

Factor H-binding protein is important for meningococcal survival in human whole blood and serum, and in the presence of the antimicrobial peptide LL-37.

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Running title: fHBP as a survival factor of *N. meningitidis*

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Abstract

Factor-H binding protein (fHBP; GNA1870) is one of the antigens of the five-component recombinant vaccine against serogroup B *Neisseria meningitidis*, which has been developed using 'Reverse Vaccinology' and that is the basis of a meningococcal B vaccine entering phase III clinical trials. Binding of factor H (fH), an inhibitor of the complement alternative pathway, to fHBP enables *N. meningitidis* to evade killing by the innate immune system. All fHBP null mutant strains analyzed were sensitive to killing in *ex vivo* human whole blood and serum models of meningococcal bacteremia, with respect to the isogenic wild type strains. The Δ fHBP strains of MC58 and BZ83 (high fHBP expressors) survived in human blood and serum less than 60 minutes (decrease of $>2 \log_{10}$ CFU), while NZ98/254 (intermediate fHBP expressor) and 67/00 (low fHBP expressor) showed a decrease of $>1 \log_{10}$ CFU after 60-120 minutes incubation. In addition, fHBP is important for survival in the presence of the antimicrobial peptide LL-37 (decrease of $>3 \log_{10}$ CFU after 2 h of incubation), most likely due to electrostatic interactions between fHBP and the cationic LL-37. Hence, the expression of fHBP by *N. meningitidis* strains is important for survival in human blood and human serum, and in the presence of LL-37 even when expressed at low levels. The functional significance of fHBP in mediating resistance to the human immune response, in addition to its widespread distribution and its ability to induce bactericidal antibodies, indicates that it is an important component of the serogroup B meningococcal vaccine.

INTRODUCTION

Disease caused by *Neisseria meningitidis* remains a major worldwide cause of morbidity and mortality even after the development of vaccines to protect against several meningococcal serogroups. *N. meningitidis* colonizes the mucosa of the nasopharynx of 5-10% of the population, and in susceptible individuals the bacterium can cross the epithelial layer into the bloodstream, causing septicemia and/or meningitis (36). The development of meningococcal serogroup A, C, W-135, Y capsular polysaccharide based vaccines, as well as tailor made meningococcal serogroup B outer membrane vesicle (OMV) vaccines, has dramatically reduced disease in areas where these vaccines have been widely used (reviewed in 40). Serogroup B (MenB) meningococcal strains cause one third of meningococcal disease in the United States of America, and up to 80% of cases in Europe, yet there is no comprehensive vaccine available to protect against this serogroup. However, the *in silico* genome-based approach, termed "Reverse Vaccinology", has led to the development of a recombinant 5-component vaccine against Meningococcal serogroup B strains (5CVMB) which forms the basis of a MenB vaccine entering phase III clinical trials. Initial results indicate that this vaccine is well tolerated and induces bactericidal antibodies against several genetically diverse serogroup B *N. meningitidis* strains (10, 33). The MenB vaccine contains the Genome-derived *Neisseria* Antigens GNA1870 (fHBP), GNA1994 (NadA), GNA2132, GNA1030 and GNA2091 (10).

GNA1870 is a 28kDa surface exposed lipoprotein that binds factor H (fH), a key inhibitor of the complement alternative pathway (AP), leading to evasion of killing by the innate immune system. GNA1870 has thus been named factor H binding protein (fHBP) (25). This antigen is expressed by all *N. meningitidis* strains studied to date, however the level of expression varies between strains (high, intermediate, or low expressors). fHBP has also been found in the culture supernatant of strain MC58 (26). fHBP induces high levels of bactericidal antibodies, and even the very low expressors are efficiently killed in serum bactericidal activity (SBA) assays (44), which are used as the main correlate of protection for meningococcal disease (42). In addition, anti-fHBP

antibodies can block binding of fH, increasing the susceptibility to killing by the complement AP (2, 25). However, it has recently been suggested that unlike fHBP of high expressors, fHBP of low expressors is not required for survival in nonimmune human blood or in the presence of certain anti-fHBP monoclonal antibodies (mAbs) (43). Antibodies to fHBP (mAbs (44) and polyclonal antibodies (26) to the recombinant protein, or antibodies elicited by an fHBP enriched OMV vaccine (16)), also confer passive protection against *N. meningitidis* bacteremia in an infant rat model.

fHBP can be classified into three allelic groups (variant 1, 2 or 3) based on its amino acid sequence. Conservation within the allelic groups ranges from 91.6% - 100%, while conservation between these groups is as low as 62% (19, 26). fH can bind to all three fHBP variants (25), but bactericidal antibodies induced by fHBP are variant-specific. Variant 1 fHBP is found in approximately 60% of disease-producing serogroup B isolates (44) and is present in the 5CVMB MenB vaccine (10). fHBP is the principal antigen of another MenB vaccine containing recombinant proteins from two different variant sub families, which is currently in clinical trials (called LP2086) (reviewed in 3, 45). Additional vaccines being investigated have been focused on OMVs isolated from a strain overexpressing variant 1 fHBP (16, 21) as well as on recombinant chimeric fHBP (1).

