Restored functional immunogenicity
of purified meningococcal PorA by
incorporation into liposomes

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Abstract

The impact of the conformation, LPS-depletion and the presentation form of outer membrane protein PorA from Neisseria meningitidis (PorA) subtype P1.7-2,4 on the immune response in mice was studied. Native PorA was purified from outer membrane vesicles (OMV) derived from meningococci and reconstituted into liposomes. The conformation of PorA after purification from OMV and reconstitution in liposomes was monitored by use of electrophoretic and spectroscopic techniques and compared with the conformation of PorA in outer membrane complexes (OMC) and heat-denatured PorA. The antigenicity of the PorA formulations was measured by ELISA by using a bactericidal anti-P1.4 monoclonal antibody. Immunogenicity was determined in Balb/c mice. PorA-specific IgG, isotype distribution and bactericidal activity were measured after subcutaneous immunization. In all formulations except in heat-denatured OMV, PorA was present as trimers. The lipopolysaccharide (LPS) content was reduced by 96% in the purified protein with respect to the original OMV. The antigenicity of purified PorA (i.e. ELISA response) was substantially higher as compared to PorA in liposomes, OMV or OMC. The results of the immunogenicity studies showed that all formulations were able to induce comparable IgG titers. However, whereas the antibodies raised by OMV were bactericidal, the antibodies elicited by immunization with purified PorA were unable to kill meningococci.Remarkably, the ability to induce bactericidal antibodies was fully recovered by incorporation of the purified PorA into liposomes, in the absence of other adjuvants, as compared to LPS-containing OMV.
**Introduction**

*Neisseria meningitidis* is one of the major causes of bacterial meningitis [1]. Vaccines based on capsular polysaccharides offer no protection against serogroup B meningococci, the predominant cause of meningococcal infection in the western world. Other major surface components, such as the outer membrane protein PorA (class 1 protein, a porin) have been evaluated as vaccine candidates [2,3,4,5,6], mostly formulated in outer membrane vesicles (OMV). Among the PorA subtypes, the subtype P1.7-2,4 is present in up to 43% of the strains causing bacterial meningitis [7]. Thus, a PorA-based vaccine should cover at least this subtype. On the other hand, the immunogenicity of PorA P1.7-2,4 is not yet optimal in its present formulation as OMV vaccine [5,6].

The vaccines against type B meningococci that are being developed by various groups [8,9,10] consist of OMV extracted from (sometimes genetically modified) meningococcal strains, with one or more PorA subtypes embedded in it [11,12]. Moreover, OMV contain neisserial lipopolysaccharide (LPS), which could play a role as immunogen, adjuvant [13] and stabilizer [14], but is also responsible for reactogenicity due to the presence of lipid A [37]. This last characteristic makes the presence of LPS in the vaccine undesired. Another drawback of OMV is the difficulty to optimize the immunogenicity by manipulating the composition, because the latter is governed by the strain from which they are purified. Components in the outer membrane may affect the immunogenicity by inducing non-bactericidal antibodies.

Besides OMV, outer membrane complexes (OMC) have been extensively used in preclinical studies. These OMC are obtained by a simple outer membrane extraction with sarcosyl [15]. However, the structure and composition of these OMC is poorly defined and, like OMV, they contain LPS.

During the past few decades, as a consequence of improved immunological insights and more strict regulatory requirements, there is a continuous trend towards better-defined vaccines in order to guarantee safety and efficacy [16]. Our aim is to obtain PorA vaccines that are better defined than OMV and OMC, in the absence of toxic components (e.g. LPS) and that induce an adequate immune response.

Physicochemical characteristics of PorA and PorB (class 3) of *Neisseria meningitidis* have previously been studied but only in their purified form [17,18]. Native PorA forms relatively stable trimers [17]. Although the crystal structure of PorA...
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has not yet been elucidated, PorA is believed to resemble the PhoE protein of *E. coli* [19], consisting of a β-barrel formed by 16 membrane spanning regions. Protruding loops contain functional (i.e. bactericidal) epitopes. In PorA the epitopes on loops 1 and 4 are immunodominant [20].

It has been previously shown that SDS-denatured recombinant PorA fails to induce a bactericidal immune response in the absence of LPS as adjuvant [14]. This indicates that monitoring the preservation of the PorA conformation should be an integral part of formulation studies. Preservation of the native structure alone, however, is not sufficient to warrant the immunogenicity of antigens. For instance, the immunogenicity of purified PorA is lower as compared to PorA in OMV [35] and the use of adjuvants is often required [21]. A reduced immunogenicity of purified PorA is expected to be reversible, provided that the protein is reconstituted in its native conformation in an adequate presentation form (e.g. liposomes). The incorporation of membrane proteins into liposomes has frequently been used with success, also for other (recombinant) PorA subtypes [21,39]. Liposomes offer various advantages with respect to other formulations. First, lipid bilayers resemble the ‘natural’ environment of membrane proteins such as PorA. Second, liposomes are well-defined systems and their characteristics are easily varied. This would allow systematic studies on the effect of incorporated adjuvants (e.g. detoxified lipid A derivatives, CpG, cytokines, QuilA) or targeting devices (e.g. to direct the antigen efficiently to antigen-presenting cells) attached to the liposome surface.

