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Immunization with the Recombinant PorB Outer Membrane Protein Induces a Bactericidal Immune Response against *Neisseria meningitidis*

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Infections with *Neisseria meningitidis* are characterized by life-threatening meningitis and septicemia. The meningococcal porin proteins from serogroup B meningococci have been identified as candidates for inclusion in vaccines to prevent such infections. In this study, we investigated the vaccine potential of the PorB porin protein free of other meningococcal components. The *porB* gene from a strain of *Neisseria meningitidis* expressing the class 3 outer membrane porin protein (PorB3) was cloned into the pRSETB vector, and the protein was expressed at high levels in a heterologous host *Escherichia coli*. The recombinant protein was purified to homogeneity by affinity chromatography and used for immunization after incorporation into liposomes and into micelles composed either of zwitterionic detergent or nondetergent sulfobetaine. The immunogenicity of these preparations was compared to recombinant PorB protein adsorbed to $Al(OH)_3$ adjuvant as a control. Although sera raised against the protein adsorbed to $Al(OH)_3$ reacted with the purified recombinant protein, sera raised against liposomes and micelles showed greater activity with native protein, as measured by enzyme immunoassay with outer membranes and by whole-cell immunofluorescence. Reactivity with native protein was considerably enhanced by incorporation of the adjuvant monophosphoryl lipid A into the liposome or micelle preparations. Recognition of the native protein was in a serotype-specific manner and was associated with the ability of the antisera to promote high levels of serotype-specific complement-mediated killing of meningococci. These results demonstrate that the PorB protein should be considered as a component of a vaccine designed to prevent serogroup B meningococcal infection.

The development of an effective vaccine against life-threatening episodes of meningitis and septicaemia, caused by serogroup B strains of *Neisseria meningitidis*, is a continuing healthcare priority. The lack of an effective vaccine against serogroup B is further emphasized by the development of conjugate polysaccharide vaccines against serogroup C strains and the recent demonstration of their efficacy (http://www.phls.org.uk). Similar strategies to produce conjugate vaccines incorporating serogroup A, W135, and Y capsular polysaccharides are likely to succeed. However, the serogroup B polysaccharide mimics human neural cell adhesion molecules and is nonimmunogenic in purified form (14). The development of an effective vaccine against serogroup B strains, which have been responsible for the majority of infections in temperate countries, is therefore likely to require alternative approaches.

The nonimmunogenicity of the serogroup B capsular polysaccharide has led to the development of outer membrane vesicle (OMV) vaccines, based on outer membranes that have been depleted of toxic lipopolysaccharide. Experimental OMV vaccines have undergone phase III trials in humans (3, 4, 11, 31) and have been shown to produce limited protection in adults (3). However, the immune responses were of limited duration (32), and no protection was shown in children under 2 years of age, the group at greatest risk of infection (11, 28). In addition, because of the multicomponent nature of the OMVs, the relative responses varied between individuals and only a proportion of the antibodies were protective (32).

The two major components of the OMV vaccine are the meningococcal porin proteins encoded by the *porA* and *porB* genes. The product of the *porA* gene, the class 1 or PorA protein, varies between strains and is responsible for meningococcal serosubtype specificity. The *porB* gene exists as one of two alleles encoding the proteins responsible for serotype specificity, the class 2 (PorB2) and class 3 (PorB3) proteins, the expression of which is mutually exclusive. The PorA and PorB proteins are members of the family of *Neisseria* porins: cloning of the encoding genes has permitted structural and immunological studies of the proteins and has led to a structural model for the organization of the porins, in which a series of β-sheets traverse the membrane to form eight surface-exposed hydrophilic loops (38). Major variations between the proteins are largely restricted to the loops, which vary in length and amino acid sequence, generating differences in immunological specificity both within and between the porin classes (5, 26).

The cloning of genes encoding the outer membrane proteins has facilitated the production of pure proteins free from other

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Neisseria antigens for investigation as potential vaccine candidates. The generally accepted correlate of protection against meningococcal infection is the presence of antibodies with the ability to activate complement-mediated killing of meningococci (15). Several studies with recombinant meningococcal PorA and Opc proteins, as well as with purified PI porin from *Neisseria gonorrhoeae*, have clearly demonstrated that the production of bactericidal antibodies was dependent on refolding of the protein to induce native conformation, which could be achieved by incorporation either into artificial membranes (liposomes) (8, 20, 22, 30, 29, 42, 44) or detergent micelles (21, 22, 43).

