Cocrystal structure of meningococcal factor H binding protein variant 3 reveals a new crossprotective epitope recognized by human mAb 1E6

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ABSTRACT: The 4 component meningococcus B vaccine (4CMenB) vaccine is the first vaccine containing recombinant proteins licensed for the prevention of invasive meningococcal disease caused by meningococcal serogroup B strains. 4CMenB contains 3 main recombinant proteins, including the Neisseria meningitidis factor H binding protein (fHbp), a lipoprotein able to bind the human factor H. To date, over 1000 aa sequences of fHbp have been identified, and they can be divided into variant groups 1, 2, and 3, which are usually not crossprotective. Nevertheless, previous characterizations of a small set (n = 10) of mAbs generated in humans after 4CMenB immunization revealed 2 human Fabs (huFabs) (1A12, 1G3) with some crossreactivity for variants 1, 2, and 3. This unexpected result prompted us to examine a much larger set of human mAbs (n = 110), with the aim of better understanding the extent and nature of crossreactive anti-fHbp antibodies. In this study, we report an analysis of the human antibody response to fHbp, by the characterization of 110 huFabs collected from 3 adult vaccinees during a 6-mo study. Although the 4CMenB vaccine contains fHbp variant 1, 13 huFabs were also found to be crossreactive with variants 2 and 3. The crystal structure of the crossreactive huFab 1E6 in complex with fHbp variant 3 was determined, revealing a novel, highly conserved epitope distinct from the epitopes recognized by 1A12 or 1G3. Further, functional characterization shows that human mAb 1E6 is able to elicit rabbit, but not human, complement-mediated bactericidal activity against meningococci displaying fHbp from any of the 3 different variant groups. This functional and structural information about the human antibody response upon 4CMenB immunization contributes to further unraveling the immunogenic properties of fHbp. Knowledge gained about the epitope profile recognized by the human antibody repertoire could guide future vaccine design.—Bianchi, F., Veggi, D., Santini, L., Buricchi, F., Bartolini, E., Lo Surdo, P., Martinelli, M., Finco, O., Masignani, V., Bottomley, M. J., Maione, D., Cozzi, R. Cocrystal structure of meningococcal factor H binding protein variant 3 reveals a new crossprotective epitope recognized by human mAb 1E6. FASEB J. 33, 12099–12111 (2019). www.fasebj.org

KEY WORDS: Neisseria meningitidis • fHbp • human antibodies • vaccine

ABBREVIATIONS: 3D, three dimensional; 4CMenB, 4 component meningococcus B vaccine; BSA, bovine serum albumin; CDR, complementarity determining region; fH, factor H; fHbp, factor H binding protein; hfH, human factor H; huFab, human Fab; MenB, meningococcal serogroup B; PDB, Protein Data Bank; rSBA, rabbit serum bactericidal assay; SBA, serum bactericidal activity; SPR, surface plasmon resonance; VH, variable region of the IgG heavy chain; VL, variable region of the IgG light chain

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Neisseria meningitidis is an exclusively human pathogen, able to colonize the mucosal surfaces, proliferate, and (under some instances) invade the bloodstream, causing morbidity and mortality in infants, children, and young adults worldwide (1–3). Bacterial strains have been classified in 12 serogroups based on the composition of their capsular polysaccharide (4, 5), but only serogroups A, B, C, W, X, and Y are responsible for almost all cases of invasive meningococcal disease (1, 2, 6).

To survive in the human host, the meningococcus has evolved several strategies to evade bactericidal killing, such as capsular polysaccharides, which mimic human cell components or by the expression of proteins are able to recruit immune system inhibitors (7). Indeed, whereas several capsular polysaccharide–based vaccines against serogroups A, C, W, and Y have been developed and
licensed (8–10), the capsular polysaccharide of meningococcal serogroup B (MenB) is composed of polymers of α (2–8)-linked N-acetylneuraminic acid, which is also found in many tissues, especially in the CNS, the human neural cell adhesion molecule, and is therefore not considered a suitable vaccine target due to the risk of autoimmunity (11).

Two vaccines specific for MenB [termed 4 component meningococcus B vaccine (4CMenB) and bivalent rLP2086] have been developed and are licensed for use in numerous countries worldwide. One of the main antigens included in both vaccines is the factor H binding protein (fHbp), a 28-kDa membrane-anchored surface lipoprotein able to bind the human factor H (hfH) protein, one of the most abundant serum negative regulators of human complement. Recruitment of hfH to the meningococcal surface helps the bacteria to evade the complement-mediated killing and renders them more resistant to the immune system (12, 13).

To date, more than 1000 distinct amino acid sequences of fHbp have been identified, and they can be divided into 3 variant groups (v1, v2, and v3). Within the fHbp variant groups, the sequence identity is usually above 87%; instead, between variant groups, the sequence identity can be as low as 62% (14).