Some non-bactericidal anti-fHBP antibodies are able to confer passive protection against meningococcal infection in the infant rat animal model, most likely via opsonophagocytosis (OP) (16) or interactions with other components of the immune response. LL-37, a cationic antimicrobial peptide, is a key component of the human innate immune system that is constitutively expressed by leukocytes including monocytes, neutrophils, T cells, NK cells, and B cells, and inducibly expressed by epithelial cells (7), including the epithelial cells of the nasopharynx (24). LL-37 is a short, positively charged, α -helical peptide that has a broad spectrum of antimicrobial activity, through its direct targeting and disruption of the negatively charged bacterial surface membrane (31) as well as its immunoregulatory roles (4). The MtrCDE efflux pump, lipid A modification, the type IV pilin secretion system and the capsule (39, 41) are all involved in protection of

N. meningitidis against killing by antimicrobial peptides. Two hypothetical genes of *N. meningitidis*, NMB0741 and NMB1828, have recently been shown to be upregulated in the presence of antimicrobial peptide (along with 19 other gene that are differentially expressed in the presence of CRAMP) and play a role in cathelicidin resistance (9).

To better characterize the functional role of fHBP, the survival of four strains of *N. meningitidis* shown to express different levels of fHBP variant 1 (26), and their Δ fHBP mutant derivative strains, was investigated in *ex vivo* human whole blood and serum models of meningococcal bacteremia, as well as *in vitro* survival assays in the presence of antimicrobial peptides (LL-37 and polymyxin B) and other environmental stresses. The results presented show that the expression of fHBP by *N. meningitidis* strains is important for survival in human blood and human serum regardless of the level of its expression. We also show that fHBP increases meningococcal survival in the presence of the antimicrobial peptide LL-37, which is most likely mediated by electrostatic interactions between fHBP and LL-37 at the cell surface.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. meningitidis* strains used in this study are described in Table 1. *N. meningitidis* strains were routinely grown on GC agar (Difco™) or Mueller Hinton (MH) agar (Difco™) at 37°C/5% CO₂ overnight. For liquid cultures, overnight growth was used to inoculate GC or MH broth. *Escherichia coli* strains used for cloning were cultured in Luria-Bertani (LB) broth or on LB agar (Difco™). When required, erythromycin and/or chloramphenicol were added to achieve a final concentration of 5 µg/ml.

Recombinant DNA techniques. Recombinant DNA techniques were routinely performed as described by Sambrook *et al.* (37). Plasmid DNA preparations and purification of DNA fragments from PCR samples and agarose were performed using QIAGEN kits according to the manufacturer's instructions. PCR amplification was carried out in a 50µl reaction consisting of 1x

reaction buffer, 1.5mM MgCl₂, 0.2mM dNTPs, 1 unit of Platinum Taq polymerase (Invitrogen), and 10pmol of each forward and reverse primer, an appropriate amount of chromosomal or plasmid DNA and sterilized deionised water. All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (NEB; Beverly, MA).

Construction of fHBP knockout *N. meningitidis* strains. The isogenic MC58 wild type and MC58ΔfHBP (previously called MC58Δ*gna1870*) knockout mutant, in which the *gna1870* gene was truncated and replaced with an erythromycin antibiotic cassette, have been described previously (26). The pBSΔ*gna1870*ERM knockout plasmid was used to generate isogenic knockout mutants in BZ83, NZ98/254 and 67/00 as described previously (26). Complementation of the MC58ΔfHBP null mutant was achieved by insertion of the *gna1870* gene, under the control of the *P_{tac}* promoter, along with the chloramphenicol resistance gene into a noncoding chromosomal location between the two converging open reading frames (ORFs) NMB1428 and NMB1429 through transformation of the MC58ΔfHBP strain with pComfHBP complementation plasmid that was generated as follows. The *P_{tac}* promoter and ribosome binding site were amplified using the Tac1 (ATTCGGGTACCGGCGCACTCCCGTTCTGGATA) and Pind-R (AATGCATGCATGGTCATATGTGTTTCCTGTGAATTG) primers and cloned as a 234 bp KpnI-NsiI fragment adjacent to the chloramphenicol cassette into pSLComCmr plasmid (17) generating pComP_{RBS}. The *gna1870* gene was amplified with primers 741-F2 (GGAATTCCATATGGTGAATCGAACTGCCTTC) and 741-R2 (CCAATGCATTTATTGCTTGGCGGCAAG) from the MC58 genome and cloned as a 760 bp NdeI-NsiI fragment into the pComP_{RBS} plasmid, generating pComfHBP. Transformants were selected on chloramphenicol and checked by PCR, and complementation of the mutant strain was verified by Western blot analysis. The resulting complemented strain was named MC58ΔfHBP_C.

Western blot analysis. Expression of fHBP in whole cell samples and culture supernatants was performed essentially as described previously (26). Briefly, strains were grown at 37°C /5% CO₂ in MH broth plus 0.25% glucose from an optical density at 600nm (OD₆₀₀) of 0.05 to 0.5. Bacterial cells were collected by centrifugation, washed once with PBS, and resuspended in SDS-PAGE loading buffer. Culture supernatant was filtered using a 0.2 µm filter and 1 ml precipitated by the addition of 250 µl of 50% trichloroacetic acid (TCA). The sample was incubated on ice for 2 h, centrifuged for 40 min at 4°C and the pellet washed with 70% ice cold ethanol, and resuspended in PBS. Samples were run on 4-12% polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Ponceau staining was performed to ensure equal loadings of the samples. Western blot analysis was performed according to standard procedures using anti-fHBP polyclonal antibodies at a 1:1,000 dilution (raised in mice against the recombinant fHBP purified protein (rfHBP), as described previously (26)), followed by a 1:5,000 dilution of HRP-labeled anti-mouse IgG (Sigma-Aldrich).