The meningococcal porin proteins induce an antibody response during infection, which correlates with the development of bactericidal and opsonic activity (17, 18, 24), and the PorA protein has been identified as making a major contribution to the development of bactericidal activity after immunization of volunteers with the experimental OMV vaccine (32). However, it has been suggested that the reduced size of the surface-exposed loops of PorB, particularly PorB3, compared with PorA make it less accessible to antibodies and hence less susceptible to immunological attack after immunization with OMV (27). Nevertheless, some monoclonal antibodies directed against PorB3 demonstrate bactericidal activity (33), suggesting that it remains a potential vaccine candidate. In order to study the vaccine potential of individual outer membrane proteins, we previously utilized purified recombinant proteins after high-level expression in *Escherichia coli*. We have investigated here the ability of recombinant PorB3, after incorporation in liposomes and micelles, to induce a protective, bactericidal immune response against serogroup B meningococci.

MATERIALS AND METHODS

Bacterial strains, vectors, and growth conditions. *N. meningitidis* strains H44/76 (B:15:P1.7,16), MC114 (B:2a:P1.2), MC139 (C:4,21:P1.1), and MC167 (B:15:P1.1) have been described previously (9, 10, 25). All strains were grown on protease peptone agar at 37°C for 18 h in an atmosphere of 5% (vol/vol) CO_2. Outer membranes were prepared by extraction of whole cells by lithium acetate as previously described (37).

The pRSETB vector and M13/T7 bacteriophage from the Express System (Invitrogen, Gronigen, The Netherlands) were used for expression of recombinant protein. *E. coli* JM101 (Promega, Southampton, United Kingdom) was transformed with recombinant plasmids as previously described and the transformants were grown in Luria-Bertani medium (Difco, West Molesey, United Kingdom), containing 50 μg of ampicillin ml^{-1} (42). Bacteriophage M13/T7, containing the gene for T7 RNA polymerase, was propagated by infecting a 25 ml of fresh culture of *E.coli* JM109 (Promega) with 50 μl of M13/T7 phage stock. The culture was incubated at 37°C, with overnight with shaking at 180 rpm. Cellular debris was removed by centrifugation, and the supernatant solution was stored at 4°C until required. The phage concentration was determined by titration.

Cloning and expression of the *porB* gene in *E. coli*. DNA was isolated from an overnight growth of *N. meningitidis* H44/76. A single colony was resuspended in 10 μl of H_2O; the bacteria were lysed by the addition of 10 μl of 0.25 M KOH, followed by boiling for 5 min, and the suspension was neutralized by the addition of 10 μl of 0.25 M HCl. The pH was adjusted by the addition of 10 μl of 0.5 M Tris-HCl (pH 7.5), and the solution was diluted with 160 μl of H_2O and stored at −20°C. Two primers were designed to amplify, by PCR, only the mature PorB protein with the omission of the 19-amino-acid signal sequence (41). To ensure correct orientation into the multiple-cloning site of the vector, the forward primer (H44f1, 5′-GCG ATT GGA TCC TGA CGT TAC CCT GTA CGG CAC-3′) incorporated a *Bam*HI restriction enzyme site, whereas the reverse primer (H44b1, 5′-CAG TGC AAG CTT GAT TAG AAT TTG TGG CGC AAA CC-3′) incorporated a *Hin*dIII site. Amplification of the DNA was achieved by using the enzyme Bio-X-Act (Bioline, London, United Kingdom); a 50-μl reaction mixture contained 250 ng of each primer, 5 μl of 10× buffer, 2 μl of 50 mM $MgCl_2$, 5 μl of 2 mM deoxynucleoside triphosphates (Boehringer Mannheim), 2 U of Bio-X-Act enzyme, and 5 μl of template DNA. Amplification was performed by using 30 cycles of denaturation at 96°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The pRSETB plasmid and the amplified 942-bp product were digested with *Bam*HI and *Hin*dIII restriction endonucleases (Promega). The DNA fragments were separated by electrophoresis, and the DNA was recovered from agarose and purified by using the Wizard DNA Miniprep kit (Promega). The *porB* DNA and pRSETB vectors were ligated with T4 DNA ligase (Promega) and transformed into *E. coli* JM101. Transformants were analyzed by restriction endonuclease digestion of the resultant plasmid (pPORB3) and sequencing of the junctions and the coding region of the *porB* gene.

For expression of recombinant PorB3 protein, the pPORB3 plasmid was transformed into *E. coli* JM101. An overnight culture (15 ml) was used to inoculate nine flasks containing 750 ml of superbroth media (25 g of Bacto-Tryptone [Difco], 15 g of yeast extract [Difco], and 5 g of NaCl [Sigma] per liter plus 50 μg of ampicillin ml^{-1}). The flasks were incubated at 37°C with vigorous shaking (200 rpm) until an A_{600} of 0.3 was reached. To induce protein expression, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM; the cultures were then incubated for 1 h and infected with M13/T7 phage (10 PFU ml^{-1}) and incubated for a further 5 h. The resulting phage lysate was centrifuged at 6,000 × *g* for 30 min at 10°C, and the insoluble material, which contained the recombinant PorB (rPorB) protein was stored at −20°C.