Several three-dimensional (3D) structures of fHbp have been solved by NMR (15) and X-ray crystallography (16–18), and despite sharing amino acid identity as low as 62%, the structures of the fHbp variants 1, 2, and 3 are well conserved and consist of 2 domains. The C-terminal domain adopts a canonical β-stranded β-barrel conformation, whereas the fHbp N-terminal domain shows a more unusual tacco-shaped β-barrel fold characterized by higher intrinsic flexibility. Differential scanning calorimetry profiles showed independent unfolding of the 2 barrels (17–19). The C-terminal β-barrel melts at temperatures above 80°C in all 3 variants; in contrast, the N-terminal β-barrel exhibits highly variable melting temperatures at 70°C in v1, 61°C in v3, and at 37°C in v2 (20). This intrinsic thermal instability and a noted susceptibility to protease cleavage (21) could explain why the structural characterization of the N-terminal domain of wild-type v2 was unsuccessful so far, although the structure of a stabilized mutant form was determined (21). The variability in the amino acid composition between the fHbp variants results in relevant chemical and physical differences that regulate the molecular and dynamic properties of fHbp. All fHbp variants bind to hfH with high affinity, with the equilibrium dissociation constant, \( K_d \), in the nanomolar range, but large differences in the stability of the complexes were observed (22). The crystal structures of domains 6 and 7 of hfH with fHbp v1 and fHbp v3 were determined by X-ray crystallography, confirming that hfH binding to fHbp is mediated by the same region of the protein but through a distinct set of v1 or v3 residues (17, 23). Vaccination with fHbp elicits a robust immune response in mice, rabbits, macaques (14, 24, 25), and humans (26, 27), conferring protection by 2 pathways: directly engaging C1q to activate the classic complement-mediated killing, or blocking hfH binding to the bacteria to increase the susceptibility of the bacterium to killing by the alternative complement pathway (28–31). To date, several anti-fHbp monoclonal antibodies were described, but probably due to the high amino acid sequence variability, the majority of them are variant-specific (14, 32–37). However, a few anti-fHbp antibodies that were able to recognize more than 1 variant were reported (18, 38–40).

In a recent study aiming to characterize the human B-cell repertoire in response to vaccination with 4CMenB, single plasma blasts from 3 vaccinated adults were isolated, and the genes encoding Ig variable regions were sequenced. Human mAbs against fHbp, Neisserial Heparin Binding Antigen, and Neisseria adhesin A were identified (41). The resulting panel of 10 anti-fHbp antibody fragments (Fabs) was analyzed by 2 different research groups, and both teams found 2 anti-fHbp antibodies (1A12 and 1G3) to be crossreactive with the 3 main fHbp variants (41, 42). Moreover, the crystal structure of one of these (1A12) in complex with fHbp v1 was determined, revealing a conserved epitope localized exclusively on the C-terminal β-barrel (18, 41).

As an extension of the first investigations by Giuliani et al. (41) and Beernink et al. (42), a new longitudinal study is in progress analyzing a much larger repertoire of anti-fHbp human mAbs from 3 additional vaccinees. In this study, 110 human FabS (huFabs) targeting fHbp and isolated at different time points were produced in Escherichia coli and analyzed in terms of antigen binding specificities. Although the 4CMenB vaccine contains the variant 1 form of fHbp, many crossreactive antibodies were identified. Here, in order to better understand the human immune response to 4CMenB and the possibility that immunization with fHbp v1 raises crossreactive antibodies, we present a structural and functional characterization of one of these human antibodies called 1E6 in complex with fHbp v3.

**MATERIALS AND METHODS**

**Human samples**

Human samples were collected from 3 adults immunized with Bexsero, also referred to as 4CMenB, in a clinical trial conducted in Krakow, Poland, approved by the Bioethics Committee of the District Medical Doctors Chamber in Krakow and conducted in accordance with the Declaration of Helsinki. The use of samples was performed upon written informed consent obtained from participants before the study-specific procedures.

**Recombinant Fab production in E. coli**

Heavy and Light chain variable regions of single plasma blasts, isolated from peripheral blood, were amplified and cloned into pET22 as a bicistronic expression cassette encoding for Fab antibody fragments. FabS anti-fHbp were expressed as recombinant proteins with a C-terminal hexahistidine tag in E. coli Rosetta 2 (DE3). Cultures were grown in Enpresso B or human trabecular meshwork cell (HTMC) medium (autoinduced medium developed in house), and protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 mM for 24 h at 25°C. Cell lysis was performed using various techniques: chemical lysis, osmotic shock, and sonication. The recombinant antibodies were purified by immobilized metal ion chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Germantown, MD, USA), according to the manufacturer’s instruction. Recombinant FabS were quantified by bicinchoninic
Recombinant Fab production in mammalian cells

For the expression of recombinant Fab, the variable regions of the heavy and light chains were cloned into a human pRS5a expression vectors encoding the Fab constant fragment with a fused C-terminal Strept-tag and expressed as previously described for mAbs production. To further purify the Fabs, the supernatant, after medium exchange with PBS, was loaded onto a StrepTrap HP Column and eluted by 2.5 mM desthiobiotin buffer (IBA solution for life). The Strept-tag was removed by tobacco etch virus (TEV) protease cleavage in a ratio of 1:100.