***Ex vivo* human whole blood and human serum models of meningococcal bacteremia.** Cells were harvested into MH broth containing 0.25% glucose and 0.02mM Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (CMP-NANA) to an OD₆₀₀ of 0.05 and grown to mid-log phase (OD₆₀₀ 0.5-0.6) then diluted in MH broth to approximately 10³ colony forming units (CFU)/ml. The assay was started by the addition of 240µl whole human blood, human serum or heat inactivated human serum (56°C 30 min) to 10µl of bacterial suspension. Cultures were incubated at 37°C/5%CO₂ with gentle agitation, and at various time points an aliquot of the sample was removed and the number of viable CFU were determined by plating serial dilutions onto MH agar and incubating overnight at 37°C/5% CO₂. Experiments were performed in triplicate on several occasions. The students' t-test was used to determine the statistical significance of survival of each mutant strain with respect to the isogenic wild type strain, with a p-value <0.05 considered to be significant.

Whole venous blood, collected from healthy individuals (unimmunized against *N. meningitidis*) and anti-coagulated with heparin (10U/ml), was used for whole blood experiments as described by Ison et al. (18). For preparation of human serum, whole blood was coagulated at 25°C for 30 min, centrifuged at 1,000xg for 10 min at 4°C and the supernatant retained.

***In vitro* antimicrobial peptide and environmental stress assays.** Overnight cultures were harvested into GC broth and diluted to approximately 10⁴ CFU/ml. Each assay was started by the addition of 10µl of a compound to 90µl of the bacterial suspension. Compounds used are as follow: LL-37 (final concentration: 2-7 µM; Innovagen, Sweden), polymyxin B (5-50 µg), detergent (0.01-0.1% SDS, Tween, Triton X), reactive oxygen species (2-10mM H₂O₂, 0.005-0.02% cumene hydroperoxide), or generators of reactive oxygen species (2.5-10mM paraquat, xanthine (1mM) /xanthine oxidase (200-400mU)). For osmotic stress assays, 10µl of bacterial suspension was added to 90µl of either NaCl (2-5M) or sucrose (10-30%) solutions. Cultures were incubated at 37°C/5%CO₂ with gentle agitation, and CFU were determined as described above.

For some experiments, GC broth with a pH of 5, 6, 7, 8, or 9 or 2% NaCl was used to investigate the influence of pH and %NaCl on LL-37 mediated killing. In some cases, LL-37 was also incubated in PBS with varying concentrations of rfHBP or cell free extract for 15 min prior to the assay as described below.

Growth in the presence of LL-37 was monitored using MC58 wild type, MC58ΔfHBP and MC58ΔfHBP_C strains inoculated into GC broth to an OD₆₀₀ of 0.05. Bacteria were incubated at 37°C/5%CO₂ with or without 1µM LL-37 and growth was followed for 5 h by reading the OD₆₀₀.

Investigation of LL-37 – fHBP interactions: peptidase activity assay. The effect of fHBP on LL-37 was investigated using either rfHBP (26), or cell free extracts from strains MC58 and MC58ΔfHBP. Cell free extracts of overnight cultures of *N. meningitidis* were prepared by resuspending the cells in PBS, followed by three cycles of freezing and thawing. Unbroken cells

and cell debris were removed by centrifugation at 13,000 rpm for 10 min and the supernatant collected and passed through 0.2µm filters. LL-37 (10 µg) was incubated with rfHBP (10µg) or 10µl of cell free extract in PBS at 37°C and samples were taken at 1 min, 5 min, 15 min, 1 hr and 15 hr and the reactions were stopped by boiling for 5 min. Samples were analyzed by SDS-PAGE (4-12% Tris-tricine gel) and stained with 0.25% Coomassie brilliant blue.

Far-Western dot blot. 5µl spots containing 1.25 or 2.5µg of BSA, rfHBP or LL-37, or 5µl bacterial suspension in PBS (OD₆₀₀ of 1) were applied to a nitrocellulose filter. The filter was air dried for 15 min, blocked in 10% milk powder in PBS-0.05% Tween (PBST) overnight and then was incubated with 4.5µg LL-37 in 8ml PBS for 1 hr at room temperature. The membrane was washed 3 times in PBST, then treated as per a normal Western blot, anti-LL-37 polyclonal antibody (Innovagen) was added at a 1:1,000 dilution, followed by a 1:5,000 dilution of HRP-labeled anti-rabbit IgG (Sigma-Aldrich).

Size-exclusion gel chromatography. Purified recombinant fHBP (98 µg) (26) was incubated with LL-37 (156 µg (1-10 molar ratio) or 78µg (1:5 molar ratio)) in PBS for 1 h and loaded onto a High-Load Superdex_75_10/300 gel filtration column (GE Healthcare) equilibrated with PBS, and 0.5-mL fractions were eluted at a flow rate of 0.8 mL/min. Fractions were analyzed by Western blot using anti-fHBP and anti-LL-37 polyclonal antibodies as described above.

