The Development of an Experimental Multiple Serogroups Vaccine for *Neisseria meningitidis*

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**Abstract**

A native outer membrane vesicles (NOMV) vaccine was developed from three antigenically diverse strains of *Neisseria meningitidis* that express the L1,8, L2, and L3,7 lipooligosaccharide (LOS) immunotypes, and whose synX and lpxL1 genes were deleted. Immunogenicity studies in mice showed that the vaccine induced bactericidal antibody against serogroups B, C, W, Y and X. *N. meningitidis* strains. However, this experimental NOMV vaccine was not effective against serogroup A. *N. meningitidis* capsular polysaccharide (PS) from serogroups A, C, W and Y were effective at inducing bactericidal antibody when conjugated to either tetanus toxoid or the fHbp1-fHbp2 fusion protein fHbp1(1+2). The combination of the NOMV vaccine and the *N. meningitidis* serogroup A capsular polysaccharide (MAPS) protein conjugate was capable of inducing bactericidal antibodies against a limited number of *N. meningitidis* strains from serogroups A, B, C, W, Y and X tested in this study.

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This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to other federal statutes and regulations relating to animals and experiments involving animals.

**Materials and Methods**

**Ethics Statement**

This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to other federal statutes and regulations relating to animals and experiments involving animals.
principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition. The animal care and use protocol number 1999-28 was approved by the IACUC/ethics committee of CBER/FDA.

**Bacterial Strains used to make the Vaccine, and used as Targets in the Bactericidal Assay**

The bactericidal target strains used for serogroups B, C, Y and W were mostly isolated from U.S. Army personnel prior to routine vaccination with the tetravalent polysaccharide vaccine (about 1982). The four serogroup A target strains were obtained from other investigators and originated in Egypt, Africa, Finland, and Germany. The serogroup X strains were obtained from the Naval Medical Unit-3 (NAMRU-3) in 2009, and were isolates from a meningococcal meningitis outbreak in western Kenya [8]. Only meningococcal strains for which human complement was available at the WRAIR were used in the bactericidal assay.

**Genetic Modifications of the Bacterial Strains used to make the Vaccine**

The only two modifications made to all three vaccine strains were deletion of the capsule by disrupting the synX gene with the KanR resistance marker and changing acylation of the LOS from hexa-acetylation to a penta-acetylation by disrupting the lpxL1 gene with the TetR marker. The two plasmids used for the modification, pMin5 (TetR) and pZero-synX-Kan (KanR) were kindly provided by Dr. Wendell D. Zollinger. Transformation was performed by adding 10 μl of plasmid DNA to 5–10 colonies of overnight cultures plated on 1 cm² area of a GC agar plate and incubated for 4–6 hours at 37°C in 5% CO₂. The cells were suspended in 1.0 ml of Mueller-Hinton broth and plated on GC agar plates supplemented with the appropriate antibiotic. Transformation was done sequentially: the first bacteria were transformed with the plasmid pMin5 (TetR), and selected on GC-tetracycline plates (tetracycline concentration of 5 μg/ml), followed by transformation by pZero-synX-Kan (KanR), and selected on GC-Kanamycin plates (Kanamycin concentration of 5 μg/ml). The *lpxL1* knockout was confirmed by PCR using primers hrtr(†) 5'-GACGGCTTCT-GAAAAGATGATTTATTTTTTGTAC-3' and hrtr(‡) 5'-TCAGTAAATTCGCGGCTGCTCCGCG-3'. PCR was carried out in 50 μl reaction volumes (45 μl Platinum Blue PCR Supermix (Invitrogen), 2 μl genomic DNA, 1.5 μl each primer. The PCR conditions were as follows: an initial step of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1.5 min, 72°C for 4 min, and a final step of 72°C for 10 min. The synX knockout was confirmed by PCR using primers SynX5 (†) 5'-CCGGGTCCGACCGACGGCTCGTCTGAAACCGCAAAGCTAAAAAC-3' and SynX6(‡) 5'-CCGGGTCCGACCGACGGCTCGTCTGAAACCGCAAAGCTAAAAAC-3'.

