Well-defined and potent liposomal meningococcal B vaccines adjuvated with LPS derivatives

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(submitted)
Abstract

Potent liposomal PorA formulations containing various lipopolysaccharide (LPS) derivatives were developed. The following adjuvants were compared: the commonly used aluminum phosphate (AlPO₄), and three LPS-like adjuvants: monophosphoryl lipid A (MPL), lipopolysaccharide (galE LPS) and the less toxic LPS mutant lpxL1. The immunogenicity in mice was evaluated and compared with that against an outer membrane vesicle (OMV) vaccine. The IgG isotype distribution and bactericidal activity were determined. Furthermore, PorA-specific proliferation of lymph node cells after immunization and re-stimulation in vitro was studied with selected formulations.

Both AlPO₄ and MPL were unable to improve the functional immunogenicity (i.e. bactericidal response) of liposomal PorA. Besides, when these adjuvants were used, the percentage of responders in the groups did not reach 100%. This was also observed with non-adjuvated PorA-liposomes or OMV. Of the adjuvants studied, only galE LPS and lpxL1 LPS were capable of increasing the immunogenicity and avoid non-responsiveness against PorA-liposomes. Importantly, the adjuvant activity of lpxL1 LPS was accompanied by an improved PorA-specific proliferation of lymph node cells and a concomitant increase in IL-2 production. In conclusion and considering its lower toxicity, lpxL1 LPS-adjuvated liposomes are superior to other formulations tested.
Introduction

*Neisseria meningitidis* serogroup B is the predominant cause of meningococcal infection in the Western world [1,2]. Traditional vaccines based on capsular polysaccharide [3] offer no protection against type B meningococci. Vaccines against this serogroup that are being developed by various groups [4,5] consist of outer membrane vesicles (OMV) extracted from (sometimes genetically modified) meningococcal strains, with one or more Porin A (PorA) subtypes embedded in them [6]. These vaccines have already been tested in clinical trials [7,8,9]. We previously demonstrated that purification of PorA from OMV induces a reversible loss of its capability to induce a protective (i.e. bactericidal) immune response. By incorporation of purified PorA into liposomes, the ability to induce bactericidal antibodies was fully recovered: liposomal PorA, in the absence of adjuvants, elicited a bactericidal response similar to that of (LPS-containing) OMV [10]. However, both with OMV and PorA-liposomes, the percentage of responding mice (i.e. with bactericidal activity in serum) in the immunization groups was below 100%. The occurrence of non-responders should be avoided before introduction of new vaccines into human vaccination programs.

Liposomes are very suitable as delivery vehicles for antigen, but they show a relatively low adjuvant activity [26]. The multimeric, particulate structure promotes uptake by antigen-presenting cells (APC) and co-incorporation of adjuvants may further improve the immune response. Bacterial cell wall components, such as LPS, activate APC through receptors on their membrane (e.g. CD14, TLR4) [11]. LPS is also responsible for reactogenicity due to the presence of lipid A [16]. Non-toxic alternatives for LPS with retained adjuvant activity are under development. Among them is monophosphoryl lipid A (MPL), a LPS derivative from *Salmonella minnesota* that has been tested as adjuvant in numerous human trials [12]. The removal of a phosphate and fatty acid group from lipid A (see Fig. 1) resulted in a molecule with decreased toxicity and retained adjuvant properties [13,14]. More recently, an *lpxL* mutant of *N. meningitidis* has been constructed containing penta- instead of hexaacylated lipid A (Fig. 1). The adjuvant activity of this *lpxL1* LPS was similar to that of wild type *N. meningitidis* LPS, but its toxicity was substantially reduced. [16]. *LpxL1* LPS is therefore an interesting candidate for inclusion in meningococcal vaccines.
Liposomal PorA adjuvated with LPS derivatives

Figure 1. Lipid A structure of LPS and derivatives used in this study. A: wild type LPS of Neisseria meningitidis, B: lpxL-mutant of Neisseria meningitidis, C: monophosphoryl lipid A from Salmonella minnesota.