RESULTS

Analysis of fHBP expression and localization in various *Neisseria* strains. Four strains of *N. meningitidis* shown to express different levels of fHBP variant 1 (26) were used in this study to investigate the role of meningococcal fHBP in response to various components of the human immune system (see Table 1 for strain details). Western blot analysis of whole cell extracts of these strains confirms the different levels of fHBP expression (MC58 and BZ83 (high expressors) > NZ98/254 (intermediate expressor) > 67/00 (low expressor))(Figure 1A). The culture supernatant of these strains was also investigated, and like MC58 (26), fHBP is present in the culture supernatant

of all strains studied (Figure 1B). No fHBP was detected in the whole cell extract or the supernatant for the isogenic Δ fHBP mutant strains. Furthermore, fHBP is once again expressed in the complemented strain MC58 Δ fHBP_C, in which the gene encoding fHBP is inserted *in trans* under the control of the P_{tac} promoter, however expression is not fully restored to wild type levels (Fig 1A, B).

fHBP of *N. meningitidis* is important for survival in *ex vivo* human whole blood and human serum models of meningococcal bacteremia. *N. meningitidis* MC58, BZ83, NZ98/254, 67/00 wild type strains, and the isogenic Δ fHBP mutants of these strains, were used to investigate the role of meningococcal fHBP in *ex vivo* human whole blood and human serum models of meningococcal bacteremia. The human blood assay was used to assess both cellular and humoral mechanisms of killing (including the action of complement, antibody-mediated serum bactericidal activity (SBA), and opsonophagocytosis (OP), as well as killing by neutrophils, macrophages and antimicrobial peptides) while the serum assay was used to assess killing of *N. meningitidis* mediated by the humoral immune response. The wild type and mutant strains were incubated with human whole blood or serum for two hours, and samples taken at various time points to assess survival. All of the Δ fHBP mutant strains were highly sensitive to killing by both human whole blood and human serum when compared to their isogenic wild type parent strains (1-2 \log_{10} less survival for Δ fHBP strains with respect to wild type, P value ≤ 0.04 ; Figures 2 and 3). The MC58 Δ fHBP and BZ83 Δ fHBP mutant strains (high expressors) survived less than 60 minutes (panels A and B) while NZ98/254 Δ fHBP (intermediate expressor) and 67/00 Δ fHBP (low expressor) survived 120 minutes or longer (panels C and D). The level of survival of mutant and wild type strains varied between different donors and strains, most likely due to differences in complement activity, cellular killing, levels and types of antibodies in each donor, in combination with different levels of intrinsic sensitivity to killing of the different strains. In addition, a higher level of killing was seen in blood than serum in certain cases (donors 1 and 2 for NZ98/254 strains; donor 1 for 67/00 and MC58

strains) indicating the involvement of leukocytes and OP in bactericidal activity. On the other hand, serum from donor 2 mediated more killing of BZ83 and 67/00 strains than blood, suggesting that antibody-mediated SBA is the predominant mode of killing of these strains by this donor.

The phenotype of MC58ΔfHBP in whole blood and serum is reversible when a single copy of the fHBP allele is provided in *trans* (MC58ΔfHBP_C; Figures 2A and 3A), indicating that this phenotype is specifically mediated by fHBP. The lower fHBP expression seen in the MC58ΔfHBP_C strain compared to the wild type strain (Figure 1) is consistent with the finding that the complemented strain is not fully rescued from killing by whole blood or serum. The wild type and ΔfHBP strains all behave in a similar manner when incubated for the duration of the assay in GC broth, indicating that differences in survival are not due to intrinsic growth or survival defects (approximately 3 fold increase in CFU over 120 min; data not shown). The wild type and ΔfHBP strains also behave in a similar manner when incubated in heat-inactivated serum, with no growth or killing seen over 120 min (data not shown). These results indicate that bacterial killing in serum is mediated by complement. This is consistent with the role of fHBP in providing resistance to complement mediated killing by binding the complement inhibitor fH (25); there is no binding of fH to fHBP in the ΔfHBP strain, enabling increased killing relative to the wild type strain. These results show that the expression of fHBP by *N. meningitidis* strains is important for survival in human blood and human serum even in strains with low levels of expression.

fHBP of *N. meningitidis* is involved in resistance to killing by the antimicrobial peptide LL-37.

The presence of an additional role of fHBP, in protection against the antimicrobial peptide LL-37, was discovered during investigation of survival of wild type and ΔfHBP strains in the presence of various antimicrobial compounds. Antimicrobial peptides are an important part of the human innate immune system, which are constitutively produced by leukocytes, and are also present in epithelial cells of the nasopharynx (24), the primary site of meningococcal colonization and infection. In order to determine whether fHBP is involved in protection against killing by antimicrobial peptides,

in vitro survival and growth assays were performed with wild type and Δ fHBP strains in the presence of LL-37, a short cationic alpha-helical peptide. MC58, BZ83, NZ98/254 and 67/00 Δ fHBP strains were sensitive to LL-37 with respect to their wild type parent strain ($>1 \log_{10}$ less survival than wild type at 120 min, $P \leq 0.01$; Figure 4). Moreover, the MC58 wild type phenotype is restored in the genetically complemented strain, MC58 Δ fHBP_C (Figure 4A). Killing of *N. meningitidis* by LL-37 is dose-dependent, with increasing concentrations of LL-37 between 2-5 μ M mediating increased killing of the wild type strains, and also causing increased sensitivity of the Δ fHBP strain with respect to its wild type parent strain (data not shown). The *N. meningitidis* strains NZ98/254 and 67/00 are intrinsically more resistant to LL-37 than MC58 and BZ/83 (3.5 μ M and 5 μ M LL-37 used in killing assays shown in Figure 4AB and 4CD, respectively). For NZ98/254 and 67/00, less than 1 \log_{10} decrease in CFU was seen over 2 hours in the presence of 3.5 μ M LL-37 compared to an almost 3 \log_{10} decrease in CFU seen for MC58 and BZ/83 (data not shown). These results show that also in the case of LL-37-mediated killing, fHBP is involved in protection regardless of the expression level of fHBP.