In this study, native PorA P1.7-2,4 has been purified from OMV and incorporated into liposomes. A detailed characterization of the protein in OMV, OMC (i.e. membrane-embedded), in purified (i.e. micellar) form and in denatured OMV has been carried out. We pay special attention to the preservation of the protein conformation upon purification and reconstitution into liposomes. The effects of purification, protein conformation and presentation form on the immune response are discussed. It is demonstrated that reconstitution of purified native PorA leads to a functional immune response similar to that of (LPS-containing) OMV.

**Materials and methods**

*Bacterial strains and growth conditions*

*N. meningitidis* strain F91 (P1.7-2,4, PorB⁺, RmpM⁺, low expression of Opa/Opc) was
used for the production of monovalent PorA-OMV, PorA-OMC and purified PorA. Bacteria were grown in a defined medium, with as main components L-glutamic acid, glucose, L-cysteine and ammonium chloride. Cells were cultivated at 35ºC for 15 h in a 40-L stirred fermentor under pH and pO2 control.

**PorA-OMV and OMC**

PorA-OMV were prepared according to a modification of the procedure described by Claassen et al. [8]. Briefly, PorA-OMV were extracted from bacteria by EDTA extraction. DNA was partially removed by DNase treatment. LPS depletion was achieved by gel permeation chromatography (GPC) in the presence of deoxycholate (DOC). DOC was removed by a second GPC step.

PorA-OMC from *Neisseria meningitidis* strain F91 were isolated by sarcosyl extraction as described by Poolman et al. [15]. To obtain denatured (monomeric) PorA, OMV were heated in a boiling waterbath for 5 min.

**Purification of PorA**

PorA-OMV were used as starting material to obtain pure PorA P1.7-2.4, by using a modification of the procedure described by Poolman et al. [22]. In short, 1 volume of OMV containing 0.5 mg/ml protein was disrupted by incubation overnight in 1 volume of 50 mM Tris-HCl, 10 mM EDTA, 0.05% (w/v) Zwittergent 3,14, pH 8.0. The mixture was centrifuged at 120 000 g and 4ºC for 20 min. The supernatant was treated overnight with DNase (Benzonase, Merck) and centrifuged at 2900 g and 4ºC for 1 h. The supernatant was further purified by anion-exchange chromatography on a Q-Sepharose column (Pharmacia), which had been equilibrated with 50 mM Tris-HCl, 0.05% (w/v) Zwittergent 3,14, pH 8.0. The protein was eluted with a linear gradient of 0-1 M NaCl in the same buffer. Fractions containing protein were pooled and dialyzed overnight at 4ºC against 50 mM Tris-HCl, 0.05% Zwittergent 3,14, pH 8.0 before use.

**Reconstitution of PorA into liposomes**

In order to incorporate the purified protein into liposomes, PorA was precipitated in 80% (v/v) ethanol and dissolved in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) containing 150 mM n-octyl β-d-glucopyranoside (OG) to a final concentration of 200 µg/ml. Liposomes were made of dimyristoylphosphatidylcholine
Restored immunogenicity of purified meningococcal PorA (PC, Rhône-Poulenc Rorer, Köln, Germany), dimyristoylphosphatidyglycerol (PG, a gift from Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Chol, SIGMA, Zwijndrecht, The Netherlands) in a 8:2:2 mol ratio. PorA-liposomes were prepared according to the detergent dilution method [23]. In short, appropriate amounts of each lipid were dissolved in chloroform/methanol (2:1, vol:vol) in a round bottom flask and a lipid film was obtained by solvent evaporation in a rotavapor under reduced pressure. The film was resuspended in the PorA solution (lipid concentration 6 mM) and the resulting mixed micelles were rapidly diluted 11-fold in TBS, allowing the formation of liposomes. Subsequently, the liposomes were pelleted by ultracentrifugation (160,000 g, 1 hour) and resuspended in TBS, yielding PorA-containing liposomes with a protein/lipid ratio of 25 (µg/µmol).

Characterization of PorA formulations

The particle size of PorA-OMV, OMC and liposomes was measured by dynamic light scattering (DLS) at 25ºC with a Malvern 4700 system equipped with a 75 mW Argon ion laser (488 nm, Uniphase, San José, CA, USA), a remote interface controller and PCS software, version 1.35 (Malvern Ltd., Malvern, UK). For refractive index and viscosity the values of pure water were used. The particle size distribution was reflected in the polydispersity index (PD), which ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion. Electron microscopy was performed as described by Claassen et al. [8].

The protein content was measured according to Peterson [24] with bovine serum albumin as standard. LPS was measured by keto-deoxyoctonate (KDO) determination [25] with KDO as standard (Sigma). The corresponding amount of Neisseria meningitidis LPS was calculated (2 mol KDO / mol LPS) for a LPS molecular weight of 3,380 Da. DNA content was determined by an ethidium bromide assay [26] with salmon sperm DNA (ICN, Rotterdam, The Netherlands) as standard.