**Preparation of fHbp1, fHbp2 and Fusion Protein of Variant 1 and Variant 2 fHbp1**

The gene of fHbp1 was amplified by PCR using strain H44/76 genomic DNA as a template and the oligos 5'-AACGCTTTCTCGAGTGGAGGAGGGGTTGTGGTGC-3' (forward) and 5'-GGCGGGGATCTACTATCTTATTGGTGGCGCGCAATCCG-3' (reverse). The gene of fHbp2 was amplified by PCR using strain 7608 genomic DNA as a template and the oligos 5'-AATCACTATTATCTTATTGGTGGCGCGCAATCCG-3' (forward) and 5'-GGCGGGGATCTACTATCTTATTGGTGGCGCGCAATCCG-3' (reverse). The PCR products were cloned in the XhoI - EcoR1 site of pT7-MAT-Tag-Flag-1 vector (Sigma) which were then used to transform E. coli cells BL21(DE3). The transformed cells were grown in LB broth, induced with 1mM IPTG, the cells were lysed and the His-tagged proteins purified on Ni columns. Serogroup A meningococcal polysaccharide (MAPS) was produced by SynCo BioPartners, Amsterdam, Netherlands. Serogroup C meningococcal polysaccharide (MCPS) was from BioCruz, BioMaguinos, Brazil. Serogroup W-135 and Y meningococcal polysaccharides (MWPS and MYPS) were from Chiron, Tetanus toxoid (TT) was obtained from Wyeth Vaccines and Serum Institute of India. 4-cyano-dimethylamino pyridinium tetrafluoroborate (CDAP) was purchased from Sigma Chemical Company (St. Louis, MO).

**Spot Blotting/Colonial Blotting**

The phenotype of the vaccine strains and the bactericidal target strains for serogroup A, C, Y, W and X N. meningitidis were determined by colony blotting as previously described [17]. Spot blotting, a variation of colony blotting where a suspension of the culture is spotted onto nitrocellulose rather than lifting colonies directly from an agar plate, was used to determine the LOS immunotype of target strains from the other groups. Colony blotting was performed as previously described [23]. The following monoclonal antibodies were used in the blotting procedures to determine the capsular polysaccharide (serogroup), PorA (serotype), and LOS immunotype of the target strains: serogroup B capsular polysaccharide (mAb2-1-B); PorA P1.16 (mAb 3–1-P1.16); PorA P1.7 and P1.7-2 (mAb 3–1-P1.7); PorA P1.2 (mAb 3–1-P1.2); PorA P1.5 (mAb was obtained from NIBSC) and P1.4 (mAb MN20B9.34); L8 LOS (mAb 2–1-L8); L3,7 LOS (mAb 9–2-L37); L1 LOS (mAb 17–1-L1); L2 LOS (mAb 27–1-L2). The monoclonal antibodies Jar5 (fHbp1) and Jar11 (fHbp2) were obtained from Dr. Dan Granoff.

**NOMV Preparation**

The serogroup B N. meningitidis vaccine strains (H44/76, NZ5247 and B16B6) were grown in liquid culture using modified Catlin's Medium in which the individual amino acids were replaced by 1% casamino acids (Becton Dickenson, Franklin Lakes, NJ, USA) and iron (ferric sulfate) was reduced to 10% of the normal level (0.5 mg/L) to induce expression of iron uptake proteins. No antibiotics were added to the growth medium. Bacterial cultures (1L), were grown in 2800 ml baffled Fernbach flasks at 37°C with rotary shaking at 160–180 RPM. NOMV were extracted from packed cells as previously described [24].
template and the oligos 5’-AAGCTTCTCAGTGAGTCGGAGCCGGGTGTCGCC-3’ (forward) and 5’-CGATGGCCACCATGGCCGACGGCCGACCCCTCGCT- GAACAGCCTTCACCTGTCGTTGCAAGGCGGAT-3’ (reverse). The PCR product was reamplified using the oligos 5’-AAGCTTCTCAGTGAGTCGGAGCCGGGTGTCGCC-3’ (forward) and 5’-GCCGCCTCCTCTAGAACCGCCACCGCCAGAGCCACCGCCAGAGCCAC-3’ (reverse). The gene of hIbp2.16 was amplified by PCR using strain 7608 genomic DNA as a template and the oligos 5’-GGTTGAGCGGCTTGTCGCC-3’ (forward) and 5’-GGCGGGGAATTCACTTACTACTGTTTGCCGGC-3’ (reverse). The PCR product was reamplified using the oligos 5’-GGTTGAGCGGCTTGTCGCC-3’ (forward) and 5’-GGCGGGGAATTCACTTACTACTGTTTGCCGGC-3’ (reverse). The hIbp1 PCR product was digested with XhoI and EcoRI and the capsular conjugates used for immunization were 1 μg/mL, and the concentration of both the fusion protein and the capsular conjugates used for immunization were 1 μg/mL each. Data reported were obtained using the final (10 week) sera.