Epitope mapping of anti-fHbp mAbs by protein microarray

To identify the regions of fHbp where the binding site is localized, we used a protein microarray developed in house that contains the full length of fHbp v1, v2, and v3 and several overlapping fragments covering the entire fHbp variants sequences (41). Genes were expressed in E. coli as either glutathione S-transferase – or His-tagged fusions or thioredoxin fusions purified from the cytoplasmatic fraction as soluble forms, and then the recombinant antigens were spotted on nitrocellulose-coated slides.

Non-specific binding was minimized by preincubating protein microarray slides with a blocking solution (BlockIt, ArrayIt) for 1 h. mAbs were diluted 1:50 1:2000 in BlockIt and overlaid on the protein arrays for 1 h at room temperature. AlexaFluor 647–conjugated anti-Human IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used at 25 nM. All Fabs were analyzed using Gyrolab Bioaffy 200 CDs and the standard Gyrolab 3-step method (capture-analyte-detection). The threshold for positive Fab binding was defined when the fluorescent signal was higher than 10.

Protein crystallization and diffraction data collection and processing

To form the fHbp/Fab complex, 7.5 mg of fHbp and 5 mg of Fab were incubated overnight at 4°C and further purified by size exclusion chromatography to remove the protein in excess, using a prepacked HiLoad 26/60 Column Superdex 75 Preparation Grade (GE Healthcare). Fab/fHbp complexes concentrated at 25 mg/ml in 50 mM Tris-HCl were screened using prepped 96 deep-well blocks commercialized by Molecular Dimensions (Newmarket, United Kingdom) using Crystal Gryphon robot (Art Robbins Instruments, Sunnyvale, CA, USA). The purified 1f5-fHbp complex (25 mg/ml) was screened using ~500 different crystallization conditions. The largest crystals were found in 1.0 M bicine, pH 8.5 as precipitant 40% PEG 500 MME and 20% PEG 2000. Crystals were soaked in the original mother liquor supplemented with 15% ethylene glycol prior to cryo-cooling in liquid nitrogen.

Structure determination and refinement

Diffraction of the crystals was tested at beamline ID29 of the European Synchrotron Radiation Facility, and several full data sets were collected at 100 K, at wavelength $\lambda = 0.983 \text{Å}$, on a
Pilatus 6 M detector. Diffraction data sets were indexed and integrated using iMosflm and reduced using Aimless via the Collaborative Computational Project No. 4(CP4) suite (43). Crystals of the fHbp-1E6 complex belong to space group P 2_1 2_1 with the asymmetric unit containing 1 complex and a solvent content of 64.4% (Matthews coefficient of 3.57 Å³/Da). The structure of the complex was solved at 2.66 Å resolution by molecular replacement with Phaser (44) using as model templates for fHbp, light chain and heavy chains the Protein Data Bank (PDB; http://www.rcsb.org/ entries 4AY1, 3PIQ, and 5I17, respectively. The coordinates of the 1E6/fHbp v3 structure determined herein have been deposited in the PDB with accession code 6HY2.

Surface plasmon resonance

Surface plasmon resonance (SPR) was used to measure and compare the binding affinities of the tested mAb with the 3 variants of fHbp recombinant proteins. All SPR experiments were performed in running buffer pH 7.4 containing 10 mM HEPES, 150 mM NaCl, and 3 mM EDTA supplemented with 0.05% (v/v) P20 surfactant using Biacore T200 (GE Healthcare) at 25°C. In total, 9000–10,000 response units of an anti-huFab binder were immobilized on CM5 sensor chip, using the Human Fab Antibody Capture Kit (GE Healthcare). To determine the K_d and kinetics parameters, the experiments were divided into 3 main steps. First, 800–1200 response units of the tested mAb at 20 μg/μl was captured by anti-Fab binder on the surface of the chip in running buffer. An anti-Fab binder coated flow cell without captured mAb was used as blank reference.

A single-cycle kinetics experiment was then performed where the fHbp analyte was injected in 5 incremental concentrations (0.39, 0.78, 1.56, 3.13, 6.25 nM) at a flow rate of 40 μl/min. Finally, the chip surfaces were regenerated using a buffer containing 10 mM glycine pH 2.1 (180 s, flow rate 10 μl/min). Each experiment was performed in duplicate.

A blank injection of buffer only was subtracted from each curve, and reference sensorgrams were subtracted from experimental sensorgrams to yield curves representing specific binding. SPR data were analyzed using the Biacore T200 Evaluation software (GE Healthcare). Each sensorgram was fitted with the 1:1 Langmuir binding model, including a term to account for potential mass transfer, to obtain the individual k_on and k_off kinetic constants; the individual values were then combined to derive the single averaged K_d values reported.