SDS-PAGE

The protein composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in ‘native’ gels as described by Jansen et al. [17]. Prior to electrophoresis, samples were incubated for 10 min at room temperature or 100ºC in sample buffer [27] containing 0.05% SDS. Polyacrylamide gels were prepared as described [27], except that SDS was omitted.
from the stacking and running gels. The acrylamide concentration of the running gel was 12%. Gels were run at 20 mA in a temperature-controlled room (4\(^\circ\) C) to prevent denaturation of the formulations containing folded PorA. Gels were stained with Coomassie Brilliant Blue.

**Circular dichroism measurements**

Circular dichroism (CD) was used to detect differences in secondary structure of PorA. Spectra were recorded at ambient temperature with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). The subtractive double-grating monochromator was equipped with a fixed disk and holographic gratings. Gratings with 2400 lines/mm (blaze wavelength 230 nm) and 1.24-mm slits were used. Spectra were recorded from 260-200 nm (path length 0.5 mm). Each measurement was the average of at least ten repeated scans (step resolution 1 nm) from which the corresponding buffer spectrum was subtracted. The measured CD signals were converted to molar extinction difference (\(\Delta\varepsilon\) based on an average amino acid residue weight of 115).

**Fluorescence measurements**

The local environment of tryptophan in PorA was monitored with fluorescence spectroscopy. Steady-state fluorescence measurements were performed with an LS-50B Luminescence spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

Fluorescence emission spectra after excitation at 295 nm were recorded from 300-450 nm, with excitation and emission bands 4 and 8 nm, respectively. Samples containing 10 \(\mu\)g/ml protein were measured at 150 nm/min. Sets of 5 spectra were averaged and corrected for buffer background.

Quenching of tryptophan fluorescence by acrylamide in the range of 0 to 0.7 M was performed by adding aliquots of acrylamide (1.4 M in the corresponding buffer) to samples containing 100 \(\mu\)g/ml protein. In order to avoid interference with acrylamide absorption, the excitation wavelength used was 300 nm. The fluorescence intensity was monitored at 350 nm and corrected for buffer background, with excitation and emission bands 4 and 20 nm, respectively. The data were analyzed by a modified form of the Stern-Volmer equation: \(F_0/\Delta F=1/f_a+1/(f_aK_{SV}[Q])\), where \(F_0\) is the fluorescence intensity in the absence of
the quencher (acrylamide) and $\Delta F$ is the difference between fluorescence intensities in the absence and presence of the quencher at a concentration $[Q]$. $K_{SV}$ is the Stern-Volmer constant for dynamic quenching and $f_a$ is the fraction of tryptophan fluorescence that is accessible to the quencher [28].

**PorA inhibition ELISA**

Antigenicity of OMV, OMC, liposomes and purified PorA was tested by an inhibition ELISA analogous to a method previously described for *Bordetella pertussis* antigens [29]. In short, samples were serially diluted in 96-well microtiter plates and incubated overnight at room temperature with a fixed dilution (1:1000, v:v) of the bactericidal monoclonal antibody MN20B9.34 directed against the P1.4 epitope of PorA. OMV of strain F91 containing 2 µg/ml protein in phosphate-buffered saline (PBS), were used for coating a separate plate overnight at room temperature. After incubation, the OMV-coated plate was washed and aliquots of the pre-incubation plate were added. After incubation for 2 h at room temperature, plates were washed again and incubated for 1.5 h at room temperature with peroxidase labeled goat anti-mouse (GAM) IgG, IgA, IgM (Cappel, Organon Teknika, dilution 1/5000). Plates were washed and the color reaction was performed as described [29]. The reference OMV batch contained at least 90% PorA, as determined by SDS-PAGE. The results are shown as the ratio between the protein concentration determined by ELISA and the protein concentration determined by Peterson. As a reference, the antigenicity of OMV was arbitrarily set at 1.

**Immunization experiments**

Procedure A: Balb/c mice, 6 to 8 weeks old (8 animals in each group) were immunized subcutaneously on day 0, 14 and 28. Either 1.5 µg or 10 µg PorA in OMV, OMC, purified micelles or denatured OMV were administered (0.25 ml/mouse) (see Table 3). The mice were bled on day 42, and sera were collected and stored -20ºC until analysis. AlPO$_4$ (RIVM) was added to all preparations (1.5 mg/dose).

Procedure B: Balb/c mice, 6 to 8 weeks old (8 animals in each group), were immunized subcutaneously on day 0, 14 and 28 with PorA in OMV or liposomes. Samples (0.25 ml/mouse) were administered in the absence of other adjuvants and increasing protein doses were tested for the liposomal formulation (see Table 4).
Anti-PorA whole-cell and OMV-ELISA

The antibody titers (total IgG and individual isotypes) in mouse sera were determined by whole-cell ELISA as described [30]. *N. meningitidis* strain H44/76(15P1.7b,4:L3,7,9) and the H44/76-derived mutant strain HI5 (lacking PorA) were used. The titer is defined as the dilution of the serum where 50% of the OD_{max} in the assay is reached. Isotypes were determined with goat anti-mouse (GAM) Ig isotype-specific conjugates, labeled with horseradish peroxidase (HRP) (Southern Technology Associates; dilution 1/5000, except for IgG1 1/2500). In a similar manner an OMV-ELISA was performed, for which plates were coated with OMV purified from several strains, diluted to a protein concentration of 3 µg/ml.