In this study we investigated the possibility to improve the immune response induced by PorA formulated in liposomes. Either MPL, galE LPS or lpxL1 LPS were incorporated in the liposomal bilayer of PorA-containing liposomes, whereas aluminum phosphate (AlPO₄) was mixed with liposomes. The effect of these adjuvants on the PorA-specific humoral immune response was investigated. Furthermore, the in vivo activation of T-cells by selected liposomal formulations was determined after re-stimulation in vitro.
CHAPTER 6

Materials and methods

Materials

All phospholipids used were synthetic. Dimyristoyl phosphatidylcholine (PC) was purchased from Rhône-Poulenc Rorer (Köln, Germany). Dimyristoyl phosphatidylglycerol (PG) was a gift from Lipoïd GmbH (Ludwigshafen, Germany). Cholesterol (Chol) and n-Octyl β-d-Glucospyranoside (OG) were obtained from Sigma (Zwijndrecht, The Netherlands).

Meningococcal H44/76 galE LPS [15] and lpxL1 LPS [16] were isolated from whole cells by the hot phenol extraction method described by Westphal and Jann [17]. Monophosphoryl lipid A from Salmonella minnesota was purchased from Sigma and AlPO₄ was obtained from Superfos Biosector (Veldbaek, Denmark).

OMV preparation and PorA purification

OMV were isolated from Neisseria meningitidis strain F91 (P1.7-2,4, PorB⁺, RmpM⁺, low expression of Opa/Opc) as previously described [10]. The obtained OMV were used for purification of PorA as described [10]. Prior to incorporation of PorA into liposomes, the protein was precipitated with 80% (v/v) ethanol at −20°C and solubilized in 150 mM OG in 50 mM Tris-HCl, 150 mM NaCl pH 7.4 (TBS).

Liposome preparation and characterization

Liposomes were made of PC, PG and Chol in a 8:2:2 mol ratio by detergent dilution as previously described [18]. In short, appropriate amounts of each lipid were dissolved in chloroform/methanol (2/1, v/v) in a round bottom flask and a film was obtained by solvent evaporation in a rotavapor under reduced pressure. The film was solubilized in the PorA solution forming mixed micelles. The initial protein/lipid ratio used was 25 µg/µmol. For liposomes containing adjuvants in the bilayer (LPS, MPL or lpxL) the adjuvant dissolved in 150 mM OG in TBS was added to the mixed micelles in an initial protein/adjuvant ratio of 2 (w/w). Mixed micelles were rapidly diluted 11-fold in TBS, allowing the formation of liposomes. Subsequently, liposomes were pelleted by ultracentrifugation (160,000 × g, 1 h) and resuspended in TBS. Liposomes were filtered through sterile 0.45-µm filters. The particle size of PorA-liposomes was measured by dynamic light scattering (DLS) as previously described.
Liposomal PorA adjuvanted with LPS derivatives

[10]. Protein content was determined according to Peterson [19] with BSA (Pierce, Rockford, IL) as standard. The phospholipid content was determined according to Rouser [20] with sodium phosphate as standard (Merck, Darmstadt, Germany). The amount of LPS-derived adjuvants (MPL, galE LPS, lpxL1 LPS) present in the liposomes was determined by gas chromatographic quantification of fatty acids as described in Chapter 2 of this thesis [21]. The correct folding of PorA into liposomes (i.e. trimerization) was analyzed by SDS-PAGE in ‘native’ gels as previously described [22]. Antigenicity of the P1.4 epitope of PorA P1.7-2,4 in liposomes was tested by an inhibition ELISA, with the monoclonal antibody MN20B9.34 as previously described [10].

Immunization studies

Balb/cOlaHsd mice were obtained from Harlan (Horst, The Netherlands) and maintained under conventional conditions at the Central Animal Laboratory of the Netherlands Vaccine Institute. All experiments were done with 8-12 weeks old animals and were approved by the Animal Ethics Committee of the National Institute for Public Health and the Environment.