Serum bactericidal activity assay

Serum bactericidal activity (SBA) against N. meningitidis strains was evaluated as reported by McCoy et al. (45). Bacteria grown until early log phase [optical density (OD)_{600} of ~0.25] were diluted in Dulbecco PBS (D8662; MilliporeSigma) containing 1% bovine serum albumin (BSA) and 0.1% glucose at the working dilution of 10^5–10^6 and incubated with serial 2-fold dilutions of test mAb starting from a concentration of 125 μg/ml. Serum bactericidal titers were defined as the mAb dilution, resulting in a 50% decrease in colony forming units (CFU) per milliliter after a 60-min incubation of bacteria with the reaction mixture compared to the control CFU per milliliter at time 0. Pooled baby rabbit sera from Cedarlane or human serum, obtained from volunteer donors under informed consent, have been used as a complement source for rabbit or human SBA, respectively.

Inhibition of binding of hFh

The ability of the mAb to inhibit binding of factor H (fH) to live bacteria was measured by flow cytometry. Bacterial cells grown until midlog phase (OD_{600} of ~0.5) were incubated with anti-fHbp mAb (50 μg/ml for UK320, 10 μg/ml for MC58, and UK104 in PBS-1% BSA buffer) for 30 min at room temperature, followed by the addition of purified human fH (25 μg/ml for UK320, 50 μg/ml for MC58 and UK104), which was incubated for an additional 30 min at room temperature in a final reaction volume of 100 μl. fH binding was detected with a goat polyclonal antiserum to fH (341276; MilliporeSigma) diluted 1:100 and incubated for 30 min at room temperature, followed by additional 30 min incubation with a donkey anti-goat IgG fluorescein isothiocyanate conjugate (705.095.003; Jackson ImmunoResearch Laboratories) diluted 1:100 in PBS-1% BSA buffer.

Data availability

All data generated or analyzed during this study were included in this published article (and in the Supplemental Data). The data sets generated during protein chip and PepScan and were analyzed and are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) under series accession no. GSE98883.

RESULTS

Thirteen crossreactive huFabs targeting fHbp identified

To investigate the anti-fHbp repertoire of 3 adults immunized with 4CMenB, the variable region of the IgG heavy chains (VH) and variable region of the IgG light chains (VL) were isolated from peripheral blood mononuclear cells and cloned into an E. coli expression construct as previously described by Beernink et al. (42), obtaining a library of 110 anti-fHbp huFab-expressing plasmids. To determine the fHbp variant binding specificity of each huFab, we set up a protocol (Supplemental Data S1) suitable to produce the recombinant 110 anti-fHbp huFabs in E. coli in a small scale suitable for rapid and efficient screening. After miniaturized immobilized metal affinity chromatography purification, all huFabs were analyzed using a nanoliter-scale immunoassay system (Gyros Protein Technologies, Uppsala, Sweden), a highly sensitive platform for screening crossreactivity in a qualitative way even for samples with suboptimal purity. As expected, the vast majority of the 110 huFabs tested recognized the fHbp variant 1 (Supplemental Figs. S2 and S3). Moreover, although only the fHbp v1 is included in the 4CMenB vaccine, 13 huFabs resulted to be crossreactive between the 3 main variants of fHbp (Fig. 1). The latter finding was of particular interest, because previously only 2 anti-fHbp huFabs were reported to be crossreactive (41).

Protein microarray epitope mapping suggests mAbs have different binding profiles

To map and identify the epitope binding regions and to further confirm crossreactivity with different fHbp variants, each antibody of the 13 crossreactive Fabs subset was produced in mammalian cells as recombinant full-length IgG1 because this is the most abundant Ig
subclass in human sera (46). The recombinant mAbs were then tested in a protein microarray containing full-length and different overlapping recombinant fragments covering the entire length of the fHbp v1, v2, and v3. The data of this analysis, summarized in Table 1, suggest that all mAbs recognize conformational epitopes on the full-length fHbp variants, except for mAb 5C6, which recognized only v1. Three mAbs (4F9, 4B3, 3G7) react with the isolated C-terminal β-barrel domain (in addition to the full-length protein), suggesting that their epitope could be localized in that fHbp region. The mAb 5F12 is unique in being able to bind the isolated N-terminal domain of the fHbp variants. The other mAbs were neither able to bind the separate N- or C-terminal domains of fHbp nor to smaller fragments.

Protein microarray data, including the data regarding the smaller fragments and domains that are not recognized and are not reported in Table 1, have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi).

Crystal structure of huFab 1E6 in complex with fHbp v.3

To obtain detailed information about the conserved epitopes in the fHbp variants, a subset of the 13 mAbs was selected for further structural studies and produced as Fabs in mammalian cells. The crystal structure of the 1E6 in complex with fHbp v.3 was determined at 2.7 Å resolution. X-ray data collection, processing, and refinement statistics are shown in Table 2. The structure was solved by the molecular replacement method, and the resulting electron density maps allowed unambiguous model building. Considered alone, the 2 proteins maintained their typical structural characteristics (Fig. 2); the fHbp assumed the canonical 3D structure of 2 β-barrels connected by a short linker, and the Fab exhibits the standard Ig domain fold (47, 48). The structure of the 1E6 Ig domains consists of antiparallel β-sheets arranged in a sandwich fashion, with the heavy chain folding into VH and CH domains and the light chain folding into VL and CL domains. The 2 sides of each β-barrel were covered by α-helices.