Serum bactericidal assay

The serum bactericidal activity was measured as previously described [31] against the *N. meningitidis* strains: H44/76 7b,4 and HI5 (PorA⁻) (see section 2.11 for details). Sera from mice were heat inactivated for 30 min at 56°C prior to use. For blocking experiments, 5 µl of bactericidal antiserum (i.e. from mice immunized with PorA-OMV) were mixed with increasing volumes (ranging from 1 to 45 µl) of non-bactericidal (blocking) antisera (i.e. from mice immunized with purified PorA). The total volume was adjusted to 50 µl with Gey’s balanced salt solution (Sigma) containing 0.5% (w/v) bovine serum albumin (BSA, Sigma) and the samples were incubated at room temperature for 10 min before inactivation. Serum samples and bacteria were incubated for 10 to 15 min at room temperature before the addition of complement. A final concentration of 80% (v/v) rabbit complement was used. As controls, the bactericidal anti-P1.4 (MN20B9.34) and anti-LPS (MN15A17F12) monoclonal antibodies were used. Also, test sera were incubated without complement as a negative control. The serum bactericidal titer was measured as the reciprocal serum dilution showing more than 90% killing of the number of bacteria used.

Statistical methods

Before statistical analysis, antibody and bactericidal titers were log_{10} converted. Antibody titers are expressed as the mean log_{10} titer of eight independent observations. Analysis of variance was used for statistical evaluation of the data. The significance of the differences between the mean values of the antibody titers was
determined by the least-significant-difference (LSD) test at a confidence level of 95%. This test could not be used for bactericidal titers, as some groups included both responders and non-responders. Values given are the average of the indicated responders ± SD.

Results

*PorA in OMV, OMC and denatured OMV*

The composition of the PorA-containing OMV, OMC and denatured PorA-OMV is given in Table 1. Lipids, LPS and other bacterial components such as DNA are still present in both OMV and OMC. OMV were further characterized by electron microscopy, which yielded images similar to those published before [8], i.e. partly aggregated vesicles with a diameter of around 80 nm (not shown). The presence of aggregated material was confirmed by DLS: OMV typically showed an average particle size of 270 nm with a relatively high polydispersity index (PD) of 0.43. OMC showed a larger size than OMV (i.e. 612 nm) and the PD was also increased (Table 1). In denatured PorA samples, both intact OMV and amorphous material were visualized by electron microscopy (data not shown). Apparently, in addition to aggregated OMV, fragmented material was formed upon heat treatment, which explains the decrease in the observed averaged size (DLS) and the highly increased PD (Table 1).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LOS content (%)</th>
<th>DNA content (%)</th>
<th>Particle size (nm)</th>
<th>PD</th>
<th>Antigenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMV</td>
<td>4.6</td>
<td>1.3</td>
<td>270</td>
<td>0.43</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>OMC</td>
<td>0.5</td>
<td>0.4</td>
<td>612</td>
<td>0.58</td>
<td>0.77 ± 0.17</td>
</tr>
<tr>
<td>Purified PorA</td>
<td>0.3</td>
<td>n.d. d</td>
<td>- e</td>
<td>- e</td>
<td>1.64 ± 0.31</td>
</tr>
<tr>
<td>Liposomes</td>
<td>0.3</td>
<td>n.d. d</td>
<td>215</td>
<td>0.30</td>
<td>1.01 ± 0.18</td>
</tr>
<tr>
<td>Denatured OMV</td>
<td>5.3</td>
<td>4.6</td>
<td>105</td>
<td>0.83</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

a Percentage refers to the total amount of protein present in the formulation.

b Polydispersity index (see materials and methods section).

c Antigenicity (relative to that of OMV) as determined with MN20B9.34 monoclonal antibody in an inhibition ELISA. The results are shown as mean values (±SD) of at least 3 measurements.

d n.d.: not detected (i.e. <0.25 µg/ml).

e Signal too low for measurement.
**Purification of PorA from OMV**

Purified PorA elutes from the anion-exchange column in one peak (Fig. 1). The purification of PorA, with OMV as starting material, yielded protein trimers (see 3.3) with a high degree of purity (Table 1). The DNA present in the original OMV was below the detection limit in the purified (micellar) form. The amount of LPS present after purification was reduced with 94% with respect to the original OMV (Table 1).