Procedure A: mice (8 animals in each group) were immunized subcutaneously (1.5 µg protein, 0.25 ml/mouse) on day 0, 14 and 28. The mice were bled on day 42, and sera were collected and stored at -20ºC until analysis.

Procedure B: mice (6 animals in each group) were immunized subcutaneously (0.25 ml/mouse) on day 0 and day 21 with 1.5 µg liposomal PorA (+/- lpxL1 LPS) or with liposomes (+/- lpxL1 LPS) without PorA. On day 31, inguinal lymph nodes located near the injection site were removed and pooled per two mice (3 samples/group) for T-cell stimulation and cytokine production assays.

Anti-PorA whole cell ELISA

The antibody titer (total IgG and individual isotypes) of each individual mouse serum was determined by whole cell ELISA as described [23]. Neisseria meningitidis isogenic H44/76-based strain (B:15P1.7-2,4:L3,7,9) expressing P1.7-2,4 and the H44/76-derived mutant strain HI5 (lacks PorA) were used. Isotypes were determined with goat anti-mouse (GAM) Ig isotype-specific conjugates, labeled with horseradish peroxidase (HRP) (Southern Technology Associates, Birmingham, AL; dilution 1/5000, except for IgG1 1/2500). A four-parameter curve fit was made for the
optical density at 450 nm values obtained with serial dilutions of the sera, and the antibody titers were calculated as the reciprocal dilutions that gave 50% of the maximum absorbance.

**Serum bactericidal assay**

The serum bactericidal activity was measured as previously described [24] against the *N. meningitidis* strains: H44/76 (B:15P1.7-2.4:L3,7,9) and H15 (PorA-). Sera from individual mice were heat inactivated for 30 min at 56°C prior to use. 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) baby rabbit serum was used as complement source (Pel-Freez Biologicals, Rogers, AR). As positive 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 expressed as the reciprocal serum dilution showing more than 90% killing of the number of bacteria used.

**Cell culture and proliferation assay**

Lymph nodes of mice immunized according to procedure B were maintained in culture medium consisting of Iscove’s modified Dulbecco’s medium with 10% fetal calf serum, 50 µM β-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin and 292 µg/ml glutamine. Pools of lymph nodes (2 mice/pool, 3 pools/group), were forced through a 70-µm filter. Cells were washed and cultured in U-bottom 96-wells plates at $2 \times 10^5$ cells/well in 200 µl medium, 0.5 µg liposomal PorA/ml or control liposomes with equivalent amounts of lipid (based on phosphorous content). ConA (Sigma), 8 ng/well, was used as positive control for proliferation. Cells were cultured at 37°C. On day 3, 50 µl aliquots of the supernatants were taken and stored at -70°C for cytokine determination, and replaced by culture medium (50 µl) containing 10 U/well of recombinant IL-2 (Cetus Corp., Emerville, CA). Cells were further cultured for another 24 hours. On day 4, 0.5 µCi $^3$H-thymidine (Amersham, UK) was added for 18 h, and incorporation of the radiolabel was determined using a liquid scintillation β-counter (LKB, Wallac, Turku, Finland). The stimulation index was calculated as the ratio of the radioactivity (cpm) obtained in the presence of PorA-liposomes to that obtained in the presence of control liposomes (without PorA).
Cytokine production during antigen-induced proliferation

The pattern of cytokine production by lymph node cells after stimulation for 3 days with PorA-liposomes was assessed with a Luminex assay adapted to murine cytokines [25]. The concentration of IL-2 (i.e. a Th1 cytokine) and IL-10 (i.e. a Th2 cytokine) was determined in supernatants of lymph node cultures using calibration curves of the corresponding cytokines.

Statistical methods

Before statistical analysis, antibody and bactericidal titers were log_{10} converted. Antibody and bactericidal 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%. Bactericidal titers were compared with the Turkey-Kramer multiple comparisons test at a confidence interval of 95%.