The ability of MC58, MC58 Δ fHBP, and MC58 Δ fHBP_C to grow in the presence of 1 μ M LL-37, a concentration that only marginally affects the growth of the MC58 wild type strain (9), was also investigated. All strains exhibited similar growth rates in the absence of peptide (doubling times between 55-60 min), however the growth of MC58 Δ fHBP was significantly decreased in the presence of LL-37 (110 min doubling time), with respect to the wild type (58 min doubling time) and the complemented (68 min doubling time) strains (Figure 4E).

In order to determine whether the Δ fHBP mutation altered the integrity of the bacterial membrane or induced a complex phenotype, leading to non-specific sensitivity to LL-37, we performed several additional stress assays. Survival of MC58 and MC58 Δ fHBP was investigated in the presence of: (1) polymyxin B, a cationic cyclic lipopeptide that is believed to have a similar mechanism of action as that of LL-37 (23, 39); (2) oxidative stress to mimic the oxidative burst of phagocytic cells; and (3) envelope stress using osmotic stress and detergent stress. The

MC58 Δ fHBP strain did not display increased sensitivity with respect to the wild type strain during these *in vitro* killing assays (data not shown), indicating that the Δ fHBP mutation does not significantly alter the integrity of the bacterial membrane and does not cause a pleiotropic phenotype.

The bacterial mechanisms generally described for evading killing by antimicrobial peptides include active extrusion, protease digestion, sequestration or electrostatic repulsion of the peptide (32). We investigated whether one of these mechanisms could mediate the resistance of fHBP to LL-37 and results show that rfHBP does not have proteolytic activity towards LL-37, it does not bind LL-37 in Far-western dot blot and gel filtration analyses, nor does it sequester LL-37 if added to cells prior to the LL-37 killing assay (data not shown). However, MC58 and MC58 Δ fHBP have increased resistance to killing by LL-37 in the presence of 2% NaCl (disrupts electrostatic interactions), and low pH (increases positive charge of surface-exposed proteins) indicating the presence of significant electrostatic interactions between LL-37 and the meningococcal cell surface (data not shown). The solution structure of the BC domain of fHBP revealed well-defined patches of negative and positive charges, which could mediate electrostatic interaction with, or repulsion of, LL-37 (26).

DISCUSSION

N. meningitidis, a commensal of the nasopharyngeal mucosa and the causative agent of life-threatening meningococcal septicemia and meningitis, is exposed to components of mucosal and systemic immunity during colonization and infection, respectively (22, 34). The ability of meningococci to colonize the mucosal epithelium as well as to survive and multiply within human blood are key factors in the development of fulminant meningococcal disease. The importance of various factors of the immune system in preventing development of meningococcal disease is evidenced by the increased incidence and recurrence of infection and disease in people with immune disorders, including deficiencies of the terminal complement factors (C5-C9) or

complement regulator proteins factors H and I (14, 27, 29, 34). *N. meningitidis* has developed many mechanisms to evade the human immune response (28) and fHBP (also known as GNA1870, LP2086) is emerging as an important player in mediating resistance to host defenses.

fHBP is widely distributed throughout the circulating meningococcal serogroup B population and is a component of the multivalent MenB vaccine that is currently in human clinical trials as a comprehensive meningococcal serogroup B vaccine (10, 33). fHBP elicits a strong bactericidal immune response and induced anti-fHBP antibodies have two identified modes of action; firstly, by directly mediating bacteriolysis via the complement classical pathway (CP) through SBA and OP (26, 43), and secondly by blocking binding of fH, a key inhibitory regulator of the complement AP, increasing the susceptibility to killing by the complement AP (2, 25). fHBP-mediated interactions with the host immune response, and mechanisms by which vaccine induced anti-fHBP antibodies contribute to protection against meningococcal infection, are outlined in Figure 5.

Here we show that fHBP of *N. meningitidis* is important for survival in *ex vivo* models of bacteremia using normal human blood and serum, regardless of the level of its expression by different strains. Recent findings indicate that unlike fHBP of high expressors, fHBP of low expressors is not required for survival in ‘nonimmune’ human blood (43). The differences between the results of these two studies are likely due to donor selection. In comparison to nonimmune blood where wild type strains grow or maintain the initial bacterial counts during the experiment, the assay reported herein uses human blood and serum which mediate some killing of the wild type strain (0.5-1 log₁₀ decrease in CFU over 2 hr), most likely due to background levels of antibodies to *N. meningitidis*, or cross-reactive antibodies, within the blood of the donors. Using the same ‘nonimmune’ donor serum from that study, we were able to replicate the finding that NZ98/254ΔfHBP did not have greatly reduced survival relative to the wild type strain (43); data not shown).