Purification of recombinant PorB protein. The insoluble material containing the rPorB protein was resuspended in buffer B containing 8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris (pH 8.0) at a concentration of 0.2 g ml^{-1}. The suspension was subjected to repeated sonication at 10 to 12 μm in 1-min bursts (Soniprep 150 sonicator; MSE, Crawley, United Kingdom), followed by cooling on ice, until a clear lysate was achieved. Insoluble material was removed by centrifugation. The crude rPorB protein was purified by affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) gel matrix (Qiagen, Crawley, United Kingdom) under denaturing conditions. A column containing 5 ml of Ni-NTA resin was equilibrated with 10 volumes of buffer B, and the cleared cell lysate was loaded onto the column. The column was washed with 5 volumes of buffer B containing 20 mM imidazole, followed by 5 volumes of the same buffer, but at pH 6.3. The rPorB protein was then eluted by increasing the imidazole concentration to 250 mM. Fractions of the eluate were collected and analyzed for protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and BCA Protein Assay (Pierce, Chester, United Kingdom). Fractions containing the rPorB protein were pooled, and the protein was precipitated at −20°C for 18 h after the addition of ethanol to a final concentration of 80% (vol/vol). The precipitate, which contained pure rPorB as protein aggregates, was recovered by centrifugation at 12,000 × *g* for 15 min, washed with water, and stored at −20°C.

Incorporation of rPorB into liposomes. Liposomes were prepared by dialysis-sonication as previously described (42). Briefly, liposomes were prepared with L-α-phosphatidylcholine and cholesterol (Sigma, Poole, United Kingdom), which were combined at a 7:2 molar ratio and dissolved (20 mg total) in 3 ml of chloroform in a glass round-bottom flask. The solvent was removed under vacuum at 25°C with rotation to achieve an even lipid film. Purified rPorB protein was solubilized in 10 mM HEPES buffer (pH 7.2) containing 2% (wt/vol) SDS to a final concentration of 1 mg of protein ml^{-1} and then boiled for 5 min. The solution (1 ml) was diluted by the addition of 4 ml of 100 mM HEPES buffer (pH 7.2) containing 2% (wt/vol) octyl glucoside and left at room temperature for 1 h. This protein solution was then used to solubilize the lipid film. The detergent-protein solution was extensively dialyzed against phosphate-buffered saline (PBS; pH 7.2) for 72 h at 4°C and then subjected to sonication to induce vesicle formation. Insoluble material was removed by centrifugation at 1,000 × *g* for 10 min. Liposomes were also prepared incorporating the adjuvant monophosphoryl lipid A (MPLA; Ribi Immunochem, Hamilton, Mont.) at an adjuvant/protein ratio of 1:1. Control liposomes with or without MPLA were also prepared. The presence of small unilamellar liposomes was determined by electron microscopy as previously described (42). All liposome preparations were stored in aliquots at −20°C until used.

Incorporation of rPorB into micelle-adjuvant mixtures. Purified rPorB protein was solubilized in 50 mM Tris buffer (pH 8.0) containing 100 mM NaCl and 0.2% (wt/vol) SDS to a stock concentration of ca. 4 mg ml^{-1}. Protein-Zwittergent mixtures were prepared that contained 1 mg of rPorB protein ml^{-1}, 8 mg of Zwittergent 3-14 (Calbiochem, Beeston, Nottingham, United Kingdom) ml^{-1}, and 0.2% (wt/vol) SDS, with or without MPLA (1 mg ml^{-1}), and then incubated overnight at room temperature. Protein-NDSB preparations were similarly pre-

pared with the nondetergent sulfobetaine, dimethylethylammonium propane sulfonate (NDSB-195; Calbiochem) at a final concentration of 8 mg ml^{-1}. For immunization, the solutions were diluted with saline to a final concentration of 100 μg of protein ml^{-1}.

As a control, the rPorB protein was adsorbed to aluminium hydroxide gel. The stock rPorB protein solution was diluted with saline to a concentration of 200 μg ml^{-1} and mixed with an equal volume of 2% (wt/vol) Alhydrogel (Superfos; Biosector A/S, Vedback, Denmark) at 4°C overnight.