Serological Assays

The serum bactericidal assay was performed as previously described [27] using normal human serum prescreened for lack of intrinsic bactericidal activity against the test strain as a source of complement. The pooled mouse sera were heat inactivated and tested using a starting dilution of 1:2. The reciprocal of the highest dilution of serum that killed ≥50% of the bacteria was taken as the end point titer of the serum. If there was less than 50% killing at a dilution of 1:2, the serum was assigned a titer of 1:1. The highest dilution tested was 1:512. All the pre-vaccination sera from the mice lacked bactericidal antibodies against any of the test strains and were assigned a titer of 1:1. The bactericidal depletion assay was performed as described [27] by coating wells of a 96-well microplate with 100 μg of antigens to be tested, then blocking, and washing the wells. The sera to be tested were diluted to the 50% kill endpoint and incubated in the coated wells for 4 hours, after which the sera were transferred to a fresh plate and tested for residual bactericidal activity. The inhibition was calculated by comparing the bactericidal activity of the unabsorbed sera (100% killing, 0% inhibition) to that of sera absorbed with antigens before the bactericidal assay. Whole cells (wild-type vaccine strains) served as a positive control since they absorbed all the bactericidal antibodies whereas BSA and Salmonella LPS served as negative controls absorbing very little (<5%) of the bactericidal antibodies from the test sera.

Measurement of Endotoxicity by Whole Blood Cytokine Release Assay

The endotoxic activity of the NOMV was measured using the whole blood cytokine release assay as previously described [28]. The two cytokines measured in the assay were IL-6 and TNF-α.

Results

Characterization of the Vaccine Strains and the NOMV Vaccine Derived from the Three Strains

The phenotypic characterization of the three serogroup B N. meningitidis strains H44/76, B16B6 and NZ9547 by colony blotting for the serotype (PorA) and immunotype (LOS) is shown in Figure 1. The data in Figure 1 show that the strain H44/76 expresses the P1.16–2,17 Tag-Flag-1 vector (Sigma) which were subsequently used to transform E. coli cells BL21 (DE3). The transformed cells were grown in LB broth, induced with 1mM IPTG, the cells were lysed and the His-tagged proteins purified on Ni columns.

Preparation of PS-hIbp(1+2) and PS-TT Conjugates

Conjugation of polysaccharide to hIbp(1+2) fusion protein was carried out using CDAP conjugation method of Lee et al. [25] with modification. On ice, 10 μL PS (10 mg/mL; Mn A, C, W or Y) is mixed with 1 μL CDAP (100 mg/mL in acetonitrile). In a cold room, the PS solution was mixed with 1 μL triethylamine (TEA, 0.2 M). After 1.5–2 hours incubation at 4°C, 4 mL of 0.1 M phosphate buffer, pH 5.5 was added to the reaction mixture followed by addition of 0.1 mg hIbp or hydrazide-modified TT prepared according to reference [26]. The conjugation reaction proceeded overnight in the cold room with agitation. After dialysis against 50 mM NaCl, 10 mM HEPES, buffer, pH 7.2 at 4°C, the conjugation product was adjusted to (PS) = 0.2 mg/mL with phosphate buffer, pH 5.5 was added to the reaction mixture followed by addition of 0.1 mg hIbp or hydrazide-modified TT prepared according to reference [26]. The conjugation reaction proceeded overnight in the cold room with agitation. After dialysis against 50 mM NaCl, 10 mM HEPES, buffer, pH 7.2 at 4°C, the conjugation product was adjusted to (PS) = 0.2 mg/mL with dialysis buffer and stored at 4°C.

High Performance Size-exclusion Liquid Chromatography (HPSEC)

Samples of proteins, polysaccharides and conjugate products (25 μL, 0.1 mg/mL) were applied to a Waters Ultrahydrogel 2000 column with 0.9% NaCl, 10 mM Tris (pH 7.2), 1 mM EDTA, at 0.5 mL/minute in a Dionex HPLC system using Chromeleon software with a UV detector at 280 nm for monitoring protein signal, and a refractive index (RI) detector for detecting protein, polysaccharide and conjugate.

Immunization of Animals

Vaccines were thawed and formulated at the desired concentration prior to vaccination. Groups of 10 CD-1 mice were vaccinated intraperitoneally with various combinations of antigens. In all experiments three doses of the antigens (0.1 mg per mouse) were administered at 0, 4 and 8 weeks and blood was taken at 0, 7, and 10 weeks following the initial vaccination. The concentration of NOMV used for immunization was 9 μg of protein 0.1 mL, and the concentration of both the fusion protein and the capsular conjugates used for immunization were 1 μg in 0.1 mL each. Data reported were obtained using the final (10 week) sera.
vaccine (which contains the lpxL1 deletion) is between 10 and 100 fold less toxic than the parental H44/76 NOMV based on the induction of TNF-α and IL-6. This is consistent with our earlier observations that NOMVs containing penta-acylated LOS are less endotoxic than the wild type hexa-acylated LOS variant [28]. Furthermore, NOMV containing the penta-acylated variant were not found to be toxic when administered intramuscularly in humans [29]. Figure 4a shows the results of the SDS-PAGE gel of the NOMV from the three strains used for the immunogenicity studies. The presence of the two major outer membrane antigens, LOS and PorA from each of the three strains in the NOMV vaccine was confirmed by Western blot (Fig. 4b and Fig. 4c).