![Figure 1. Typical anion-exchange elution chromatogram of disrupted and DNase-treated OMV containing PorA P1.7-2.4. Squares: protein elution pattern, solid line: NaCl gradient, expressed as the percentage of elution buffer containing 1 M NaCl. Fractions 16 to 24 were pooled and dialyzed overnight against elution buffer without NaCl.](image)

**Effect of purification on the conformation of PorA P1.7-2,4**

In order to observe the folding into trimers of the protein in all the formulations, SDS-PAGE electrophoresis was performed under mild conditions (Fig. 2) [17]. Purified PorA incubated with loading buffer at room temperature shows the presence of a high molecular weight band (~120 kDa) corresponding to PorA trimers (lane 3-a). Upon incubation of the purified PorA at 100°C (lane 3-b), the trimers were converted to monomers (43 kDa). This pattern is also observed in the OMV and OMC (lanes 1, 2). The denaturation of PorA in OMV was confirmed by SDS-PAGE, as only monomers were present at both incubation temperatures (lane 4). In samples containing LPS (OMV, OMC) a smear is observed above the position of the trimers (lanes 1,2,4), which disappears upon purification of the protein (lane 5). This suggests that the smear represents assembled PorA-LPS complexes.
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Figure 2. SDS-PAGE of PorA P1.7-2.4 in different formulations. The samples were dissolved in sample buffer containing 0.05% SDS and incubated either at room temperature (a) or 100°C (b). The molecular weight of reference proteins are indicated at the left (kDa). Lane 1: PorA-OMV, lane 2: PorA-OMC, lane 3: purified PorA, lane 4: denatured PorA-OMV, lane 5: liposomes.

Figure 3. Fluorescence emission spectra of PorA P1.7-2.4 formulations, excited at 295 nm. Closed diamonds: PorA-OMV, open diamonds: purified PorA, closed triangles: denatured PorA-OMV, open triangles: PorA-OMC.

Fluorescence spectroscopy allows to study the local tryptophan environment and the accessibility of tryptophan to a quencher, in our case acrylamide. The intrinsic fluorescence of the four tryptophan residues that the protein contains showed a similar fluorescence emission spectrum in all the formulations (Fig. 3), with an emission maximum ($\lambda_{\text{max}}$) at 342 nm for PorA-OMV and PorA-OMC and at 340 nm for the purified protein and denatured PorA-OMV, which corresponds with a relatively hydrophobic environment [28]. This is in accordance with the topology model
described by van der Ley et al. [32], where the four tryptophan residues are located in or near the highly conserved trans-membrane regions: two in the membrane-spanning regions and the other two in the vicinity of the bilayer. None of the tryptophans are located in the subtype specific epitopes. The intensity of the fluorescence signal was decreased in the PorA-OMV (both native and heat denatured) as compared with the other formulations (Fig. 3). This suggests that in the OMV formulation quenching compounds are present, which are removed after purification.

Figure 4. Modified Stern-Volmer plots of the quenching of PorA fluorescence by acrylamide. Quenching experiments were performed by adding aliquots of acrylamide to PorA formulations (ca. 100 µg/ml). The data were fitted by linear regression of 4 sets of measurements and analyzed with a modification of the Stern-Volmer equation: $F_0/\Delta F=1/f_s+1/(f_s*K_{SV}[Q])$. See for more details materials and methods. Closed diamonds: PorA-OMV, open diamonds: purified PorA, closed triangles: denatured PorA-OMV, open triangles: PorA-OMC.

Table 2. Summary of fluorescence quenching studies with acrylamide

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$K_{SV} (M^{-1})$</th>
<th>$f_s$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMV</td>
<td>3.47 ± 0.84</td>
<td>0.72 ± 0.06</td>
<td>0.9985</td>
</tr>
<tr>
<td>Purified P1.4</td>
<td>3.95 ± 0.45</td>
<td>0.62 ± 0.07</td>
<td>0.9995</td>
</tr>
<tr>
<td>OMC</td>
<td>3.95 ± 0.48</td>
<td>0.59 ± 0.01</td>
<td>0.9981</td>
</tr>
<tr>
<td>Denatured OMV</td>
<td>4.91 ± 0.81</td>
<td>0.65 ± 0.05</td>
<td>0.9989</td>
</tr>
</tbody>
</table>

1) Data were fitted by linear regression. From the fitted line and according to $F_0/\Delta F=1/f_s+1/(f_s*K_{SV}[Q])$, $f_s$ and $K_{SV}$ were obtained. The tryptophan residues were excited at 300 nm, and the intensity was read at an emission wavelength of 350 nm. The results are shown as mean values (±SD) of 4 measurements. See materials and methods for further details.

To examine the accessibility of the tryptophan residues in PorA P1.7-2.4, acrylamide was used as a non-charged quencher. The modified Stern-Volmer plots for the quenching of PorA-OMV, PorA-OMC and purified PorA do not show major differences (see Fig. 4), indicating that the accessibility of the tryptophan residues to
acrylamide is comparable in the different formulations. Only for heat-denatured PorA-OMV, a slightly different slope of the modified Stern-Volmer plot was obtained. As shown in Table 2, the accessible fraction \( f_a \) calculated from the modified Stern-Volmer plot suggests that, on the average, 59-72% of the tryptophan fluorescence can be quenched. As the fractional contribution of the individual tryptophans to the total fluorescence is not known, these results do not show which of them are accessible to acrylamide. Logically, the two tryptophans that are located near the trans-membrane region of the protein are fully accessible, whereas the tryptophan residues inside the trans-membrane region are partly or not accessible to acrylamide. In the purified form, PorA is formulated with a detergent in a micellar form (without a membrane). Nevertheless, even in the solubilized (trimers) and in the denatured (monomers) form the quencher could not reach all tryptophan residues. This indicates that, although only monomers are observed by SDS-PAGE for the heat-denatured OMV (see Fig. 2, lane 4), these are not completely unfolded.