Immunization of animals. Seven-week-old, BALB/C (*H-2*^{d}) haplotype female mice were used for immunizations. Mice were divided into groups of five and prebled before each was immunized intraperitoneally with 20 μg of rPorB protein in each of the above preparations, or in an equivalent control, in a 200-μl volume on days 0, 14, 28, and 42. At 2 weeks after the last immunization, mice were terminally bled by cardiac puncture, and sera were stored at −20°C.

SDS-PAGE and Western immunoblotting. SDS-PAGE was performed by using a 10 to 25% linear gradient gel at 200 V for 18 h at 4°C. Purified rPorB protein was loaded at 10 μg per well; whole-cell lysates were loaded at 15 μg per well. Separated proteins were transferred to nitrocellulose by semidry blotting at 100 mA for 1 h and, after incubation with murine sera, immunological reactivity was detected by using anti-mouse immunoglobulin-alkaline phosphatase conjugate (Bio-Rad, Hemel Hempstead, United Kingdom) as described previously (9).

Detection of immune response. (i) ELISA. Flat-bottom polystyrene microtiter plates were coated overnight at 37°C with either rPorB protein or outer membrane in 0.05 M sodium carbonate buffer (pH 9.6; 1 μg of protein ml^{-1}). The plate was blocked with diluent solution containing 50 mM Tris, 0.15 M NaCl, 0.05% (vol/vol) Tween 20, and 1% bovine serum albumin (wt/vol) at pH 7.4 for 1 h at 37°C and then incubated for 1 h with serial dilutions of the murine sera. Antibody binding was detected by using anti-mouse immunoglobulin-horseradish peroxidase (HRP) conjugate (1:2,000 dilution from Zymed, Cambridge, United Kingdom) with 3,3′,5,5′-tetramethylbenzidine and H_2O_2 as an enzyme substrate. Absorbance was measured at 450 nm, and the enzyme-linked immunosorbent assay (ELISA) titer, extrapolated from the linear portion of the serum titration curve, was taken as the dilution which gave an increase in absorbance of 0.1 U h^{-1} (9).

(ii) Subclass-specific ELISA. Outer membrane ELISA with rat monoclonal anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgM-HRP conjugates (Zymed) were used to quantify antibody subclasses present. For IgG3, a biotin-conjugated rat monoclonal anti-mouse IgG3 antibody (Pharmingen/Becton Dickinson, Crawley, United Kingdom) and HRP-streptavidin (Zymed) were used. All conjugates were used at a 1 in 1,000 dilution (22).

Immunofluorescence (IF). *N. meningitidis* strains were grown overnight and harvested into PBS; suspensions (30 μl) were placed on microscope slides and left to air dry. The slides were fixed with 70% ethanol for 15 min and then blocked with 1% (wt/vol) bovine serum albumin solution at room temperature for 30 min. Pooled murine anti-sera, diluted 1 in 10, were reacted with the fixed organisms for 1 h at room temperature with gentle shaking. After a washing step with PBS, antibody bound to the meningococci was detected by reacting with anti-mouse immunoglobulin-fluorescein isothiocyanate conjugate (Dako; diluted 1 in 1;000 in PBS) for 1 h at room temperature in the dark. After being washed in PBS, the meningococci were counterstained with propidium iodide (25 μg ml^{-1}; Sigma) for 5 min before a rewashing in PBS. Slides were viewed by using a fluorescent microscope (22).

Bactericidal assays. The bactericidal activities of antisera were determined with 5% (vol/vol) baby rabbit serum as a source of exogenous complement, as previously described (9). Murine antisera raised to purified outer membranes were used as a positive control. Statistical analysis was performed as previously described (9).

RESULTS

Cloning of the meningococcal *porB* gene in *E. coli* by using the Express system: expression and purification of PorB protein. In order to obtain PorB3 protein free from other meningococcal components, the pRSET vector system (Invitrogen) was employed. This system has previously been used to express high levels of recombinant meningococcal PorA and Opc outer membrane proteins (8, 22). The plasmid pPORB3 was constructed to contain the entire coding sequence for the mature PorB3 protein, omitting the 19-amino-acid leader sequence, under the control of a bacteriophage T7 promoter and in frame with a sequence that encodes an N-terminal fusion peptide that contains six histidine residues, which function as a metal-binding domain. After expression trials with different media, strains, and time courses, optimal expression was achieved by transforming pPORB3 into *E. coli* JM101 and inducing expression by infecting the culture with the bacteriophage M13/T7 in the presence of IPTG. After the crude protein was solubilized in 8 M urea, the rPorB was readily purified by affinity chromatography on a Ni^{2+} column. From a typical culture volume of 7 liters, 75 mg of purified rPorB protein was obtained. SDS-PAGE showed a single homogeneous band of ca. 32 kDa that reacted on Western blot with the antiserum R141, which specifically detects *Neisseria* porin proteins (2). In addition SDS-PAGE and silver staining detected no lipopolysaccharide.