The findings from this study indicate the importance of a third mode of action by which anti-

fHBP antibodies induce a bactericidal response (Figure 5B). fH, by controlling the key steps in the complement AP amplification cycle, also dampens activation that has been initiated via the complement CP or the lectin pathway (13, 15). Hence, antibody-mediated blocking of fH binding to fHBP enables amplification of the complement CP and enhances CP-mediated killing in the presence of other antibodies. Therefore, even when expressed at low levels, fHBP likely plays a role in resistance to *N. meningitidis* by dampening activation of the complement CP that occurs in the presence of anti-meningococcal antibodies in normal blood. Conversely, in the absence of fH binding (Δ fHBP strains or blocking of binding by antibodies (2, 25)), AP complement-mediated killing of *N. meningitidis* is derepressed, and the effect of the complement CP may be amplified. These findings are particularly interesting in the context of the general population or a population vaccinated with a multivalent vaccine containing fHBP, both of which would be expected to have an array of anti-meningococcal antibodies. Colonization by *N. meningitidis*, *N. lactamica* or certain enteric bacteria, has been reported to induce an immunologic response so that by young adulthood the majority of people have measurable levels of antibody to the pathogenic meningococcal serogroups (8, 12, 35). In addition, more than 50% of newborn infants have bactericidal maternal antibodies to the major meningococcal serogroup, the prevalence of which wanes after birth and is lowest from 6 - 24 months of age (11). This correlates with the highest rate of serogroup B meningococcal disease occurring in infants under the age of 1 year (5). This age group is the main target of a comprehensive serogroup B vaccine. The MenB vaccine was recently administered to 150 healthy UK infants concomitantly with routine immunizations at 2, 4 and 6 months of age, with a booster dose at 12 months (30).

From studies using mAbs, it has been suggested that multiple sites on the surface of fHBP may interact with fH (2). Hence vaccine-induced polyclonal antibodies raised to fHBP variant 1, even if not bactericidal or directly protective against strains carrying other fHBP variants, may still interfere with fH binding thus enabling amplification of the immune response induced by the other antigens of a multicomponent vaccine.

We have identified an additional role of fHBP in protection against killing by the cationic antimicrobial peptide LL-37, in high, intermediate and low expressing strains (Figure 5A). Since LL-37 is produced by cells that interact with *N. meningitidis* during infection, including the nasopharyngeal epithelia and phagocytic cells of the blood, it may be involved in innate host defenses against meningococcal disease. The in vivo-role of LL-37 in antimicrobial host defense has been demonstrated for several pathogens (reviewed in 20). The direct mechanisms of LL-37 dependent microbial killing are not completely understood, however LL-37 is believed to act through a blanket effect by interaction of its positive charges with the negatively charged bacterial surface, causing destabilization and permeabilization of the membrane. In response, bacteria have evolved several mechanisms for evading killing by antimicrobial peptides, including 1) active extrusion via an export complex, 2) protease digestion of the peptide, 3) sequestration by secreted proteins, and/or 4) alteration of the surface charge to prevent electrostatic interactions (32). While the mechanism of fHBP-mediated resistance to antimicrobial peptides remains elusive, our data suggest that the sensitivity of fHBP-deficient strains is not attributable to decreased outer membrane stability, nor is fHBP involved in protease activity, efflux, or sequestration of LL-37. We have not ruled out the possibility of an indirect effect of fHBP in resistance to LL-37, however it is unlikely that fHBP has a secondary effect on MtrCDE efflux pump since wild type and mutant strains display the same sensitivity to polymyxin B, unlike meningococcal MtrCDE mutant strains that have increased sensitivity to both LL-37 and polymyxin B (42). fHBP mediated protection against LL-37 is most likely mediated by electrostatic interactions which prevent interaction of LL-37 with the cell surface. The solution structure of the immunodominant BC domain of fHBP revealed an extensive hydrophobic region on one side of the molecule that may interact with the outer membrane or other molecules, while the other face has a well-defined negative patch and diverse patches of positive charge (26), which could mediate electrostatic interaction with LL-37 preventing contact with the cell membrane.

LL-37 may contribute to the sensitivity of the Δ fHBP strains seen in the *ex vivo* blood and serum assays described above. Since fHBP binds specifically to human, but not rat or mouse, factor H (38), the fact that nonbactericidal antibodies raised against fHBP can provide passive protection in an infant rat model (16) supports the presence of an additional role of fHBP.

fHBP is an attractive vaccine antigen due to its complex interactions with the immune system and the fact that anti-fHBP antibodies have multiple modes of inducing a bactericidal response. In addition, the functional role of fHBP in survival of *N. meningitidis* in *ex vivo* (human serum and whole blood) and *in vitro* (antimicrobial peptide LL-37) infection models may decrease the likelihood of vaccine-induced selection of mutants lacking fHBP.

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Figure Legends.

Figure 1. fHBP expression by *N. meningitidis* strains. Western blots of (A) whole cell lysates and (B) culture supernatants (S/N) of strains expressing high (MC58 and BZ83), intermediate (NZ98/254), and low (67/00) levels of fHBP. The Δ fHBP isogenic mutants of these strains and the complemented strain MC58 Δ fHBP_C are also shown.

Figure 2. Survival of wild type and Δ fHBP strains in the *ex vivo* human whole blood model of meningococcal septicemia. Results show survival of strains in human whole blood from two different donors over two hours. (A) MC58 wild type strain, MC58 Δ fHBP mutant strain and MC58 Δ fHBP_C complemented strain. (B) BZ83 wild type strain and BZ83 Δ fHBP mutant strain. (C) NZ98/254 wild type strain and NZ98/254 Δ fHBP mutant strain. (D) 67/00 wild type strain and 67/00 Δ fHBP mutant strain. P values using Student's T-test for the survival of the mutant strain, or the MC58 Δ fHBP_C complemented strain, with respect to the isogenic wild type parent strain are ≤ 0.04 for 120 min time point in all assays.