![Figure 5. Far-UV CD spectra of PorA P1.7-2,4. Closed diamonds: PorA-OMV, open diamonds: purified PorA, closed triangles: denatured PorA-OMV, open triangles: PorA-OMC.](image)

Far-UV circular dichroism measurements were performed to study the secondary structure of PorA P1.7-2,4. The far-UV CD spectra (Fig. 5) of PorA are similar in all native formulations, and resemble the spectra obtained for PorB class 3 protein of Neisseria meningitidis [28]. In all formulations containing trimeric PorA the
spectrum has a similar shape, with a minimum around 220 nm and a maximum around 200 nm, indicative of the predominant presence of antiparallel β-sheets [33]. Heat-denatured PorA-OMV, however, show a different shape, with a minimum shifted down to 214 nm, indicating an altered, but not random secondary structure.

For PorA, 8 surface-exposed, variable loops have been predicted [32,34]. In bacteria containing PorA P1.7-2.4, loop 1 is hidden (i.e., not surface-exposed), leaving as the most immunogenic region loop 4, where the epitope P1.4 is located. The antigenicity of the P1.4 epitope in the different formulations was quantified by ELISA. The results (Table 1) show that both OMV and OMC have a lower antigenicity than the purified PorA. This may be due to the steric hindrance caused by other components than PorA present in OMV and OMC. On the other hand, the interaction in solution of the anti P1.4 monoclonal antibody with denatured PorA-OMV is (almost) completely absent.

Reconstitution of purified PorA into liposomes

Purified, LPS-depleted PorA was incorporated into liposomes. The bilayer composition was PC:PG:Chol with a molar ratio of 8:2:2. PorA-containing liposomes had a particle size around 200 nm and a lower polydispersity as compared to OMV (Table 1). Fluorescence and CD measurements of liposomal PorA were not performed because of too much interference from light scattering by the liposomes, as a result of relatively low protein/lipid ratios as compared to OMV.

The trimeric structure of purified PorA was preserved upon reconstitution in liposomes (Fig. 2, lane 5a). The increase in antigenicity of PorA was decreased to the level of OMV by its incorporation into liposomes (Table 1).

Effect of purification on the immunogenicity of PorA

Balb/c mice (eight mice per group) were immunized with Por A-OMV, PorA-OMC, purified PorA P1.7-2.4 and denatured PorA-OMV according to procedure A. AlPO₄ was used as adjuvant (see materials and methods). The purified protein was expected to have a lower immunogenicity as compared to the formulated PorA, as previously shown with other PorA subtypes [14,35]. Therefore, two doses of purified PorA were tested: 1.5 and 10 µg/mouse. The results of the serum analysis for this immunization are shown in Table 3. All sera showed significant IgG titers. The antibody titers raised by PorA-OMV, PorA-OMC and denatured PorA-OMV were
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comparable. Purified PorA at the same protein concentration (1.5 µg/mouse) induced the lowest amount of total IgG. The titer was restored when the dose was raised to 10 µg/mouse.

Table 3. Humoral immune response induced by PorA P1.7-b,4 adjuvated with AlPO₄; effect of the presentation form.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dose (µg)</th>
<th>Whole-cell ELISA titer</th>
<th>Bactericidal titer</th>
<th>Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgG1</td>
<td>IgG2a</td>
</tr>
<tr>
<td>OMV</td>
<td>1.5</td>
<td>4.5</td>
<td>3.7</td>
<td>4.2</td>
</tr>
<tr>
<td>OMC</td>
<td>1.5</td>
<td>4.0</td>
<td>2.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Pure PorA</td>
<td>1.5</td>
<td>3.3</td>
<td>3.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Pure PorA</td>
<td>10</td>
<td>4.2</td>
<td>4.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Denatured</td>
<td>1.5</td>
<td>4.3</td>
<td>4.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Group 5 was measured in a separate experiment. As bridging group OMV were used, giving similar results as in the previous experiment.
  
* The titer of each anti-P1.7-2,4 IgG isotype was determined by whole-cell ELISA and is expressed as the mean log₁₀ titer. The means were compared by the LSD test with a confidence level of 95%:
  
* The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of strain H44/76 7-2,4 and is expressed as the averaged mean log₁₀ titer of the responders ± SD.

Determination of the relative subclass distribution of the anti-PorA specific antibodies in the sera showed that PorA-OMV and PorA-OMC induced relatively high IgG2a and IgG2b titers (Table 3). Both purified PorA and denatured PorA-OMV showed a shift towards the production of IgG1.

To make sure that the antibody response as measured by whole-cell ELISA was PorA-specific, an OMV-ELISA was performed. In this way, cross-reactivity of the sera with capsular polysaccharide, other proteins and LPS present on the bacterial surface is largely avoided. The titers obtained with this method were slightly lower, but showed the same trend as those obtained with whole-cell ELISA (not shown). Cross-reactivity of the antisera with OMV from the PorA negative strain HI5 was almost completely absent. Only purified PorA at the highest dose induced antibodies that interacted weakly with these OMV (not shown).