Humoral murine immune response to rPorB protein. The purified protein was used to immunize mice in formulations that have the potential for human use. To attempt to present the protein in its native conformation, the rPorB protein was incorporated into artificial membranes (rPorB-liposomes) (42) and micelles either by solubilization with the zwitterionic detergent Zwittergent 3-14 (rPorB-Zwit) (34) or the nondetergent sulfobetaine NDSB-195 (rPorB-NDSB) (39). In an attempt to increase the immunogenicity of the rPorB protein, the adjuvant MPLA was incorporated into rPorB-liposomes (rPorB+MPLA-liposomes), rPorB-Zwit micelles (rPorB+MPLA-Zwit), and rPorB-NDSB micelles (rPorB+MPLA-NDSB). In addition, rPorB-liposomes were mixed with liposomes incorporating MPLA (MPLA-liposomes). As a control, the rPorB protein was adsorbed to $Al(OH)_3$, the standard adjuvant routinely licensed for human use.

The immunogenicity of the preparations were tested by the reactivity of the resulting sera with the purified rPorB protein in ELISA (Table 1). All of the antisera raised by immunization with formulations containing the rPorB protein reacted strongly against the homologous protein. Immunization with $Al(OH)_3$ produced a mean titer of ca. 10^6, whereas the titers with antisera from the rPorB-liposome preparation were ca. 80-fold lower. However, incorporation of MPLA into the liposomes increased the titer ca. 40-fold. In contrast, mixing MPLA-liposomes with rPorB-liposomes had only a marginal effect. Immunization with the two micelle preparations induced a considerably greater antibody response than liposomes, although this was still lower (two- to sevenfold) than observed with $Al(OH)_3$. However, addition of MPLA to the micelle preparations further increased the immune response to levels even higher than those observed with $Al(OH)_3$ (two to fivefold).

Humoral murine immune response to PorB protein in outer membranes. The antisera raised against rPorB protein were also tested in ELISA against outer membranes from three strains: H44/76, the homologous PorB3 strain (serotype 15); MC139, a heterologous PorB3 strain (serotype 4); and MC114, a heterologous PorB2 strain (serotype 2a) (Table 1). In each case the titers against the homologous outer membrane were significantly lower than observed against the recombinant protein. Only weak reactivity could be seen with sera raised against rPorB-liposomes. However, the addition of MPLA to the liposomes resulted in an immune response that was four-

TABLE 1. ELISA reactivity of antisera raised against recombinant PorB protein preparations[a]

Formulation	Geometric mean ELISA titer (10^{-3}) measured against:			
	Homologous rPorB protein	OMs from strain:		
		H44/76	MC139	MC114
rPorB-Alum	984	14	–	–
Liposome preparations				
rPorB-liposomes	12	1	–	–
rPorB+MPLA-liposomes	506	67	5	–
rPorB-liposomes+MPLA-liposomes	31	10	–	–
Micelle preparations				
rPorB-Zwit micelles	420	28	3	–
rPorB+MPLA-Zwit micelles	2,139	105	9	–
rPorB-NDSB micelles	145	4	–	–
rPorB+MPLA-NDSB micelles	4,915	143	17	–

[a] Mice were immunized with recombinant PorB protein in the preparations as described in Materials and Methods. Pre- and postimmune sera were tested by ELISA against homologous protein and against outer membranes from the homologous PorB3 strain (H44/76), a heterologous PorB3 strain (MC139), and a heterologous PorB2 strain (MC114). Data are the geometric mean titers from groups of five mice. –, No reactivity was detected. Preimmune sera and antisera raised against control preparations lacking rPorB showed no reactivity (<1) against the antigens in ELISA.

TABLE 2. Antibody subclass-specific response to the homologous class 3 protein in the outer membrane of H44/76 after immunization of mice with rPorB protein preparations[a]

Formulation	Mean concn ($\mu g\ ml^{-1}$) of antibody isotype:				
	IgG1	IgG2a	IgG2b	IgG3	IgM
rPorB-Alum	13	4	3	<0.1	2
Liposome preparations					
rPorB-liposomes	<1.1	5	1	1	1
rPorB+MPLA-liposomes	9	38	42	2	4
rPorB-liposomes+MPLA-liposomes	2	6	2	<0.1	1
Micelle preparations					
rPorB-Zwit micelles	18	7	2	<0.1	2
rPorB+MPLA-Zwit micelles	60	54	23	<0.1	3
rPorB-NDSB micelles	7	8	1	<0.1	5
rPorB+MPLA-NDSB micelles	20	252	56	<0.1	8