Bactericidal Activity against Serogroup B N. meningitidis Strains

Since the NOMVs were obtained from serogroup B N. meningitidis strains, the first set of target strains analyzed for killing by the bactericidal activity of the immunized mouse sera were the wild type parents of the vaccine strains and a diverse panel of serogroup B strains taken from the repository of case strains at the Walter Reed Army Institute of Research. The strains in the panel share either LOS and or PorA with the vaccine strains. An immunizing dose of 9 μg per mouse was used since earlier studies showed this to be optimal in inducing a good bactericidal response against a diverse panel of serogroup B N. meningitidis strains [17]. The results in Table 1 show that the bactericidal titer for all the serogroup B N. meningitidis strains was greater than 1:4 with the exception of N. meningitidis serogroup B strain 6557. The reason for the resistance of N. meningitidis serogroup B strain 6557 is unclear since it does share LOS with the vaccine strains. The results obtained in this study are similar to results obtained using a completely different NOMV vaccine that was extensively genetically modified for both safety and increased immunogenicity purposes [17].

Bactericidal Activity Induced by PorA and LOS in the Experimental Vaccine

To study the role of PorA and LOS in the vaccine preparation, the three wild type parental strains were used as targets in the bactericidal inhibition assay as described in Materials and Methods. As expected, bactericidal antibodies present in the sera of mice immunized with the experimental trivalent vaccine were completely depleted when pre-adsorbed by whole cells (H44/76, B16B6, NZ9547) before being used in the bactericidal assay (Fig. 5). The depletion of bactericidal antibodies in the sera of mice immunized with the experimental trivalent vaccine by the controls (BSA and LPS from Salmonella enterica) was minimal. Interestingly, the bactericidal inhibition assay showed that most of the antibody directed against the target strain NZ9547 was to the surface antigens PorA (P1.7–2,4) and L8 LOS (Fig. 5a), but not the L1 LOS that is also present on the surface of the target strain. The reason why L1 LOS did not induce a bactericidal response is unclear. The bactericidal inhibition assay also showed that most of the antibody directed against the target strain H44/76 was to the surface antigens PorA (P1.7,16) and L3,7 LOS (Fig. 5b). Since L3,7 LOS has an L8 core, the depletion seen with the L8 LOS can be attributed to the presence of cross reacting antibodies induced by L3,7 present in the vaccine. The bactericidal inhibition assay showed that most of the antibody directed against the target strain B16B6 was to the surface antigens PorA (P1.3,2) and the L2 LOS (Fig. 5c). The data obtained from the bactericidal inhibition assay shows that most of the bactericidal antibody is directed to the two dominant surface antigens LOS and PorA.

Bactericidal Activity against non serogroup B N. meningitidis Strains

The bactericidal activity of the pooled mouse sera was tested on a limited number of strains from serogroups A, C, Y, W and X in the WRAIR collection for which human complement was available. The phenotype (PorA and LOS) and genotype (MLST) of the strains in this panel are shown in Table 2. As seen in Table 2, the strains in serogroup A were highly resistant to killing in the bactericidal assay. Since the serogroup A strains do not share the major outer membrane antigens such as PorA and LOS with the vaccine strains, it is not surprising that the experimental trivalent vesicle vaccine was unable to induce a protective response to these strains.

All three serogroup C strains in the panel share the major outer membrane antigen, L3 LOS, with the vaccine strains. Furthermore, two of the three strains also express the P1.5 PorA epitope
that is also expressed by one of the vaccine strain (B16B6). As seen in Table 2, the two serogroup C strains (5416 and 5660) that share both the LOS and PorA were killed in the bactericidal assay with titers of 512 and 128 respectively. Interestingly, the two serogroup C strains (5416 and 5660) and the vaccine strain B16B6 belong to the ST11 complex. Thus it is quite possible that vaccine strain B16B6 and the two serogroup C strains 5416 and 5660 share other surface antigens besides LOS and PorA. The serogroup C strain 8837 appear to be resistant despite sharing the LOS with the vaccine strains and being part of the ST41/44 complex like the vaccine strain NZ9547. The reason for the resistance to killing in the bactericidal assay are not clear since anti-L3,7 antibodies are induced by the experimental trivalent vesicle vaccine.