The bactericidal activity of antibodies is a good measure for protective immunity [36]. Each individual mouse serum was tested for bactericidal activity with strain H44/76 7-2,4 (Table 3). The anti-OMV and OMC sera had the highest bactericidal titers (Table 3), confirming the observation that bactericidal antisera
contain significant titers of the complement-binding IgG2a and 2b isotypes [31,37]. In mice immunized with PorA-OMV, sera of six out of eight mice showed bactericidal activity. Also PorA-OMC induced bactericidal antibodies (5/8 responders). Neither the purified LPS-depleted PorA at the two doses tested in the presence of adjuvants nor the denatured protein (OMV) induced significant amounts of bactericidal antibodies. None of the formulations elicited bactericidal antibodies against strain HI5, which excludes the presence of bactericidal antibodies against components other than PorA.

In order to investigate the nature of the non-bactericidal antibodies, an inhibition study with a bactericidal serum was done. By mixing bactericidal sera (from mice immunized with PorA-OMV and AlPO₄ as adjuvant) with non-bactericidal sera containing similar antibody levels (from mice immunized with purified PorA, high dose), the bactericidal activity was completely inhibited (Fig. 6).

**Effect of reconstitution into liposomes on the immunogenicity of PorA**

From the results presented above, it can be concluded that upon purification PorA lost its capacity to induce bactericidal antibodies, despite the preservation of its conformation. In order to investigate whether the functional immunogenicity could be restored after incorporation of PorA into liposomes, a separate immunization experiment was done (procedure B, see materials and methods). Animals were vaccinated with OMV and liposomes, and a liposomal dose-response was generated. To prevent any masking effects caused by AlPO₄, immunizations were performed without adjuvant (Table 4). In the absence of AlPO₄, OMV still induced substantial
Restored immunogenicity of purified meningococcal PorA

total IgG titers. Also high titers were obtained with liposomes at all concentrations used (1.5, 3, 6 and 12 µg/mouse).

Table 4. Recovery of the immunogenicity of purified PorA by incorporation into liposomes

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dose (µg)</th>
<th>Whole-cell ELISA titer</th>
<th>Bactericidal titer</th>
<th>Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG IgG1 IgG2a IgG2b IgG3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMV</td>
<td>1.5</td>
<td>3.7 2.8 3.0 3.2 0.4</td>
<td>1.67 ± 0.86</td>
<td>5/8</td>
</tr>
<tr>
<td>Liposomes</td>
<td>1.5</td>
<td>3.7 2.6 3.3 3.1 0.3</td>
<td>1.91 ± 0.56</td>
<td>6/8</td>
</tr>
<tr>
<td>Liposomes</td>
<td>3</td>
<td>3.8 2.9 3.3 3.3 2.0</td>
<td>1.95 ± 0.74</td>
<td>7/8</td>
</tr>
<tr>
<td>Liposomes</td>
<td>6</td>
<td>3.9 3.2 3.6 3.4 1.9</td>
<td>1.94 ± 0.37</td>
<td>8/8</td>
</tr>
<tr>
<td>Liposomes</td>
<td>12</td>
<td>4.1 3.1 3.9 3.6 2.4</td>
<td>1.98 ± 0.56</td>
<td>6/8</td>
</tr>
</tbody>
</table>

a The titer of each anti-P1.7-2,4 IgG isotype was determined by whole-cell ELISA and is expressed as the mean log_{10} titer. The means were compared by the LSD test with a confidence level of 95%:
LSD_{0.05} (IgG) = 0.4; LSD_{0.05} (IgG1) = 0.5; LSD_{0.05} (IgG2a) = 0.2; LSD_{0.05} (IgG2b) = 0.2; LSD_{0.05} (IgG3) = 0.9.

b The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of strain H44/76 7-2,4 and is expressed as the averaged log_{10} titer of the responders ± SD.

Both OMV and liposomes induced high IgG2a and IgG2b titers (Table 4). Antisera raised by liposomal PorA showed a similar relative subclass distribution as compared to PorA-OMV, with only a significant increase in the IgG3 titer observed for liposomes when the dose was increased.

Sera of 5/8 animals immunized with OMV in the absence of AlPO₄ showed bactericidal activity. Remarkably, in the groups immunized with the LPS-depleted PorA incorporated into liposomes the number of responders and the bactericidal titers were at least as high as the ones obtained with LPS-containing OMV. For liposomal PorA there was no clear effect of the dose on the number of responders or average bactericidal titer (Table 4). Again, in this experiment none of the formulations elicited bactericidal antibodies against strain HI5, excluding the presence of bactericidal antibodies against components other than PorA.