Figure 3. Survival of wild type and Δ fHBP strains in the *ex vivo* human serum model of meningococcal septicemia. Results show survival of strains in human serum from two different donors over two hours. (A) MC58 wild type strain, MC58 Δ fHBP mutant strain and MC58 Δ fHBP_C complemented strain. (B) BZ83 wild type strain and BZ83 Δ fHBP mutant strain. (C) NZ98/254 wild type strain and NZ98/254 Δ fHBP mutant strain. (D) 67/00 wild type strain and 67/00 Δ fHBP mutant strain. (E) MC58 wild type strain, MC58 Δ fHBP mutant strain and MC58 Δ fHBP_C complemented strain in the presence of serum heat inactivated for 30 min at 56°C. P values using Student's T-test for the survival of the mutant strain with respect to the isogenic wild type parent strain are ≤ 0.02 for 120 min time point in all assays. The P values for survival of the MC58 Δ fHBP_C complemented strain with respect to the wild type are 0.02 and 0.07 for donors 1 and 2, respectively.

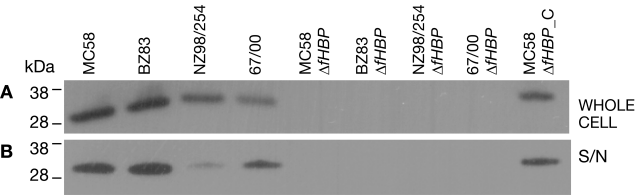
Figure 4. Survival and growth of wild type and Δ fHBP strains in the presence of the antimicrobial peptide LL-37. Results show survival of strains in the presence of 3.5 μ M (A and B) or 5 μ M (C and D) LL-37 over two hours. (A) MC58 wild type strain, MC58 Δ fHBP mutant strain and MC58 Δ fHBP_C complemented strain. (B) BZ83 wild type strain and BZ83 Δ fHBP mutant strain. (C) NZ98/254 wild type strain and NZ98/254 Δ fHBP mutant strain. (D) 67/00 wild type strain and 67/00 Δ fHBP mutant strain. P values using Student's T-test for the survival of the mutant strain with respect to the isogenic wild type parent strain are ≤ 0.01 for 120 min time point in all assays. (E) Growth of MC58 WT, Δ fHBP and Δ fHBP_C in the absence (-) or presence (+) of 1 μ M LL-37.

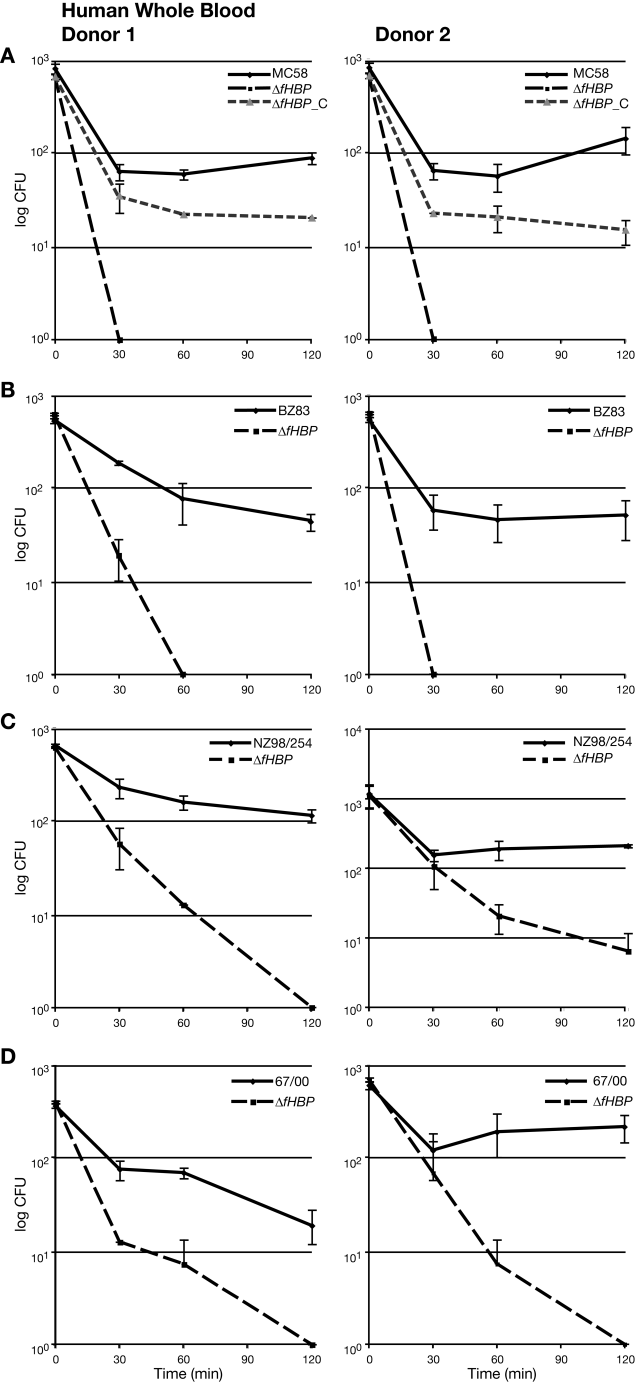
Figure 5. Model of (A) fHBP-mediated interactions with the host immune response and (B) mechanisms by which vaccine induced α -fHBP antibodies contribute to protection against meningococcal infection. (A) Binding of fH by fHBP leads to inhibition of the complement alternative pathway (AP). fHBP also provides protection against killing by the cationic antimicrobial peptide LL-37, by an unknown mechanism. (B) α -fHBP antibodies induce bactericidal activity via several mechanisms: (1) direct activation of the complement classical pathway (CP), leading to complement-dependent serum bactericidal activity (SBA) and opsonophagocytosis (OP); (2) prevention of inhibition of the AP by blocking of fH binding, leading to AP-mediated killing; (3) amplification of the CP (3a) in the absence of AP inhibition, leading to increased bactericidal activity of other anti-meningococcal antibodies (3b); (4) disruption of interactions between LL-37 and fHBP may lead to increased destabilization of the bacterial cell by LL-37.