The three strains in serogroups W and Y share the major outer membrane antigen LOS with the vaccine strain, and, as seen in Table 2, all three strains are effectively killed in the bactericidal assay at titers greater than 512. The three serogroup X strains were independent isolates from an outbreak in Western Kenya. Since all three strains were part of a single outbreak and belong to ST 5403, they are considered the same clone, and any observable differences between the independent isolates could be attributed to clonal variation. They share the major outer membrane antigen, L8 LOS with the vaccine strain, and as seen in Table 2, are killed in the bactericidal assay at titers of 32. Taken together, these results suggest that a combination of NOMV and serogroup A polysaccharide (MAPS) would result in a vaccine that protects against serogroups A, B, C, W, X and Y N. meningitidis infections.

Preparation and Characterization of PS-fHbp(1+2) Conjugate

Since vaccines based on fHbp1 and fHbp2 have also been shown to induce broad protection, the utility of using a fusion protein made of fHbp1 and fHbp2 to act as a protein carrier for N. meningitidis capsular polysaccharide was investigated. The fusion of fHbp1 and fHbp2 DNA, the expression of the fusion protein and its purification is described in Materials and Methods. An SDS-PAGE gel of the purified fHbp1 and fHbp2 fusion proteins is shown in Fig. 6a. As expected, the fusion protein fHbp1(1+2) is much larger than either of the two purified protein. As shown in Fig. 6b, the fusion protein also reacts to the two antibodies Jar 5 (specific for fHbp1) and Jar 11 (specific for fHbp2). The purified fHbp(1+2) fusion protein was conjugated to N. meningitidis PS as described in Materials and Methods. The
size-exclusion HPLC profiles of fHbp(1+2) conjugated to MAPS, MCPS, MWPS and MYPS are shown in Fig. 6c. The peak of fHbp(1+2) is at minute 23 as seen in Fig. 6c. Conjugate formation between PS and the protein is indicated by the shift of protein signal from minute 23 to the higher molecular weight position at minutes 15–19 upon conjugation with, the residual unconjugated protein unmoved.

**Bactericidal Activity Induced by PS-fHbp(1+2) Conjugates**

As shown in Table 3, immunization with fHbp(1+2) fusion protein, which is a combination of fHbp1.1 and fHbp2.16, did induce a four-fold or greater increase in bactericidal antibodies against strains 8570 (serogroup B), strain 7510 (serogroup W), strain 9557 (serogroup X) and strains 8020 and 9463 (serogroup Y). With the exception of 8570 (serogroup B) all other strains that were killed expressed alleles of fHbp that differed from the vaccine. The strains that were resistant to killing presumably did not express enough fHbp on the surface for the antibodies to be bactericidal. Immunization with The fHbp (1+2) all capsular conjugates did indeed induce specific bactericidal antibodies that killed serogroup A, C, W and Y strains (Table 3). Sera from mice immunized with fHbp(1+2) were not tested for bactericidal activity against serogroup B strains since they have a different capsule and LOS is better than fHbp (1+2) at inducing protective antibodies (Tables 2 and 3). The data shows that fHbp can serve as a carrier for the N. meningitidis capsular polysaccharides (Table 3). The data also shows that anti-fHbp bactericidal antibodies are induced upon immunization with fHbp(1+2) all capsular conjugates as evidenced by the killing of serogroup X strains at low titers. Immunization with serogroup A capsular conjugate, MAPS-fHbp(1+2) resulted in the killing of serogroup A strains at high titers because of the induction of anti-capsular bactericidal antibodies. Interestingly, the killing of 2 of 3 serogroup X strains at low titers in comparison to serogroup A strains when immunized with MAPS-fHbp(1+2).

**Table 1. Bactericidal activity against serogroup B N. meningitidis strains.**

| Strain   | MLST/Clonal complex          | PorA     | LOS  | Antigens shared with the vaccine strains | Titer |
|----------|------------------------------|----------|------|-----------------------------------------|-------|
| H44/76   | ST-32 complex/ET-5 complex   | P1.7,16  | L3,7 | vaccine strains                         | 16    |
| 8570     | ST-32 complex/ET-5 complex   | P1.19,15 | L3,7 | LOS                                     | 256   |
| 7608     | ST-8 complex/cluster A4      | P1.5-2,2-2 | L4   |                                         | 64    |
| 8047     | ST-8 complex/cluster A4      | P1.5-1,2-2 | L3,4,7 | LOS                                 | 64    |
| NZ 9547  | ST-41/44 complex/lineage 3   | P1.7-2,4 | L1,8 | vaccine strains                         | 128   |
| 81686    | ST-11 complex/ET-37 complex  | P1.5,2   | L2   | vaccine strains                         | 512   |
| 6557     | ST-41/44 complex/lineage 3   | P1.22-1,14 | L1,3,7 | LOS                                 | 2     |
| 1901     | ST-41/44 complex/lineage 3   | P1.18,25 | L1,3,7 | LOS                                 | 128   |
| 6275     | ST-41/44 complex/lineage 3   | P1.5,2   | L3,7 | LOS and PorA                           | 512   |
| M1080    | ST-41/44 complex/lineage 3   | P1.7-1,1 | L1,3,7 | LOS                                 | 8     |
| 9162     | ST-32 complex/ET-5 complex   | P1.7-2,3 | L3,7 | LOS                                     | 128   |
| 99M      | ST-11 complex/ET-37 complex  | P1.5,2   | L3,7 | LOS and PorA                           | 512   |

