbp variant 2 or 3. Against low fHbp-expressing sub-family A strains, however, there was less cross-reactive bactericidal activity (Figure 6). For example, strain RM1090 (modular group III, variant 2) was killed only by the homologous modular group-specific antisera (Figure 6), and strain M1239 (modular group II, variant 3) was killed only by the homologous modular group-specific sera and one of the three other sub-family A modular antisera. Minimal cross-reactive bactericidal activity also was observed against low fHbp expressing strains in sub-family B (variant 1). Thus, for predicting vaccine coverage, it is important to consider both fHbp sequence variability (including modular group) and level of expression of fHbp.

Previous studies measured strain fHbp expression by conventional Western blots [14,26], flow cytometry [24], or a whole bacterial cell ELISA [29]. Since the observed binding was not linear, the semi-quantitative results may not have been sufficient to discriminate between threshold levels of fHbp required for anti-fHbp cross-reactive bactericidal activity. For the present study, we developed a quantitative Western blot assay to measure fHbp expression. The method used two broadly cross-reactive anti-fHbp mAbs (Supplementary Table 3) and an infrared scanner for integration of the intensities of secondary antibody binding. The method had a wide dynamic range (Figure 1), and showed a more than 30-fold variation in fHbp expression by the different test strains (1 to 31 ng/10⁶ bacterial cells, Table 1). One may question how the strains with low fHbp expression were able to invade the host and cause disease, since the ability to bind fH is known to down-regulate complement activation and permit the organism to evade innate host defenses [13,30-33]. From previous studies, however, we can infer that some low fHbp-expressing *N. meningitidis* strains possess alternative mechanisms for resistance to alternative complement pathway activation [33]. Determining the underlying molecular basis for survival of low fHbp expressing strains in human serum will require additional studies.

In summary, the present data show that both modular group and strain expression of fHbp affect susceptibility to anti-fHbp bactericidal activity. The results confirmed the modular architecture of fHbp and underscored its importance for the design of broadly protective group B vaccines in different regions. To define the global epidemiology of meningococcal fHbp sequence variation will require additional studies of isolates from other areas of the world where genetic variability may be greater, and studies of carriage isolates in addition to invasive isolates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure. 1.

fHbp expression measured by immunoblotting with infrared detection. Panel A, Recombinant proteins in modular groups I (ID 1) or VI (ID 77), or heat-killed bacterial cells from strains expressing fHbp in the corresponding modular groups. The modular group I proteins were detected with murine anti-fHbp mAb JAR 5, which recognizes nearly all fHbps in modular groups I and IV. The modular group VI proteins were detected with anti-fHbp mAb JAR 31, which recognizes nearly all proteins in modular groups II, III, V and VI (See Supplemental Table 3). Panel B, Standard curves from the corresponding binding of the recombinant proteins shown in Panel A.

Figure 2.

Network analysis of the relatedness of the amino acid sequences of 242 unique fHbp sequence variants as generated by the program, SplitsTree [22]. The previously described classifications into sub-families A and B [16], or variant groups 1, 2 and 3 [14], are shown along with the respective nine modular groups (I to IX, see Figure 3). The most extreme modular groups (II on the left and I on the right) represent proteins for which each of the respective five segments was derived from one lineage or the other. The proteins in modular groups III to IX apparently represent recombination events that resulted in natural chimeras.

Figure 3.

Schematic representations of fHbp modular structure. Panel A, Positions of blocks of invariant residues (shown as vertical rectangles), which flank five variable segments, designated V_A to V_E . The amino acid positions of the last residue in each variable segment are shown. The numbering is based on sequences of mature proteins of three *N. meningitidis* fHbp variants (IDs 1, 77 and 26) in variant groups 1, 2 and 3, respectively, as defined by Masignani et al [14]. Panel B. Schematic of nine fHbp modular groups deduced from phylogenetic analysis of 242 unique amino acid variants. The respective Masignani variant group designations, and the number of unique sequences observed within each fHbp modular group, are shown. Adapted from Beernink and Granoff [18].

Figure 4. Summary networks of peptide sequences of each of the variant sequences in variable segments A and E as computed by the program SplitsTree [22] (See legend to Figure 2).

Figure 5.

Frequency of fHbp modular groups among systematically collected *N. meningitidis* group B case isolates. Data are from sequences of isolates collected in the United States (N=432), United Kingdom (N=536) and France (N=244) reported by Murphy et al [16], and newly obtained sequences of 143 additional U.S. isolates from California (2003-2004), Maryland (1995 and 2005), and pediatric hospitals in 9 states (2001-2005) from a collection previously described by Beernink et al [27].

Figure 6.

Serum bactericidal activity of serum pools from mice immunized with fHbps from modular groups I to VI. The black bars represent the median titers of 3 to 4 serum pools for each modular group tested against heterologous test strains. The white bars represent the median titer of the respective homologous serum pools against the test strain. +, refers to relative expression of fHbp by each of the strains; strains with +/- representing low fHbp-expressing strains (see values in Table 1).

Table 1
 Characteristics of *Neisseria meningitidis* strains used for measuring serum bactericidal activity

Strain Name	ST ¹	Clonal Complex ¹	Factor H-Binding Protein				
			Variant Group ²	Protein ID ³	Modular Group ⁴	Expression ⁵ (ng/10 ⁶ cells)	Genbank Accession Number
H44/76	32	32	1	1	I	31	AY548370
03S-0408	11	11	1	78	I	3	ACF35432
SK104	5748	162	3	99	II	11	GQ219769
M1239	437	41/44	3	28	II	2	ABF82029
03S-0673	1364	32	2	23	III	14	GU056306
RM1090	11	11	2	22	III	2	ABY26518
NM452	283	269	1	15	IV	20	ABL14232
M01573	44	41/44	1	55	IV	1	AAR84481
S3032	6874	UA ⁶	3	79	V	8	ACH48234
MD1475	162	162	3	82	V	7	GQ219772
961-5945	153	8	2	16	VI	5	AAR84453
NMB	8	8	2	50	VI	1	AY330379

All strains are from the United States except 961-5945 (Australia) and H44/76 (Norway), and are capsular group B except strain RM1090 (group C).

¹ST (sequence Types) and Clonal Complexes were defined as described on *Neisseria* Multi-Locus Sequence Typing website (<http://pubmlst.org/neisseria/>).

- ²Variant group according to classification system of Maignani et al [14];
- ³fHbp protein ID (Identification) numbers are described on the Neisseria.org website (www.neisseria.org).
- ⁴Modular Group designations and variable segments are as described by Beemink and Granoff [18].
- ⁵Relative expression of fHbp was determined by quantitative Western blot (see methods).
- ⁶UA, clonal complex not designated.