Table 1. Summary of *Neisseria meningitidis* strains used in this study.

Wild type Strains	Strain Description				fHBP information		Ref.
	Country of origin (Year)	Serological classification	ET cluster (ST)		fHBP variant ^a (% ID) ^b	fHBP expression	
MC58	United Kingdom (1985)	B:15:P1.7, 16	ET5	(ST-74)	1.1 (100)	high	6
BZ83	The Netherlands (1984)	B:15:P-	ET5	(ST-32)	1.1 (100)	high	6
NZ98/254	New Zealand (1998)	B:4:P1.4	Lineage 3 (ST-42)		1.10 (92)	medium	6
67/00	Norway (2000)	B: P1	Lineage 3 (ST-41/44)		1.10 (92)	low	6
Mutant Strains	Strain Description						Ref.
MC58ΔfHBP	fHBP mutant; derivative of MC58 in which the fHBP gene is replaced by a erythromycin cassette						25
MC58ΔfHBP_C	Complemented fHBP mutant; derivative of MC58 ² fHBP in which the fHBP gene and promoter were inserted, with a chloramphenicol cassette, in the noncoding region between NMB1074 and NMB1075						this study
BZ83ΔfHBP	fHBP mutant; derivative of BZ83 in which the fHBP gene is replaced by a erythromycin cassette						"
NZ98/254ΔfHBP	fHBP mutant; derivative of NZ98/254 in which the fHBP gene is replaced by a erythromycin cassette						"
67/00ΔfHBP	fHBP mutant; derivative of 67/00 in which the fHBP gene is replaced by a erythromycin cassette						"

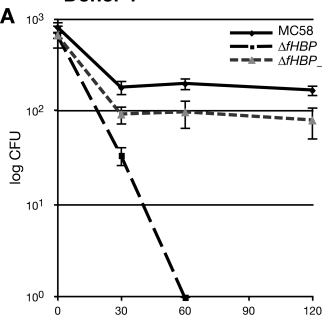
^a fHBP variant nomenclature is described in ref. 21. ^b %ID to fHBP of MC58.



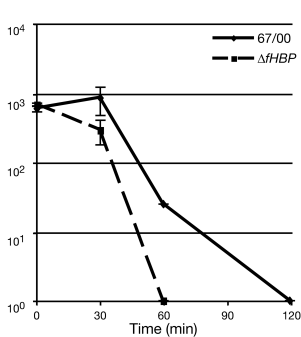
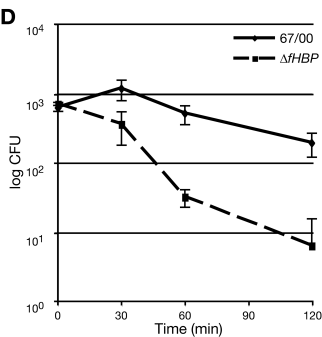
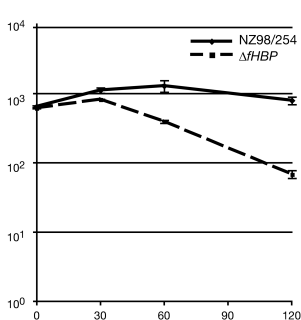
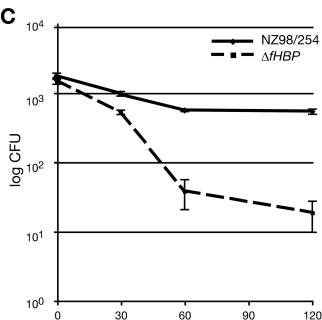
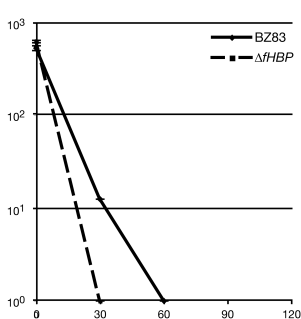
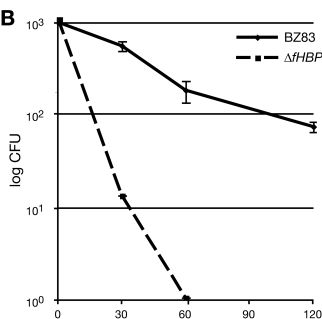
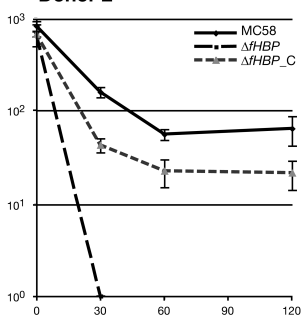


Human Serum

Donor 1



Donor 2



LL-37