Pooled sera from mice immunized with the NOMV vaccine were tested for bactericidal activity with human complement against the serogroup B N. meningitidis strains listed. The data are reciprocal titers. The MLST, PorA, and LOS (immunotype) of the target strains and antigens shared with the vaccine strains are also shown in the Table.

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**Figure 5. Characterization antibody specificity by the bactericidal Inhibition assay.** Pooled sera from mice immunized with the NOMV vaccine was analyzed for bactericidal antibody specificity using N. meningitidis wild type vaccine strains as the target strain in a bactericidal depletion assay. In Fig. 5a the bactericidal target strain used was wild type NZ9547, in Fig. 5b the bactericidal target strain used was wild type H44/76, and in Fig. 5c the bactericidal target strain used was wild type B16B6. The antigens used to for depletion are shown in the figure.

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capsular conjugate, could be attributed to the induction of anti-fHbp antibodies. Thus the results suggest that the MAPS-fHbp(1+2) capsular conjugate may serve as a good vaccine candidate for the sub-Saharan meningitis belt where strains from these two serogroups are mainly responsible for the meningitis epidemics. It is also possible that the immunogenicity of these conjugates could be further enhanced with adjuvants.

**Bactericidal Activity of a Mixture of NOMV and Either MAPS-fHbp(1+2) or MAPS-tetanus Toxoid Conjugate**

Mice immunized with either NOMV or a mixture of NOMV and fHbp (1+2) fusion protein were able to protect against serogroup B *N. meningitidis* strains, but were not able to protect against serogroup A *N. meningitidis* strains (Table 4). Table 4 also shows that the fHbp(1+2) fusion protein alone did not induce much bactericidal antibody and the MAPS-fHbp(1+2) conjugate did not induce bactericidal titers superior to MAPS-tetanus toxoid conjugate. However, the fusion protein did provide protection against *N. meningitidis* strains from serogroups Y and W (Table 3). On the other hand, mice immunized with a mixture of NOMV and MAPS-fHbp(1+2) conjugate or MAPS-tetanus toxoid conjugate produced bactericidal antibodies that were protective against not only serogroup B but also serogroup A *N. meningitidis* strains. Since the NOMV have been shown to protect against serogroups B, C, W, Y and X, one can conclude from the data shown in Tables 2 and 4 that a mixture of NOMV and a capsular-protein conjugate could induce protection against all major *N. meningitidis* serogroups.

**Discussion**

To date we do not have a single licensed vaccine to prevent disease against all *N. meningitidis* serogroups. Licensed capsular conjugate vaccines are available for individual *N. meningitidis* serogroups A, C, W and Y. In the case of *N. meningitidis* serogroup

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**Table 2. Bactericidal activity against non serogroup B *N. meningitidis* strains.**

| Strain | Group | MLST/Clonal complex | PorA | LOS | Antigens shared with the vaccine strains | Titer |
|--------|-------|---------------------|------|-----|----------------------------------------|-------|
| 5878   | A     | ST-4 complex/subgroup IV | P1.7,13-1 | L9  | none | 2 |
| 8991   | A     | ST-5 complex/subgroup III | P1.20.9 | L8,9 | none | 2 |
| 7891   | A     | ST-5 complex/subgroup III | P1.20.9 | L11 | none | 1 |
| 8822   | A     | ST-1 complex/subgroup I/III | P1.5,1-2-2 | L10 | none | 1 |
| 8837   | C     | ST-41/44 complex/lineage 3 | P1.19,15 | L3  | LOS | 2 |
| 5416   | C     | ST-11 complex/ET-37 complex | P1.5,2-1 | L3  | LOS | 512 |
| 5660   | C     | ST-11 complex/ET-37 complex | P1.5,2-1 | L3  | LOS | 128 |
| 7510   | W     | ST-178 complex | P1.19,15 | L3,7 | LOS | >512 |
| 8020   | Y     | ST-23 complex/Cluster A3 | P1.5,1-2-2 | L3  | LOS | >512 |
| 9463   | Y     | ST-167 complex | P1.5,1-10-4 | L3-5,7-5 | LOS | >512 |
| 9557   | X     | ST-5403 | P1.19,26 | L8  | LOS | 32 |
| 9558   | X     | ST-5403 | P1.19,26 | L8  | LOS | 32 |
| 9559   | X     | ST-5403 | P1.19,26 | L8  | LOS | 32 |

Pooled sera from mice immunized with the NOMV vaccine were tested for bactericidal activity with human complement against the non-serogroup B *N. meningitidis* strains listed. The data are reciprocal titers. The MLST, PorA, and LOS (immunotype) of the target strains and antigens shared with the vaccine strains are also shown in the Table.

