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A region of the N-terminal domain of meningococcal factor H-binding protein that elicits bactericidal antibody across antigenic variant groups

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ABSTRACT

Meningococcal factor H-binding protein (fHbp) is a promising vaccine antigen. Previous studies described three fHbp antigenic variant groups and identified amino acid residues between 100 and 255 as important targets of variant-specific bactericidal antibodies. We investigated residues affecting expression of an epitope recognized by a murine IgG2a anti-fHbp mAb, designated JAR 4, which cross-reacted with fHbps in variant group 1 or 2 (95% of strains), and elicited human complement-mediated, cooperative bactericidal activity with other non-bactericidal anti-fHbp mAbs with epitopes involving residues between 121 and 216. From filamentous bacteriophage libraries containing random peptides that were recognized by JAR 4, we identified a consensus tripeptide, DHK that matched residues 25–27 in the N-terminal domain of fHbp. Since DHK was present in both JAR 4-reactive and non-reactive fHbps, the tripeptide was necessary but not sufficient for reactivity. Based on site-directed mutagenesis studies, the JAR 4 epitope could either be knocked out of a reactive variant 1 fHbp, or introduced into a non-reactive variant 3 protein. Collectively, the data indicated that the JAR 4 epitope was discontinuous and involved DHK residues beginning at position 25; YGN residues beginning at position 57; and a KDN tripeptide that was present in variant 3 proteins beginning at position 67 that negatively affected expression of the epitope. Thus, the region of fHbp encompassing residues 25–59 in the N-terminal domain is important for eliciting antibodies that can cooperate with other anti-fHbp antibodies for cross-reactive bactericidal activity against strains expressing fHbp from different antigenic variant groups.

1. Introduction

Meningococcal factor H-binding protein (fHbp) is a promising vaccine candidate that is part of two vaccines in clinical development (Jiang et al., 2008; Rappuoli, 2008). The protein binds factor H (fH) (Granoff et al., 2009; Madico et al., 2006), which is a down-regulatory molecule in the complement pathway (Schneider et al., 2007). The presence of fH on the *Neisseria meningitidis* bacterial surface is critical for the organism to circumvent innate host defenses (Madico et al., 2006; Schneider et al., 2006; Welsch et al., 2008). In the absence of bound fH, the organism becomes susceptible to unregulated alternative complement activation and bacteriolysis (Granoff et al., 2009; Seib et al., 2008; Welsch et al., 2008). Recently, binding of fH was demonstrated to be specific for human fH (low for chimpanzee and negligible for baboon and rhesus monkey), which adds to a list of mechanisms by which *N. meningitidis* only infects humans (Granoff et al., 2009). Antibodies against fHbp both activate the classical complement pathway and also block binding of fH to the surface of the bacteria (Beernink et al., 2008; Welsch et al., 2008).

Among different strains of *N. meningitidis*, fHbp exists in several antigenic variant groups based on antibody cross-reactivity and amino acid sequence identity. These fHbp groups have been designated variant 1, 2 or 3 by Masignani et al (Masignani et al., 2003), or sub-families A and B by Fletcher et al. (2004). Sub-family A includes strains with fHbp in the variant 2 or 3 groups as defined by Masignani, and sub-family B includes strains with fHbp in the variant 1 group. JAR 4 is an IgG2a mAb that was isolated from a mouse immunized with recombinant fHbp in the variant 1 group (Welsch et al., 2004). The mAb was not bactericidal with human complement when tested alone, even against strains with relatively high fHbp expression (Welsch et al., 2008, 2004). However, the mAb activated C3b deposition on strains expressing fHbp in the variant 1 or 2 group (Welsch et al., 2004) and elicited cooperative, complement-mediated bactericidal activity with other non-bactericidal mAbs specific for fHbp in the variant 1 or 2 group (Beernink et al., 2008; Welsch et al., 2008).

The reasons why JAR 4 individually was not bactericidal are not understood. JAR 4 did not inhibit binding of fH (Beernink et al.,

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2008; Welsch et al., 2008). For anti-fHbp mAbs that inhibited fH binding, the absence of bound fH on the bacterial surface would be expected to result in unregulated C3 activation and enhanced susceptibility to bactericidal activity (Granoff et al., 2009) (see also Section 4). The lack of JAR 4 bactericidal activity also may be the result of insufficient amounts of JAR 4 immune complex at the bacterial surface to engage C1q and activate the classical complement pathway (Beernink et al., 2008; Welsch et al., 2008). In contrast, the combination of JAR 4 along with other anti-fHbp mAbs may provide sufficient immune complex.

In a previous study, we mapped the locations of residues involved in the epitopes recognized by eight anti-fHbp mAbs using a combination of sequence alignments and site-specific mutagenesis (Beernink et al., 2008). However, this approach was not successful for mapping the JAR 4 epitope. The objective of the present study was to identify amino acid residues in fHbp affecting the JAR 4 epitope. The long-term goals were to identify the portion of the fHbp molecular capable of eliciting cross-reactive bactericidal antibodies against strains with fHbp from different antigenic groups and to increase our understanding of the basis of cooperative cross-reactive anti-fHbp mAb bactericidal activity.