### Table 1. Crossreactivity of 13 huFabs with different fHbp variants assessed by protein microarrays containing recombinant full-length proteins and subdomains of fHbp v1, v2, and v3

| mAbs  | fHbp v1 |          | fHbp v2 |          | fHbp v3 |          |
|-------|---------|----------|---------|----------|---------|----------|
|       | Full length | C-term β-barrel | N-terminal | Full length | β-Barrel | N-terminal | Full length | β-Barrel | N-terminal |
| 3B7   | X        |          |         |          |         |          |         |         |           |
| 1D1   | X        |          |         |          |         |          |         |         |           |
| 3F1   | X        |          |         |          |         |          |         |         |           |
| 4B10  | X        |          |         |          |         |          |         |         |           |
| 1E10  | X        |          |         |          |         |          |         |         |           |
| 2G1   | X        |          |         |          |         |          |         |         |           |
| 5F12  | X        |          |         | X        |         |          |         |         | X          |
| 1E6   | X        |          |         |          |         |          |         |         | X          |
| 2C8   | X        |          |         |          |         |          |         |         | X          |
| 5C6   | X        |          |         |          |         |          |         |         | X          |
| 4F9   | X        | X        |         | X        |         | X        |         |         | X          |
| 4B3   | X        | X        |         | X        |         | X        |         |         | X          |
| 3G7   | X        | X        |         | X        |         | X        |         |         | X          |

"X" indicates which regions of fHbp were bound by the mAb, whereas empty cells indicate that no signal was detected.

CRYSTAL STRUCTURE OF HUMAN FAB AND FACTOR H BINDING PROTEIN
the sandwich motif are covalently linked by disulfide bonds. The elbow angle, defined by the relative displacement of the variable domains and the constant domains, is 138.2° (49).

The complementarity determining regions (CDRs) L1, L2, H1, and H2 of huFab 1E6 assumed canonical structures similar to Chothia class 2, 1, 1, 2 (50). The CDR-L3 shows a noncanonical conformation with standard loop length. HuFab 1E6 forms a broad network of interactions with the N-terminal region of the fHbp v.3, whereas only 3 residues of the C-terminal domain are involved in the binding. The calculated interface buries a total area of 1058 Å², which is in the typical range of the interaction surface between antibodies and Fab:antigens (51, 52), with the N-terminal β-barrel mainly involved in the interaction (Fig. 3A). Both chains of huFab 1E6 contribute to binding of the fHbp v.3 with the heavy chain that contacts only the N-terminal domain defining an interface area of 551 Å², whereas the light chain contacts both domains of fHbp (Fig. 3B, C) on a surface of 507 Å². The binding does not follow the classic “lock and key or induced fit model” (53); indeed, the epitope is localized on a rather flat area (Fig. 3A). Nevertheless, several polar and electrostatic interactions are formed, mediated by side chain atoms from all 6 CDRs (Table 3).

X-ray structures of the complex between antibodies and Fab:antigens are essential to clearly define the details of epitope:paratope interactions (54). Closer examination of the key residues revealed many of polar nature, forming hydrogen bonds and salt bridges, which contribute to the binding of this mAb to the meningococcal fHbp. Notably, some residues engaged a complex network of molecular interactions that connected more than 2 aa, such as the interconnection established between fHbp v3 E119, D166, K191, and the light chain A28, K30, D50, R92 (Fig. 4A), and fHbp v.3 residues E58, S53, and heavy chain L54, R56 (Fig. 4B).

High conservation of the epitope underlies the broad crossreactivity of mAb 1E6

Antigenic drift is the continuous process of genetic and antigenic changes that occur through point mutations. The antigenic distance between meningococcal strains increases with time as the drift increases the grade of variability to escape the host immune system. Several studies of the diversity of the fHbp gene and the encoded protein in a representative sample of meningococcal isolates confirmed high conservation of the epitope underlies the broad crossreactivity of mAb 1E6.
variability in this antigen, with sequence identities falling as low as ~62% for some pairwise comparisons (48, 55). Therefore, to qualitatively estimate the extent of cross-reactivity of the mAb 1E6, we calculated the percentage of conservation of the 15 fHbp residues within the epitope. A total of 1119 different fHbp alleles were retrieved from the public database N. meningitidis multilocus sequence typing (55), and the degree of conservation of each epitope residue was noted (Fig. 5 and Supplemental Table S1). Interestingly, 10 out of 15 residues are conserved in >99% between all currently known fHbp amino acid sequences. Remarkable instances are represented by Q55, K79, D166 present in 100% of known meningococcal fHbp sequences. Those 3 epitope residues play key roles in binding to mAb 1E6. Of the remaining 5 residues comprising the epitope, K120 and N132 are conserved in 40% of the isolates, whereas the S53, V86, and N169 are represented in <30% of meningococcal strains (Fig. 5 and Supplemental Table S1). Collectively, these observations suggest that mAb 1E6, shown to bind representatives of the 3 fHbp variant groups, might indeed display broad crossreactivity across the entire repertoire of fHbp.