Results

Preparation and characterization of liposomal PorA formulations

Depending on the formulation, the final protein/lipid ratio (µg/µmol) of the PorA-liposomes varied from 24 to 32. In the formulations containing adjuvants, the protein/adjuvant ratio (w/w) was 2 for MPL-containing liposomes and approximately 1.4 for liposomes containing meningococcal galE LPS and lpxL1 LPS (Table 1). The presence of adjuvants in the liposomal formulations resulted in a slightly larger particle size of the liposomes, ranging from 200 nm for non-adjuvated liposomes to ca. 250 nm for liposomes containing galE LPS (Table 1). The polydispersity ranged from 0.3 to 0.4, indicating a rather broad particle size distribution.

PorA in liposomes (with or without adjuvants in the bilayer) was present as trimers, as observed in ‘native’ SDS-PAGE (not shown). Under denaturing conditions, the trimers were converted to monomers. No aggregates or fragments could be detected. The interaction of PorA with specific antibodies directed against loop 4 of the protein (i.e. antigenicity) was similar for OMV and liposomal PorA (Table 1). These data indicate that the PorA conformation was preserved.
Table 1. Characteristics of PorA-liposomes.

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>protein:l lipid ratio (µg/µmol)</th>
<th>protein:adjuvant ratio (w/w)</th>
<th>Particle size (nm)</th>
<th>PD</th>
<th>Antigenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>32</td>
<td>-</td>
<td>205 ± 7</td>
<td>0.4</td>
<td>0.94 ± 0.12</td>
</tr>
<tr>
<td>MPL</td>
<td>24</td>
<td>2.0</td>
<td>242 ± 4</td>
<td>0.4</td>
<td>1.04 ± 0.11</td>
</tr>
<tr>
<td>galE LPS</td>
<td>32</td>
<td>1.3</td>
<td>248 ± 7</td>
<td>0.3</td>
<td>0.87 ± 0.04</td>
</tr>
<tr>
<td>LpxL1 LPS</td>
<td>29</td>
<td>1.4</td>
<td>219 ± 3</td>
<td>0.4</td>
<td>0.82 ± 0.05</td>
</tr>
</tbody>
</table>

aData in this table are representative for PorA-liposomes used in the experiments described.

bParticle size average ± SD of three measurements.

cPolydispersity: indication of the size distribution of the liposomes; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.

dRatio between the protein concentration determined by ELISA and the protein concentration determined according to Peterson [27]. As a reference, the antigenicity of PorA in OMVs was arbitrarily set at 1. The results are shown as averaged values ± SD of three measurements.

**Humoral immune response to (adjuvated) liposomal PorA formulations**

The humoral immune response of (adjuvated) PorA-liposomes and OMV was tested *in vivo* after subcutaneous immunization of Balb/c mice according to procedure A. All formulations induced PorA-specific mean log10 IgG titers of 3 or higher (Table 2). Significantly higher mean IgG titers were obtained with PorA-liposomes adjuvated with *galE* LPS or *lpxL1* LPS as compared to all other formulations, including OMV. No significant differences were found between the IgG titers induced by OMV, non-adjuvated PorA-liposomes, and liposomes adjuvated with AlPO4 or MPL. The IgG response against other structures than PorA was also investigated: the highest non-PorA specific titers were found in mice immunized with OMV, plain liposomes or PorA-liposomes adjuvated with *lpxL1* LPS (Table 2).