2. Experimental

2.1. Materials and methods

2.1.1. Gene cloning and site-specific mutagenesis

The methods used for cloning of *fHbp* variant 1, 2 or 3 in the expression plasmid pET21b (Novagen, Inc., Madison, WI) were described previously (Beernink et al., 2008; Masignani et al., 2003). Plasmids encoding fHbp with single or multiple amino acid substitutions were generated using the QuikChange II kit (Stratagene, La Jolla, CA) and the manufacturer's protocols. The mutagenesis reactions were performed using 10 ng of plasmid template and a PTC-200 thermal cycler (MJ Research, Waltham, MA). The forward mutagenic primers were: MC58 D25A 5′-AACCGCACCGCTCGCCCATAAAGACAAAGG-3′; MC58 H26A 5′-AACCGCACCGCTCGACGCTAAAGACAAAGGTTTGC-3′; MC58 K27A 5′-GCACCGCTCGACCATGCAGACAAAGGTTTGCAG-3′; MC58 ins KDN 5′-CTTATGGAAACGGTGACAAAGACAACAGCCTCAATACGGGC-3′; M1239 ΔKDN 5′-TTCAAAGCCGGCGACAGCCTCAACACGG-3′; M1239 FKA → YGN, CACAAGGTGCGGAAAAAACTTACGGAAACGGCGACAGCCTCAACACGGG-3′, in which the underlined sequences denote mutated codons. The reverse primers were the respective antiparallel sequences. All oligonucleotides were synthesized commercially (Integrated DNA Technologies, Coralville, IA). Plasmids encoding wildtype or mutant fHbp were verified by DNA sequence determination (Davis Sequencing, Davis, CA) using primers described previously (Masignani et al., 2003).

2.1.2. Protein purification

Recombinant fHbps representative of the variant 1, 2 and 3 groups (cloned from the genes from strains MC58, 8047 and M1239; Genbank accession numbers NC_003112, FJ422922 and DQ523569, respectively) were expressed with C-terminal hexahistidine tags in *E. coli* strain BL21(DE3). Cultures were grown at 37 °C in Super Broth (30 g/l Bacto-tryptone (BD Biosciences, San Jose, CA), 20 g/l yeast extract (BD Biosciences), 10 g/l MOPS (3-N-morpholinopropanesulfonic acid; Sigma–Aldrich, St. Louis, MO), pH adjusted to 7.0 with NaOH. Once the cultures reached an optical density at 600 nm of 0.6, fHbp expression was induced with 0.5 mM IPTG for 3 h. The proteins were purified by metal chelate chromatography as described previously (Beernink and Granoff, 2008), dialyzed against PBS (Roche Applied Science, Indianapolis, IN), sterilized using 0.45 μm syringe-tip filters (Millipore, Billerica, MA) and stored at 4 °C prior to use.

2.1.3. Phage library preparation and screening

Peptides binding to JAR 4 mAb were selected by panning four phage libraries constructed in the two-gene/phagemid vector pC89 (Felici et al., 1991) by cassette mutagenesis. The libraries carried random inserts encoding peptides of various sizes fused into the N-terminal region of the major coat protein (pVIII) of filamentous phage. The pVIII-9aa and pVIII-12aa libraries were composed of random 9-mers and 12-mers, respectively, whereas the pVIII-9aa.Cys and pVIII-Cys.Cys libraries had random inserts, each containing two cysteine residues (Luzzago and Felici, 1998).

Specific phage clones were isolated from the libraries by two rounds of affinity selection. In the first round the JAR 4 mAb (1 μg/ml) was incubated with magnetic beads conjugated with protein G (50 μl, 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 (~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. The beads were washed 10 times with 1 ml of washing solution, and bound phages were eluted with 500 μl of 0.1 M HCl, adjusted to pH 2.2 with glycine, and 10 mg/ml BSA. The solution was neutralized with 60 μl of 2 M Tris pH 9.6, and the phages were amplified by infecting *E. coli* strain TG1. The second round of panning was carried out in the same way, but using 10^{10} ampicillin-transducing units obtained from the first round of amplified phage pools.

Positive phage clones were identified through immunoscreening, performed as described (Luzzago et al., 1993). In brief, phages obtained from the affinity selection were mixed with 2 ml of a culture of *E. coli* TG1 (OD_{600} ~ 0.4). Five hundred μl of this culture was infected with M13KO7 helper phage (10^9 pfu) in a 1.5 ml tube, and incubated for 15 min at 37 °C and an additional 15 min with shaking. Serial dilutions of infected bacteria were plated on Luria–Bertani (LB) agar plates containing ampicillin, kanamycin and IPTG, and were incubated overnight at 37 °C. Nitrocellulose filters (Protran BA85, 0.45 mm, Schleicher & Schuell, Keene, NH) were layered on the plates containing 50–200 colonies, and left at room temperature for 1 h. Filters were blocked for 1 h with blocking solution, and incubated for 2 h at room temperature with mAb JAR 4 (1 μg/ml in blocking solution), and then for 1 h at room temperature with an AP-conjugated anti-mouse IgG secondary Ab (Sigma, St. Louis, MO, 1:5000) in the same solution. Filters were washed in washing solution, and developed with nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma). The positive colonies were resuspended in 50 μl of 1× PBS. The bacterial cells were killed by heating at 70 °C for 15 min. After centrifugation for 5 min in a microcentrifuge, the supernatants containing the positive phage particles were collected.

2.1.4. ELISA

Binding of mAbs to recombinant fHbp was performed in a direct binding ELISA, using purified fHbp (2 μg/ml) as the antigen on the plate, and performed as described previously (Beernink and Granoff, 2008). Binding of JAR 4 to peptides displayed by the phage library selected clones was assessed by ELISA (Dente et al., 1994). Ninety-six well plates were coated with 100 μl per well of a rat anti-pIII (coat protein III) mAb (1 μg/ml in 50 mM $NaHCO_3$, 0.02% (w/v) NaN_3, 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 cleared phage supernatant were added and the plates were incubated for 1 h at 37 °C. After washing, the mAb was added (1 μg/ml in blocking buffer), incubated for 2 h at 37 °C, and mAb binding was detected by AP-conjugated goat anti-mouse IgG antibody (Sigma, 1:5000) using *p*-nitrophenyl phosphate substrate tablets (Sigma).