**Human mAb 1E6 binds fHbp in a different region compared to mAb 1A12 and on the opposite side of hfH binding site**

To date, several human anti-fHbp antibodies have been described in functional assays (41, 42, 56); however, to our knowledge, only 2 examples of epitope mapping at the atomic level of anti-fHbp antibodies elicited in humans have been reported so far. Giuliani et al. (41) restricted the epitope localization of 1 crossreactive anti-fHbp mAb 1G3 to short fragments of fHbp v1 by hydrogen-deuterium

| fHbp v3 | Fab 1E6 | CDR | Bond type |
|---------|---------|-----|-----------|
| S53 | L54 | H2 | VdW |
| Q55 | Y32, D100 | H1, H3 | Hydrogen bond |
| G56 | D31, Y32 | H1, H1 | VdW, VdW |
| E58 | R56 | H2 | Salt bridge |
| K79 | D52 | L2 | Salt bridge, VdW |
| R82 | T101 | H3 | Hydrogen bond |
| D84 | T101, A102 | H3 | Salt bridge, salt bridge |
| V86 | L54 | H2 | VdW |
| S100 | F55 | H2 | VdW |
| E119 | R92 | L3 | Salt bridge |
| K120 | D95 | L3 | Salt bridge |
| N132 | D95 | L3 | Hydrogen bond |
| D166 | T31, G29, R92, | L1, L1, L3 | VdW, hydrogen bond, salt bridge |
| N169 | S51, S50 | L2, L2 | Salt bridge, hydrogen bond |
| K191 | D50, A28, K30 | L2, L1, L1 | Salt bridge, hydrogen bond, hydrogen bond |

VdW, Van der Waals.
exchange–MS, whereas López-Sagaseta et al. (18) were able to fully characterize at high resolution, by X-ray crystallography, the first human antibody, the 1A12, identifying the epitope on fHbp v1. Fab 1A12 targets exclusively the C-terminal β-barrel, whereas the Fab 1E6 mainly binds the N-terminal region on the opposite side compared to the binding site for hfH (Fig. 6). The latter suggested that Fab 1E6 would not inhibit the binding of fHbp to hfH. This hypothesis was confirmed using a competition assay performed by flow cytometry (Supplemental Fig. S4). Three residues of the C-terminal β-barrel of fHbp v.3 are involved in 1E6 binding, but only residue K191 engaged a strong network of interactions results to be a crucial residue in both fHbp v1:Fab 1A12 and fHbp v3:Fab 1E6 complexes, further underlining the important antigenic role of this specific amino acid.

**Human mAb 1E6 binds tightly to all 3 fHbp variants**

To connect structure with function, we decided to perform a deeper biochemical characterization of this antibody. We performed SPR analyses to investigate the interaction between mAb 1E6 and the purified recombinant fHbp variants in terms of binding affinity and association/dissociation kinetics (Fig. 7A). Although the vaccine contains only fHbp v1, the analysis confirmed that mAb 1E6 recognized all the 3 main variants of fHbp with high affinity, with $K_d$ values in the low nanomolar range for v3, and even tighter for v1 and v2 (Table 4). Kinetic constants measured suggest a different degree of stability of the complexes when mAb 1E6 bound different variants of fHbp (Table 4 and Fig. 7A). The most stable binding was measured on fHbp v2 because mAb 1E6 seems to associate rapidly and form a very stable complex with slow dissociation. On the other hand, the affinity for the v1 is 10-fold lower than with v2, and the rapid association of the mAb to fHbp followed by a fast dissociation suggests the formation of a shorter lived 1E6/fHbp v1 complex.

**Human mAb 1E6 recognizes native fHbp on live bacteria**

Meningococcal fHbp is a surface-exposed lipoprotein bound to the outer membrane by an N-terminal lipid anchor. Therefore, flow cytometry is a suitable tool to detect the anti-fHbp mAbs binding directly to the surface of the bacteria. Fluorescence-activated cell sorting analysis confirmed that human mAb 1E6 is crossreactive because it is able to bind different variants of fHbp on the surface of live meningococcal strains MC58, M08-0240104 (UK104), and M01-0240320 (UK320), which express fHbp v1, v2, and v3, respectively. We also observed that the fluorescent signal was 10-fold stronger on the meningococcal strain expressing v2 compared to v1 and v3 (Fig. 7A). The amount of fHbp present on the bacterial surface varies between meningococcal strains, and it has been previously described by Biagini et al. (57) that the strain M08-0240104 displays an fHbp surface density higher than the other strains, likely explaining the higher fluorescence intensity measured.
The 1E6 mAb is crossbactericidal in presence of rabbit complement