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**Figure 6. Analysis of purified fHbp variants and fHbp capsular conjugates.** In Fig. 6a, is the Commassie Blue stained gel (10%) showing purified fHbp1 (Lane 1), fHbp2 (Lane 2) and fHbp(1+2) fusion product (Lane 3). In Fig. 6b is the Western blot analysis using mAbs Jar 5 (specific for fHbp1) and Jar 11 (specific for fHbp2); fHbp(1+2) in lanes 1 and 4 developed with mAbs Jar 5 and Jar 11 respectively; fHbp1 in lane 2 developed with mAb Jar 5, and fHbp2 in lane 3 developed with mAb Jar 11. In Fig. 6c are shown the HPLC profiles of fHbp(1+2) and the conjugate products fHbp(1+2)MAPS, fHbp(1+2)MCPS, fHbp(1+2)MWPS, fHbp(1+2)MYPS monitored at 280 nm.

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B, while several subcapsular vaccine preparations are currently being studied for their protective efficacy, only the 4CMenB vaccine has progressed to licensing in Europe. Cost-effectiveness modeling of new meningococcal vaccines in England suggests that, given current disease levels, it is unlikely that these vaccines would be cost-effective [30]. Thus there is a need for a vaccine against all disease serogroups of *N. meningitidis*, that is also cost-effective.

fHbp which is present in both 4CMenB and the bivalent fHbp tetanus toxoid was sufficient to induce a protective response against most serogroups *N. meningitidis* vaccine. NOMV vaccines that are AlpxL1AynX have been shown to be safe and induce a good antibody response to both PorA and LOS in humans. This newly developed vaccine induced bactericidal antibodies that were specific to both PorA and LOS. This NOMV vaccine as expected induced broad cross protection against *N. meningitidis* strains not only of serogroup B, but also serogroups C, W, Y and X. Since fHbp was shown to be protective against multiple serogroups [19], it was thought that conjugating fHbp to capsular polysaccharide and combining it with NOMV would result in a vaccine formulation that would be protective against most disease causing strains of *N. meningitidis*. The fHbp conjugates by themselves were capable of inducing a protective response against serogroups A, C, W, Y and X. Interestingly, the serogroup A polysaccharide fHbp conjugate induced a protective response against both serogroup A and also serogroup X *N. meningitidis* strains, which make it a good vaccine candidate for the sub-Saharan meningitis belt. The protection induced by fHbp or the fHbp capsular conjugates against serogroup B was not impressive despite the presence of bactericidal antibodies to both fHbp1 and fHbp2. This suggests that a critical threshold of fHbp expression is needed on the surface for anti-fHbp antibodies to be effective. The fact that fHbp was a good carrier for the capsular polysaccharide suggested that a combination of the NOMV vaccine along with the fHbp capsular conjugate induced a protective response against both serogroup A and also serogroup X *N. meningitidis* strains. Recently, other investigators