Determination of the subclass distribution of the anti-PorA specific antibodies confirmed that PorA-liposomes in the absence of adjuvants induced overall lower titers as compared to other formulations. PorA-liposomes adjuvated with *galE* LPS induced the highest IgG2a titers and these titers were, together with PorA-liposomes adjuvated with *lpxL1* LPS, significantly higher than those induced by all other formulations. However, the IgG2a to IgG1 ratio was only increased for PorA-liposomes adjuvated with *galE* LPS and not for those adjuvated with *lpxL1* LPS (Table 2). This indicates that *galE* LPS induces a Th1-type response.
Table 2. Total IgG and subclass distribution in sera of mice immunized with PorA-liposomes and OMVs.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adjuvant</th>
<th>Anti-PorA titer</th>
<th>IgG2a/IgG1 ratio</th>
<th>Non-specific IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgG1</td>
<td>IgG2a</td>
</tr>
<tr>
<td>OMVs</td>
<td>galE LPS</td>
<td>3.20</td>
<td>3.32</td>
<td>3.46</td>
</tr>
<tr>
<td>liposomes</td>
<td>-</td>
<td>3.02</td>
<td>3.01</td>
<td>2.98</td>
</tr>
<tr>
<td>liposomes</td>
<td>AlPO4</td>
<td>3.21</td>
<td>3.13</td>
<td>3.34</td>
</tr>
<tr>
<td>liposomes</td>
<td>MPL</td>
<td>3.15</td>
<td>2.95</td>
<td>3.26</td>
</tr>
<tr>
<td>liposomes</td>
<td>galE</td>
<td>3.94*</td>
<td>2.96</td>
<td>4.18*</td>
</tr>
<tr>
<td>liposomes</td>
<td>LpxL1</td>
<td>3.74*</td>
<td>3.59*</td>
<td>3.76*</td>
</tr>
</tbody>
</table>

a The titer of each anti-PorA 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%: LSD0.05 (IgG)=0.29; LSD0.05 (IgG1)=0.22; LSD0.05 (IgG2a)=0.25; LSD0.05 (IgG2b)=0.27; LSD0.05 (IgG3)=0.31.
b Represented as the averaged IgG2a/IgG1 ratio of individual mice ± SD (8 mice/group).
c Antibodies against other structures that PorA were determined by whole cell ELISA in plates coated with a N. meningitidis strain lacking PorA and their level is expressed as the mean log10 titer. The means were compared by the LSD test with a confidence level of 95%: LSD0.05 (IgG)=1.11.
d OMVs contained 9.4% galE LPS relative to the total amount of protein present in the formulation.
* significantly higher (groups compared inside the same IgG subclass).

Figure 2. Serum bactericidal activity of mice immunized with OMV and (adjuvated) PorA-liposomes. Bars indicate the average bactericidal activity of sera of responder ± SEM. White bars: groups with non-responders; the number of responders is indicated above the bars. Black bars: groups with 100% responders. **: Bactericidal titers are significantly higher than those of all other groups (p ≤ 0.005).

The results of the complement-dependent bactericidal assay are summarized in Fig. 2. PorA-liposomes adjuvated with galE LPS or lpxL1 LPS gave rise to serum bactericidal antibodies in all mice. Moreover, they induced a significantly higher bactericidal immune response as compared to all other formulations (Fig. 2). No differences in the bactericidal titers were found between OMV, PorA-liposomes without adjuvants, or adjuvated with AlPO4 or MPL. However, the number of responders in three of these groups was not 100% and varied: only PorA-liposomes adjuvated with MPL induced bactericidal antibodies in all mice. The bactericidal
antibodies induced by all six formulations were PorA-specific, as these sera were not able to kill bacteria of *N. meningitidis* strain H1.5, lacking PorA (not shown).

**Cellular immune response to lpxL1-adjuvated liposomal PorA**

T-cells are important regulators of B cell responses. To investigate whether the strong adjuvating effect of *lpxL1* LPS was related to an enhanced cellular immune response to liposomal PorA, we compared the PorA-specific proliferation of cells isolated from lymph nodes of mice immunized according to procedure B using non-adjuvated PorA-liposomes, PorA-liposomes adjuvated with *lpxL1* LPS and the corresponding control liposomes.