The bactericidal activity of the human mAb 1E6 was tested against MenB strains expressing distinct fHbp v1, v2, and v3 using baby rabbit and human serum as source of exogenous complement. SBA is a functional serological surrogate of vaccine efficacy (14, 58, 59). When tested in human SBA (with human complement source) against strains carrying 1 of the 3 fHbp variants, the mAb 1E6 alone failed in eliciting positive titers (<4). In contrast, when baby rabbit serum was used as the source of exogenous complement, the mAb 1E6 showed positive bactericidal titers against meningococcal strains carrying

\[ f\text{Hbp v1} \quad K_D = 2.6 \times 10^{-10} \]
\[ f\text{Hbp v2} \quad K_D = 4.2 \times 10^{-11} \]
\[ f\text{Hbp v3} \quad K_D = 2.0 \times 10^{-9} \]

**Figure 6.** Cartoon representations of fHbp (green), bound to Fab 1A12 (red), Fab 1E6 (blue), and hfH (orange). fHbp is shown in the same orientation in all panels.

**Figure 7.** A) Histograms showing binding to live meningococci of MenB strains when incubated with 10 μg/ml of HumAbs anti-fHbp (blue line, MC58 strain; red line UK104 strain; green line, UK320 strain). Gray-filled histogram represents negative control, bacteria incubated with PBS and anti-human IgG fluoresceinisothiocyanate conjugated. B) Sensorgrams show the experimental association and dissociation traces (colored) of binding of mAb 1E6 on 5 incremental concentrations of fHbp v1, v2, and v3, respectively, performed in duplicate. The calculated fitting traces are shown in black.

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different fHbp variants. However, the extent of functionality varied between strains; the higher titers were measured against the M08-0240104 strain expressing the fHbp v2, rabbit serum bactericidal assay (rSBA) titer value of 4096, compared to lower rSBA titers of 256 and 128 when tested against M11295 expressing the fHbp v1 or UK320 expressing the fHbp v3, respectively. Notably, rSBA titers correlated with the affinity of mAb 1E6 for the recombinant proteins measured herein and with the previously reported surface density of the antigens (57).

**DISCUSSION**

*N. meningitidis* remains the most common cause of bacterial meningitis, often leading to permanent disabilities or even death (60). After many years of research and development, 2 vaccines to protect against MenB disease are now licensed, and both contain fHbp, a key meningococcal virulence factor. Recent data from the United Kingdom infant immunization campaign against MenB reported >80% 4CMenB vaccine-mediated protection (61). Here, we report detailed molecular studies that describe important elements of the human antibody response to 4CMenB.

As an extension of previous smaller-scale investigations (41, 42), the study presented here probes the repertoire of human mAbs isolated for a long time from 3 vaccine recipients following vaccine administration. From this study, a new library of huFab expression constructs was obtained. The library encodes over 100 distinct huFabs that recognize fHbp. Most notably, even though the vaccine contains specifically the fHbp variant 1, subjects immunized with 4CMenB produced at least some crossreactive antibodies, and we identified 13 (of 110 total) huFabs that were able to recognize all 3 fHbp variants (Fig. 1). Protein microarray epitope mapping experiments suggested that these 13 crossreactive huFabs bind diverse regions of fHbp, mostly involving conformational epitopes requiring the full-length fHbp, although 3 huFabs bind to the isolated C-terminal β-barrel domain and, remarkably, just 1 huFab binds to the isolated N-terminal domain (Table 1).

To date, the 3D structure of only 1 huFab (1A12) in complex with fHbp variant 1 has been reported (18). Here, we present the crystal structure of fHbp variant 3 (only 58% sequence identity with variant 1) in complex with a quite different crossreactive huFab, 1E6 to deepen our understanding of the molecular bases underlying the crossreactive antibody response in humans (Fig. 2). The structure revealed a large conformational epitope, featuring a dense network of salt bridges and hydrogen bonds, mainly localized at the N terminus of fHbp variant 3 that was not previously seen in other crystal structures of fHbp complexed with murine Fabs, nor with huFab 1A12, which targets a very different region on the C-terminal domain of fHbp (Figs. 3–6).

In biochemical SPR studies, huFab 1E6 was found to bind tightly to fHbp variants 1, 2, and 3, with highest affinity for variant 2 (Fig. 7A). Similarly, huFab 1E6 bound to live meningococcal cells expressing fHbp variant 1, 2, and 3 when examined by flow cytometry (Fig. 7B). The crystal structure enabled a clear understanding of this crossreactivity. Of 15 total fHbp variant 3 residues involved in binding to huFab 1E6 (Fig. 5), 10 are fully conserved in the variants 1 and 2 tested herein (Supplemental Fig. S5). Further, we calculated the degree of conservation of epitope residues considering the 1119 fHbp alleles (from clinical isolates and carrier strains) deposited in the Bacterial Isolate Genome Sequence Database (https://bigd.bigd.big.d [webpage]) (55). Again, we found out that 10 of the 15 epitope residues are conserved >99% in the entire known fHbp sequence database, suggesting that mAb 1E6 could have a very broad recognition of most circulating meningococcal strains.