### Table 3. Bactericidal activity induced by PS-fHbp(1+2) conjugates.

| Strain     | MLST/Clonal Complex          | Serogroup | fHbp Titer | fHbp(1+2) fusion protein Titer | all PS-fHbp(1+2) capsular conjugate Titer | MAPS-fHbp(1+2) capsular conjugate Titer |
|------------|------------------------------|-----------|------------|-------------------------------|-------------------------------------|-------------------------------------|
| H44/76     | ST-32 complex/ET-5 complex   | B         | 1.1        | 1 ND                          | ND                                  | ND                                  |
| 8570       | ST-32 complex/ET-5 complex   | B         | 1.1        | 4 ND                          | ND                                  | ND                                  |
| NZ 9547    | ST-41/44 complex/lineage 3   | B         | 2.64       | 1 ND                          | ND                                  | ND                                  |
| 81686      | ST-11 complex/ET-37 complex  | B         | 2.22       | 1 ND                          | ND                                  | ND                                  |
| 5878       | ST-4 complex/subgroup IV     | A         | 1.5        | 1 >512                        | >512                                | >512                                |
| 8991       | ST-5 complex/subgroup IV     | A         | 1.5        | 1 >512                        | >512                                | >512                                |
| 7891       | ST-5 complex/subgroup III    | A         | 1.5        | 256 >512                      | >512                                | >512                                |
| 8822       | ST-1 complex/subgroup I/II   | A         | 1.4        | 1 >512                        | >512                                | >512                                |
| 8837       | ST-41/44 complex/lineage 3   | C         | 2.72       | 1 512                         | ND                                  | ND                                  |
| 5416       | ST-11 complex/ET-37 complex  | C         | 2.22       | 1 256                         | ND                                  | ND                                  |
| 5660       | ST-11 complex/ET-37 complex  | C         | 2.22       | 1 128                         | ND                                  | ND                                  |
| 7510       | ST-178 complex               | W         | 1.34       | 128 64                        | ND                                  | ND                                  |
| 8020       | ST-23 complex/cluster A3     | Y         | 2.25       | >512                         | >512                                | >512                                |
| 9463       | ST-167 complex               | Y         | 2.23       | >512                         | >512                                | >512                                |
| 9557       | ST-5403                      | X         | 1.61       | 4 16                         | 8                                   | 1                                   |
| 9558       | ST-5403                      | X         | 1.61       | 2 4                          | 8                                   | 1                                   |
| 9559       | ST-5403                      | X         | 1.61       | 2 4                          | 8                                   | 1                                   |

*The *N. meningitidis* capsular polysaccharides used were from serogroups A, C, W, and Y.
ND = not determined.

Pooled sera from mice immunized with fHbp(1+2) and fHbp(1+2) capsular conjugates were tested for bactericidal activity with human complement against the *N. meningitidis* strains from serogroups A, B, C, W, Y and X.

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have shown that immunization with a combination of NOMV-fHbp and MenA polysaccharide would be sufficient for the prevention of meningococcal infections [32], where fHbp is mainly responsible for the protection against the various serogroups tested and the resistant strains from serogroup A being protected by MenA polysaccharide conjugate in the vaccine preparation.

While it may be possible to protect against most N. meningitidis infections by using a combination of NOMV and MenA polysaccharide as an immunogen, given the safety and efficacy of the quadrivalent polysaccharide a combination of NOMV derived from 2 strains expressing L2 and either L3,7 or L8 along with capsules from serogroups A, C, W-135 and Y conjugated to either fHbp(1+2) or tetanus toxoid may be both protective against most N. meningitidis infections, and also a safer option. The use of NOMV is crucial for the induction of bactericidal antibodies to LOS since deoxycholate extracts of the outer membrane of N. meningitidis strains used as vaccines fail to induce bactericidal antibodies to LOS [33]. While the panel of strains used in this study is fairly limited, the data provide a reason to further investigate the potential of such combination vaccines. Vaccines combining capsular and sub-capsular antigens may also be effective against pneumococcal disease where capsular polysaccharide vaccine usage has resulted in increased incidence of disease due to capsule types not present in the vaccine [34–36].

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Author Contributions

Conceived and designed the experiments: VBP CHL. Performed the experiments: VBP RB AW EEM CHL. Analyzed the data: VBP EEM CHL. Wrote the paper: VBP CHL.

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Table 4. Bactericidal activity of NOMV and either MAPS-fHbp(1+2) or MAPS-tetanus toxoid conjugate.

| Strain | Group | Titer | Titer | Titer | Titer |
|--------|-------|-------|-------|-------|-------|
| H44/76 | B     | 32    | 16    | 32    |       |
| 8570   | B     | 256   | 256   | 512   |       |
| 7608   | B     | 128   | 64    | 128   |       |
| 8047   | B     | 64    | 64    | 256   |       |
| NZ9547 | B     | 256   | 256   | 512   |       |
| 81686  | B     | 512   | 256   | 512   |       |
| 6557   | B     | 16    | 2     | 2     |       |
| 190    | B     | 128   | 256   | 256   |       |
| 6275   | B     | >512  | >512  | >512  |       |
| M1080  | B     | 32    | 32    | 32    |       |
| 9162   | B     | 128   | 128   | 128   |       |
| 99M    | B     | 512   | >512  | 512   |       |
| 5878   | A     | 2     | >512  | >512  |       |
| 8991   | A     | 2     | >512  | >512  |       |
| 7891   | A     | 1     | 128   | 512   |       |
| 8822   | A     | 1     | >512  | >512  |       |

Pooled sera from mice immunized with immunogens shown in the table and tested for bactericidal activity with human complement against the N. meningitidis strains from serogroups A and B.

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Table 4. Bactericidal activity of NOMV and either MAPS-fHbp(1+2) or MAPS-tetanus toxoid conjugate.
Meningococcal Vaccine

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