![Figure 3](image)

**Figure 3.** Proliferation and cytokine production of cells isolated from lymph nodes of mice immunized with non-adjuvated PorA-liposomes (Group 1) or PorA-liposomes adjuvated with *lpxL* (Group 2) and re-stimulated with the corresponding formulations. Panel A: Proliferation of cells. The bars indicate the stimulation index (SI) of individual pools in one group. The SI values are relative to the proliferation of cells of the same pool in the presence of liposomal formulations without PorA or adjuvants. See materials and methods for details. A stimulation index above 2 is considered significant. Panel B: Cytokine production (IL-2 and IL-10) by cells isolated from lymph nodes on day 3. White bars: IL-10; black bars: IL-2. Data are presented as averaged values ± SEM (n=3).*: p < 0.05

PorA-specific proliferation was found in two of the three pools of lymph node cells from mice immunized with plain PorA-liposomes adjuvated with *lpxL1* LPS. In contrast to this, none of the pools from animals immunized with plain PorA-liposomes (Fig. 3, panel A) or with *lpxL1* LPS- or non-adjuvated liposomes without PorA (data not shown) showed PorA-specific proliferation. In parallel to these findings, cells isolated from lymph nodes of mice immunized with *lpxL1* LPS-adjuvated PorA-liposomes produced significantly higher amounts of IL-2 but not IL-10, if specifically re-stimulated with PorA-liposomes, compared to those of mice immunized with non-adjuvated PorA-liposomes (Fig. 3, panel B). Altogether, these data indicate
that the use of \textit{lpxL1} LPS as adjuvant with PorA-liposomes results in an improved T-cell response.

**Discussion**

Adjuvants are important modulators of vaccine responses and play an important role in the selection or increase of the immune response. In the present study we investigated the benefits of liposomes as carrier and LPS derivatives as additional adjuvant to improve the immune response against a meningococcal PorA. OMV were used as vehicle alternative and AlPO$_4$ as an adjuvant alternative. Adjuvants can improve the immune response by: a) causing depot formation at the injection site; b) increasing cellular infiltration at the injection site, particularly of antigen presenting cells (APC); and c) improving antigen presentation to T-cells [26].

AlPO$_4$ is one of the most commonly used adjuvants for human vaccines [26] and is also used with PorA-based meningococcal vaccines [7]. Aluminum salts induce Th2 responses, characterized in mice by induction of IgG1 and secretion of IL-4, IL-5, IL-6 and IL-10 by cells of the immune system [27,28]. In vaccines against \textit{N. meningitidis}, however, a more Th1-directed response is preferred, as it results in B cell isotype switching to IgG2a production [29,30]. Murine IgG of the isotypes 2a and 2b are able to induce complement-mediated killing of bacteria [24], which is considered one of the correlates of protection against \textit{N. meningitidis} in humans [31,32]. Our results show that the total IgG titers of sera of mice immunized with PorA-liposomes adjuvated with AlPO$_4$ were not significantly increased compared to other groups (Table 2), nor were the IgG1 titers, in contrast with what one would be expect for a Th2 type adjuvant. In agreement with these results, the bactericidal activity of these sera was not improved when compared with non-adjuvated PorA-liposomes or OMV. Thus, the use of AlPO$_4$, although generally used in PorA-vaccines [5,7,8] does not seem to have any beneficial effect.

Significant titers against other structures than PorA were only observed for OMV, non-adjuvated liposomes or liposomes adjuvated with \textit{lpxL1} LPS. However, the PorA-unspecific IgG titers were not bactericidal. The specificity of these IgG remains unknown. The PorA-unspecific response is especially surprising for non-adjuvated PorA-liposomes. In the case of OMV or \textit{lpxL1} LPS-adjuvated...
liposomes, the PorA-unspecific IgG could be raised against galE LPS or lpxL1 LPS, but this was not investigated.