A similar relationship between kinetic binding parameters measured in an *in vitro* SPR assay were also found in an *in vivo* assay measuring binding of 1E6 to fHbp naturally exposed on MenB strains and in a bactericidal activity assay. Antibodies have 2 distinct functions: one is to bind specifically to their target antigens, and the other is to elicit an immune response recruiting other cells and molecules. Previous studies demonstrated that several factors contribute to bactericidal activity in the presence of human serum of anti-fHbp mAbs, such as human IgG subclass, alternative complement pathway activation, and epitope density (29–31). However, anti-fHbp mAbs that individually did not elicit bactericidal activity could become bactericidal when mixed (36, 41, 42). We found that 1E6 can efficiently activate the complement cascade in a rabbit serum bactericidal assay (rSBA), but is not able alone to induce human anti-FHbp bactericidal activity. A likely explanation is that fHbp present on the bacterial surface binds to hF (binding of FH to fHbp is human-specific), which protects bacteria from complement-mediated killing. Another possible explanation is species-specific differences in Clq engagement by 1E6. Our results are consistent with prior studies with anti-fHbp mAbs (murine or human), where human complement-mediated bactericidal activity was observed only if the mAb blocked fH-binding or was used in combination with other mAbs (29, 30, 41). Previous studies demonstrated that the quantity of fHbp present on the bacterial surface varies between isolates and in the strains used, MC58, M08-0240104, and UK320 the concentration of fHbp was determined to be 2900 (fHbp v1), 9390 (fHbp v2), and 1111 (fHbp v3) molecules for cells, respectively (57). The 9-fold higher antigen density displayed on the surface of strain M08-0240104 explains why the binding on v2 is higher than on v1 and v3 (Fig. 7B). The ability of meningococci to selectively bind to hFh enable the bacteria to be

**TABLE 4. Kinetic constants measured by SPR**

| Type       | fHbp v1 | fHbp v2 | fHbp v3 |
|------------|---------|---------|---------|
| $K_d$ (1/Ms) | 2.65E +0.15 | 1.2E +0.15 | 5.7E +1.8 |
| $K_a$ (1/Ms) | 1.4E -0.15 | 4.3E -1.3 | 1.3E -0.3 |
| $K_d$ (M)   | 2.65E -0.15 | 3.6E -1.1 | 2.7E -0.9 |

$K_d = k_{off}, K_a$: mean ± sd values were calculated from SPR experiments performed in duplicate for each fHbp variant and mutant. Binding affinity and association/dissociation kinetics were measured at Biacore T200.
more resistant to the immune system in 2 ways: directly down-regulating the complement alternative pathway or preventing the antibodies competitively binding for the same hfH binding site on fHbp, or both (13). The crystal structure of fHbp v3 in complex with the complement control protein domain 6 and 7 of hfH was previously determined (17). Comparing the binding site of hfH and Fab 1E6, we observe that 1E6 binds fHbp v3 on the opposite side, and accordingly, the data obtained herein by flow cytometry analyses confirm that this antibody is not able to prevent the hfH binding to fHbp (Supplemental Fig. S4A), and the hfH cannot block the 1E6 binding to bacteria (Supplemental Fig. S4B).

In addition to its antigenic crossreactivity, the functional characterization of mAb 1E6 showed that it is crossprotective in rSBA. This finding suggests that vaccination with 4CMenB can induce the production of at least a small set of crossprotective antibodies in humans. In summary, here we report the identification of a highly conserved epitope, the first epitope recognized by a human mAb and predominantly localized on the N terminus of fHbp. Improving the knowledge of the epitope profiles identified by potent human antibodies could facilitate antigen engineering aiming to induce the immune system to continuously generate novel antibodies able to recognize multiple sites for a broadly protective response. Moreover, epitope mapping is a crucial step in the development of therapeutic mAbs, allowing improvements of the affinity, recognition breadth, and bactericidal efficacy potentially for treatment of meningococcal infections. The results presented here reinforce the proof-of-concept for the use of memory B-cell–derived huFab sequences in the structural and functional analysis of the human immune response after 4CMenB vaccination. This information will be useful for future vaccine research projects and to train and potentiate capabilities of emerging computational methods for antibody modeling and B-cell epitope predictions.

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AUTHOR CONTRIBUTIONS

F. Bianchi and D. Veggi contributed equally to this work; F. Bianchi and D. Veggi performed the research, participated in data analysis, and wrote the paper; L. Santini, F. Buricchi, E. Bartolini, P. Lo Surdo, and M. Martinelli performed the research and participated in data analysis; O. Finco, V. Masiagni, and D. Maione designed research; M. J. Bottomley and R. Cozzi designed research, performed research, analyzed data, and wrote the paper; and all authors reviewed and approved the final version of the manuscript.

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