[a] ELISA with enzyme conjugates of rat monoclonal antibodies specific for murine immunoglobulin subclasses was used to determine concentrations of antibody subclasses in murine sera. Data are the geometric mean antibody isotype concentrations in serum from groups of five individual mice. A concentration of <0.1 $\mu g\ ml^{-1}$ is below the detectable concentration. Preimmune sera and antisera raised against control preparations lacking rPorB showed no reactivity.

fold greater than that seen with the $Al(OH)_3$ preparation. Similarly, addition of MPLA to micelles significantly increased the immune response to the homologous outer membrane with titers six- to tenfold greater than that seen with $Al(OH)_3$. The sera, which showed significant reactivity with the homologous outer membrane, showed approximately tenfold lower reactivity with the outer membrane from the heterologous PorB3 expressing strain and no significant reactivity with the PorB2-expressing strain.

The specificities of the antisera were also tested by Western blotting. Sera raised against rPorB-liposomes showed weak reactivity with the PorB3 protein of the homologous strain. The remaining sera all showed strong reactivity with the protein from the heterologous PorB3 protein-expressing strain. Antisera raised against rPorB+MPLA-liposomes also showed weak reactivity with PorB2 protein, whereas sera raised against rPorB+MPLA-NDSB reacted strongly with this protein and also reacted weakly with PorA protein (data not shown).

Antibody subclass-specific response to PorB in outer membranes. The antibody subclass-specific response to the homologous PorB3 protein in the outer membrane was determined by ELISA with anti-mouse immunoglobulin isotype-specific conjugates (Table 2). Immunization with rPorB adsorbed to $Al(OH)_3$ induced antibodies that were predominantly of IgG1 subclass. Immunization with liposomes and micelles also induced IgG2a antibodies, whereas the addition of MPLA substantially increased this subclass and also induced an IgG2b response.

Antibody recognition of PorB protein on meningococcal cells detected by IF. The antisera were tested by IF analyses for the ability to recognize the PorB3 protein on the surface of whole meningococci (Table 3). Antisera raised against the rPorB protein adsorbed to $Al(OH)_3$ were unable to recognize any strain of meningococci. Antisera raised against liposomes containing rPorB protein reacted only weakly against the homologous strain and did not recognize either of the heterologous strains. However, when MPLA was included in the liposomes the reactivity was significantly enhanced. In contrast, mixing rPorB liposomes with a separate liposome preparation incorporating MPLA did not increase reactivity. The antisera to rPorB-NDSB micelles recognized the homologous strain

TABLE 3. Reactivity of antisera raised against the rPorB preparations with whole, fixed meningococci as detected by immunofluorescence[a]

Formulation	Reactivity of antisera to:		
	Homologous PorB3 strain (H44/76)	Heterologous PorB3 strain (MC139)	Heterologous PorB2 strain (MC114)
rPorB-Alum	–	–	–
Liposome preparations			
rPorB-liposomes	+	–	–
rPorB+MPLA-liposomes	++	–	–
rPorB-liposomes+MPLA-liposomes	+	–	–
Micelle preparations			
rPorB-Zwit micelles	++	–	–
rPorB+MPLA-Zwit micelles	+++	+/–	–
rPorB-NDSB micelles	+	–	–
rPorB+MPLA-NDSB micelles	++	+	–

[a] Sera from groups of mice were pooled diluted 1 in 10 and reacted with whole cells of the homologous strain (H44/76) and heterologous strains that expressed either class 3 (MC139) or class 2 (MC114) porins. The patterns of reactivity have been described in a previous study (22). Preimmune sera and antisera raised against control preparations lacking rPorB showed no reactivity.

TABLE 4. Bactericidal activity of antisera raised against rPorB protein preparations[a]

Formulation	Bactericidal titer against strain:		
	H44/76 (B:15:P1.7,16)	MC139 (C:4,21:P1.1)	MC167 (B:15:P1.1)
rPorB-Alum	−	ND	ND
Liposome preparations			
rPorB-liposomes	128	ND	ND
rPorB+MPLA-liposomes	512	<4	>512
rPorB-liposomes+MPLA-liposomes	128	ND	ND
Micelle preparations			
rPorB-ZW micelles	8	ND	ND
rPorB+MPLA-ZW micelles	512	<4	512
rPorB-NDSB micelles	256	ND	ND
rPorB+MPLA-NDSB micelles	512	<4	256

[a] Pooled antisera raised against rPorB preparations were tested for their ability to induce complement-mediated killing of the homologous strain H44/76 (serotype 15), and against two heterologous strains also expressing PorB3, strains MC139 (serotype 4) and MC167 (serotype 15). Sera were tested in series of dilutions; the titer is the highest dilution of sera which produced >50% killing. −, <50% killing at the lowest dilution tested (1/4); ND, not determined.

only weakly, but the reactivity was also increased by the inclusion of MPLA in the preparation. The antisera to rPorB-Zwit micelles reacted strongly to the homologous strain, and this reactivity was increased further by the addition of MPLA. The sera raised against both micelles and incorporating MPLA were the only sera to recognize the heterologous PorB3 strain, albeit weakly.