Discussion

To determine the preferred characteristics of a PorA formulation for an effective immune response, both the conformation of the antigen and the presentation form have to be well defined. The combined physicochemical techniques used in this study indicated that the conformation of PorA P1.7-2,4 in
OMV and OMC was similar. Purification of the protein did not induce measurable conformational changes. Only a clear change in the conformation was observed upon heat treatment, as detected by circular dichroism and fluorescence quenching studies. Both circular dichroism and fluorescence studies indicated that heat-denatured PorA was misfolded rather than completely unfolded. The circular dichroism spectrum of heat-denatured PorA (Fig. 5) suggests an altered, but non-random secondary structure. The fluorescence spectrum does not show a spectral shift (Fig. 3), indicating that the average hydrophobicity of the local environments of the four tryptophans has not changed. In contrast, in the presence of 5 M guanidinium hydrochloride (a common protein denaturant) the fluorescence spectrum of PorA shifts to 353 nm (unpublished results), indicating a more hydrophilic environment of the tryptophan residues typical of fully unfolded proteins [28]. Finally, although the quenching studies with acrylamide point to enhanced quenching of heat-denatured PorA (cf. $K_{SV}$ values, Table 2; Fig. 4), yet not all tryptophan residues were accessible to acrylamide. Improper folding of heat-treated PorA explains the absence of trimers (see Fig. 2, lane 7), as these will only form through specific hydrophobic interactions between three native PorA molecules [19].

In line with a perturbation of the overall conformation as discussed above, heat-treated PorA failed to interact strongly with an anti-P1.4 antibody in vitro (Table 1), suggesting that the P1.4 epitope was largely destroyed. In contrast, purified PorA was recognized to a higher extent by the same anti-P1.4 antibody as compared to OMV and OMC. As we showed that the conformation of purified PorA was similar to that in OMV and OMC formulations, this is likely to be due to a better accessibility of the P1.4 epitope to the antibodies.

When incorporated into liposomes, PorA preserved its trimeric conformation (Fig. 2) but the interaction with anti-P1.4 antibody was reduced to the level of OMV (Table 1). This supports the idea that the protein embedded in a membrane is less accessible to the antibodies.

Immunization experiments were carried out to evaluate the impact of the purification on the immune response. Although all formulations raised significant antibody levels (Table 3), only mice immunized with the membrane-embedded OMV and OMC showed substantial bactericidal activity. In spite of its native-like conformation, micellar purified PorA did not induce bactericidal antibodies. Differences in the physicochemical properties of the presentation forms (micelles vs.
OMV and OMC) may be responsible for this, such as the absence of a bilayer structure, particle size, surface charge, as well as the reduced amount of LPS when compared to OMV and OMC (see Table 1) [14]. The lack of bactericidal activity of heat-treated PorA-OMV confirms that an appropriated conformation is necessary for induction of functional antibodies.

Not only was the bactericidal activity induced by purified PorA absent, but the antisera elicited by purified PorA also inhibited the activity of bactericidal antibodies present in antisera obtained by immunization with PorA-OMV (Fig. 6). This blocking effect was only observed when mixing sera with IgG-titers similar to those in bactericidal antisera (data not shown). In the presence of 1:1 (v/v) ratio of bactericidal/blocking antisera, the bactericidal activity of anti-OMV antiserum completely disappeared. This suggests that the affinity of non-bactericidal antibodies for bacteria is comparable to or higher than that of bactericidal antibodies. The bactericidal serum used for our blocking experiments contained relatively large amounts of the complement-binding isotypes IgG2a and 2b [31], whereas the blocking antiserum contained relatively large amounts of IgG1. It has been speculated that the blocking effect is due to steric hindrance, induction of conformational changes or to antibodies binding to the same epitope but unable to activate complement because of a different antibody isotype [38]. Our results cannot exclude the first two possibilities, but clearly indicate the importance of the antibodies affinity together with an appropriate isotype for complement binding.

Although these results might be discouraging for the further use of purified PorA, in a separate immunization and in the absence of AlPO₄ as adjuvant we demonstrated that purified native PorA is still able to induce a bactericidal immune response, provided that it is reconstituted into a membrane (i.e. liposomes). This has been previously observed with liposomal recombinant PorA [21,39]. However, in these studies either the bactericidal titer raised with recombinant PorA in liposomes was reduced with respect to native PorA in OMV [21] or the liposome-embedded recombinant PorA was not compared with the native protein [39]. Our results show that the liposomal (LPS-depleted) PorA is capable to induce a comparable bactericidal response to the one of LPS-containing OMV in the absence of other adjuvants. The discrepancies with earlier results can be due to i) the nature of the protein (native vs. recombinant) ii) the sonication method used for liposome preparation, which may negatively affect the protein conformation, or iii) the presence
of residual LPS in the purified protein. We were not able to completely deplete the purified protein from LPS. This residual LPS could be involved in the adequate folding of the protein or still act as an adjuvant. The latter, however, is not likely, as only 0.3% LPS was present in our liposomes, which is expected to be too low to exert adjuvant activity. In other studies up to 25% (w/w) was used to recover the bactericidal activity of PorA-OMC derived from an LPS-deficient mutant [37].

All together these results clearly indicate that both the conformation and the presentation form of PorA dictate the quality of the immune response. Although purification of the PorA adversely affected its capacity to elicit bactericidal antibodies, its conformation was maintained and the bactericidal response was recovered upon reconstitution of the purified protein in liposomes, without further need of adjuvants, to similar levels as LPS-containing OMV. In follow-up studies we will investigate which formulation parameters result in a further improvement of the functional immunogenicity of liposomal PorA.

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References