LPS-derived adjuvants are known to direct the immune response towards Th1 rather than Th2 [27]. Our results show that PorA-liposomes adjuvated with galE LPS or lpxL1 LPS induced significantly higher amounts of IgG2a than other formulations (Table 2). The IgG2a to IgG1 ratio, however, was only increased with galE LPS and not with lpxL1 LPS. This is in agreement with results of immunization of mice with outer membrane complexes (OMC) of the N. meningitidis mutant lpxL1 [33]. Despite the fact that only PorA-liposomes adjuvated with galE LPS showed a clear shift in the IgG2a-to-IgG1 ratio (i.e. a more Th1 type response), both galE LPS- and lpxL1 LPS-adjuvated liposomes induced higher PorA-specific bactericidal titers when compared to the other formulations, with 100% responders in the groups (Fig. 2). This indicates that IgG2a titers as such result in increased bactericidal response, irrespective of a concomitant increase in IgG1 levels. In a previous study it has been shown that in sera of mice immunized with OMC isolated from a Neisseria meningitidis mutant without LPS, the IgG2a to IgG1 ratio was much lower than the one obtained with OMC isolated from wild-type meningococci [33]. However, in our study the IgG2a-to-IgG1 ratio obtained in sera from mice immunized with non-adjuvated liposomes did not decrease when compared to other formulations such as OMV. This might be due to the presence of LPS traces in the purified PorA used for preparation of the liposomes [10]. However, the presence of LPS traces in non-adjuvated liposomes did not result in adjuvant activity. The OMV used in our study also contained galE LPS. However, the adjuvant-to-PorA ratio in OMV was much lower than in adjuvated liposomes, which may be one of the reasons why the percentage of responding mice was below 100% (Fig. 2).

The incorporation of MPL to liposomal PorA failed to increase the serum bactericidal activity of mice sera, although non-responsiveness of mice was absent. Moreover, the isotype distribution of MPL-adjuvated liposomes was comparable to that of PorA-liposomes adjuvated with AlPO₄ or OMV (Table 2). It has been previously reported that MPL did not improve the bactericidal response of a recombinant liposomal PorA formulation [34]. In another study, higher doses of MPL were needed to stimulate dendritic cells (DC), as compared to LPS. Once stimulated, DC pulsed with MPL induced a mixture of Th1 and Th2 differentiation [35]. The dose of MPL used in our formulations was relatively low. Furthermore, PorA-liposomes
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adjuvated with MPL probably induced a mixed Th1/Th2 response (at the IgG level) that did not result in increased serum bactericidal activity in mice.

In our study the best adjuvant activity for PorA-liposomes was obtained with galE LPS and lpxL1 LPS. Since lpxL1 LPS has been shown to have a 100-fold reduced toxicity as compared to LPS [16], we investigated whether the strong adjuvant activity was related to an improvement in the cellular immune response. Indeed, PorA-specific proliferation of cells in the lymph nodes was only induced by PorA-liposomes adjuvated with lpxL1 LPS, and not by non-adjuvated PorA-liposomes. Proliferation of cells from mice immunized with PorA-liposomes adjuvated with lpxL1 LPS was accompanied by increased production of IL-2 by cultured cells, whereas the production of IL-10 did not increase (Fig. 3B). This is an indication of an enhanced Th1-type response. Further studies determining the production of other cytokines (e.g. INF-γ, TNF-α, IL-12), by proliferating cells are needed to confirm the Th1 type response induced by lpxL.

In conclusion, our results indicate that both galE and the less toxic lpxL1 mutant LPS can be used as a highly effective adjuvant in well-defined PorA-liposomes. Both MPL and AlPO₄ were unable to improve the immunogenicity of PorA-liposomes. Although galE LPS was equally effective as lpxL1 LPS, its toxicity makes it less suitable for use in vaccines. The increased induction of bactericidal serum activity by lpxL1 LPS-adjuvated liposomes was accompanied by an improved stimulation of cellular immune responses. Furthermore, the use of lpxL1 LPS overcomes the problem of non-responsive subpopulations after vaccination against Neisseria meningitidis.

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References