Bactericidal activity of antisera. Antisera raised against the rPorB protein were tested for the ability to promote in vitro complement-mediated killing of the homologous meningococcal strain, H44/76 (Table 4). The antisera to rPorB adsorbed to $Al(OH)_3$ showed no significant effect. All of the antisera raised against liposomes or micelles showed bactericidal activity. Antisera to rPorB-liposomes had a bactericidal titer of 1 in 128, and inclusion of MPLA in the same liposome preparation increased the titer to 1 in 512, whereas no increase was observed when the MPLA was administered in separate liposomes. Antisera raised against the rPorB-Zwit micelles was only weakly bactericidal (titer of 1 in 8), but this level was increased to 1 in 512 by the inclusion of MPLA. In contrast, the antisera raised against rPorB-NDSB micelles showed strong bactericidal activity without inclusion of MPLA (1 in 256), and this was further increased by the inclusion of the adjuvant. Antisera from these preparations, which showed the highest titer with the homologous strain, were also tested for their activity against two heterologous strains. Similar bactericidal activity was observed with strain MC167 of the same serotype (serotype 15), but no significant activity was detected against strain MC139 expressing PorB3 of serotype 4.

DISCUSSION

Studies with experimental OMV vaccines have demonstrated their potential to induce a protective immune response to serogroup B meningococci. Although detailed analysis of the immune response has identified the contribution of the PorA and Opc proteins to the bactericidal response (28, 32), the potential contribution of other outer membrane antigens is less clear. However, when volunteers in the Norwegian trial were given a booster immunization 4 to 5 years later, an increase in the type, PorB-specific bactericidal response was observed (32). The PorB protein is the most abundant in the meningococcal outer membrane. In this study, we investigated the vaccine potential of PorB by purifying the protein free from other meningococcal antigens. High-level expression of PorB3 was achieved with the pRSET vector system, which overexpressed the antigen as a recombinant protein in the heterologous host *E.coli*. In previous studies, high levels of expression were also obtained for the PorA and Opc proteins (8, 22), confirming the utility of this expression system for the production of large amounts of meningococcal outer membrane antigens.

In the current study, recombinant PorB3 was incorporated into both liposomes and micelles containing either the detergent Zwittergent or the nondetergent sulfobetaine NDSB. Liposomes have been used extensively by the pharmaceutical industry for targeted drug delivery to humans (16), and their recent use in trials of several experimental human vaccines (1, 6, 7, 19) indicates that liposomes containing potential meningococcal protective antigens are also likely to be suitable for human vaccination. In contrast, although detergent micelles are not currently licensed for human vaccines, they offer facile methods for refolding proteins to native conformations. The choice of NSDB was based on its ability to improve both the efficiency of solubilization of membrane proteins (39, 40) and the renaturation of chemically and thermally denatured proteins. In addition, in order to enhance the immunogenicity of the PorB protein, additional liposome and micelle formulations were prepared containing MPLA, an adjuvant which has been used in clinical trials of some human vaccines (12, 35, 36).

Immunization with both rPorB liposomes and rPorB micelles induced antibodies that recognized the homologous recombinant protein in ELISA. The effect of MPLA was to increase the immunogenicity of the protein in both liposomes and micelles; indeed, with the latter, antibody levels were higher than those achieved by using conventional alum adjuvant. In previous studies with PorA and Opc proteins, the combination of recombinant protein with alum induced the highest levels of antibodies, but these reacted only poorly with the proteins present in outer membranes (8, 22). In contrast, adsorption of rPorB to alum induced antibodies that cross-reacted with the outer membrane of the homologous strain. The three preparations that contained MPLA showed greater reactivity with the homologous outer membrane, although this was lower than that observed with the recombinant protein. Although it is difficult to compare quantitatively the immune responses to native and denatured proteins this, together with the reactivity of the sera against the heterologous porB2 protein in the outer membrane and on Western blot, suggests that a significant proportion of the antibodies induced do react with nonnative protein, as well as with native protein.

Although outer membrane preparations are likely to contain proteins in a more native conformation than the purified recombinant protein, previous studies have suggested that immunofluorescent detection of antibodies bound to whole cells was a better indicator of antibodies that reacted with native

epitopes (8, 22). In the current study, the antibodies raised against rPorB-alum failed to react with the homologous strain, confirming previous observations that adsorption of meningococcal outer membrane proteins to alum does not promote native conformation. In contrast, all of the liposome and micelle preparations showed significant reactivity with the homologous strain, and in each case the addition of MPLA to the formulation increased reactivity. With liposomes the adjuvant effect was observed when MPLA was incorporated into the same liposome preparation as rPorB, but not when rPorB and MPLA were incorporated into separate liposome preparations that were subsequently mixed together. This suggests, in addition to its adjuvant effect, that MPLA may promote refolding of the protein by replacing the lipopolysaccharide that is naturally associated with PorB in outer membranes. A further effect of MPLA was to induce a broad distribution of antibody isotypes directed against PorB present in the outer membrane. This property of the adjuvant is particularly important since a broad distribution of isotypes, with different effector functions, is likely to be required for protection against infection. In the current study, most of the sera raised against rPorB in liposomes and micelles did not react with cells of the heterologous PorB3 strain, and with the two sera that did show some reactivity it was markedly reduced. Furthermore, none of the sera showed significant reactivity with the heterologous PorB2 strain. Thus, these data, together with the ELISA and Western blotting data, demonstrate that the specificity of the immune response was largely directed against type (PorB)-specific epitopes.

The effect of MPLA in the PorB-liposome preparations is similar to that previously seen with the Opc protein (22) but is in contrast to that seen with the other meningococcal porin, PorA. Incorporation of MPLA into PorA-liposome preparations boosted the immune response to the denatured protein at the expense of the ability of the resulting antibodies to react with whole cells (8). Although the two porins show significant sequence homology and both are believed to adopt a β-barrel conformation in the outer membrane, there are some significant differences. PorB is more closely related to the gonococcal PI porins (43); it forms an anion-selective rather than a cation-selective pore (34), and the regions of variability responsible for immunological specificity have been mapped to different surface-exposed loops and are not readily mimicked by synthetic peptides. Therefore, it would appear that liposomes containing rPorB are more able to accommodate MPLA without adversely affecting the conformation of the immunodominant, type-specific epitopes.

The accepted correlate of in vivo protection against meningococcal infection is the ability of antibodies in serum to promote complement-mediated killing of the relevant meningococcal strain in vitro (15). As in previous studies with recombinant proteins incorporated into liposomes, no other single parameter could be shown to correlate precisely with presence of bactericidal activity in sera (8, 22). In the present study, immunization with each of the rPorB liposome and micelle preparations induced bactericidal activity, with the most effective antisera being those raised against rPorB incorporated into MPLA-liposomes, MPLA-Zwit micelles, and MPLA-NDSB micelles. Significantly, these sera were among those which showed the greatest reactivity with the native protein in IF studies with whole cells and a broader spectrum of complement-fixing antibody subclasses. The potential protective effect was seen not only with the homologous strain but also with a heterologous stain of the same serotype, whereas no significant bactericidal activity was demonstrated against a heterologous strain expressing PorB3 of different serotype. This serotype-specific nature of the protective effect is consistent with the protein refolding to a native conformation and is similar to the restricted specificity obtained with the PorA and Opc preparations (8, 22).

The ideal vaccine against serogroup B meningococcal infection would induce bactericidal activity against a wide range of strains. The outer membrane proteins that have been reported to be recognized by the human immune response during infection are the PorA, PorB, Opc, and iron-regulated proteins (13, 32). In human trials, current experimental vaccines based on PorA induce immunity that is subtype restricted (32) and may also be influenced by further intrasubtype variations (26). Additionally, PorA incorporated into either liposomes or detergent micelles can only induce subtype-specific immunity. This subtype restriction prevents the production of a monovalent PorA vaccine that is effective against all meningococcal strains, and the efficacy of PorA may be further reduced by the occasional reports of clinical isolates lacking PorA (23). Despite the fact that liposomal and micellar preparations of Opc were able to induce bactericidal antibodies, the Opc protein is the least attractive of these outer membrane antigens for inclusion in meningococcal vaccines. The expression of Opc varies widely among strains, and the protective effect in strains expressing the homologous protein was only seen with a minority of isolates expressing high levels of the protein (22). In addition, bactericidal activity was also markedly reduced in heterologous strains by variations in the amino acid sequence of surface-exposed loops. In contrast, the PorB protein now appears to be a more promising vaccine candidate than the Opc protein and has some advantages over PorA in that clinical isolates show less variation in PorB serotype than they do in PorA subtype and no natural isolates lacking PorB have been reported. These observations, together with the results from the present study, suggest that PorB can be regarded as a potential component of new meningococcal vaccines.

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