Towards an improved *Neisseria meningitidis* B vaccine: vesicular PorA formulations

Naar een verbeterde *Neisseria meningitidis* B vaccin: vesiculair PorA formuleringen

(met een samenvatting in het Nederlands)

Proefschrijft

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Carmen Arigita Maza

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Promotores Prof. dr. D.J.A. Crommelin
Prof. dr. ir. W.E. Hennink

Co-promotores Dr. W. Jiskoot
Dr. G.F.A. Kersten

Prof. dr. D.J.A. Crommelin, prof. dr. ir. W.E. Hennink en dr. W. Jiskoot zijn verbonden aan de Faculteit Farmaceutische Wetenschappen van de Universiteit Utrecht
Dr. G.F.A. Kersten is verbonden aan het Nationale Vaccin Instituut (vroeger Rijksinstituut voor de Volksgezondheid en Milieu) te Bilthoven

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Es mejor saber después de haber pensado
y discutido, que aceptar los saberes que
nadie discute para no tener que pensar.

Fernando Savater

Review committee:

Prof. dr. M.J. Alonso, Universidad de Santiago de Compostela, Spain
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Prof. dr. G. Storm, Utrecht University
Dr. L. van Alphen, Nederlands Vaccin Instituut

Carmen Arigita Maza
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CHAPTER 1

General introduction

Neisseria meningitidis and meningococcal disease

Neisseria meningitidis is one of the major causes of bacterial meningitis and sepsis worldwide [1]. *N. meningitidis* isolates can be divided into thirteen serogroups based on the structure of the capsular polysaccharide [2]. Five serogroups (A, B, C, Y and W135) virtually account for all disease-causing isolates [3,4]. In Europe, approximately two thirds (63%) of the reported cases of meningococcal disease are attributable to serogroup B strains, and most of the remainder to serogroup C strains (30%), with very low incidence of the other serogroups (data 1999-2000) [5]. In the Netherlands the incidence of serogroup B is even higher, accounting for almost 77% of the cases, while serogroup C is found in 19% of all isolates (data 2000) [6]. In the USA the serogroup distribution differs, with 32% of the cases caused by serogroup B, 35% by serogroup C and 26% by serogroup Y (data 1996) [7]. Serogroup A isolates are predominantly found in sub-Saharan African countries (so-called meningitis belt countries) and cause frequent epidemics. Disease caused by serogroup W135 has been associated with the Haji pilgrimage in Saudi Arabia [3].

In non-epidemic situations, the nasopharynx of 10-25% of the general population is colonized by meningococci [8,9,10]. Increased carrier rates have been observed during epidemics and crowding [11]. Meningococcal carriage is a natural immunizing process resulting in the induction of systemic protective antibody responses [12]. Despite the high rate of colonization, meningococcal disease is quite

rare [13], and the mechanism by which bacteria invade the mucosal barrier and get access to the blood stream remains unknown. The highest incidence of meningococcal disease occurs in young children under the age of four [5]. An incidence peak is also observed among teenagers, probably due to an increased exposure to environmental risk factors [14,15].

Meningococcal disease is characterized by a variety of clinical symptoms [16,17] and the early phase resembles the disease caused by ordinary influenza. The mild symptoms can change rapidly (within hours) in a life-threatening disease displaying two clinical pictures, i.e. sepsis and/or meningitis. In approximately 10% of the cases of meningitis and 20% of sepsis, the disease is fatal despite antibiotic treatment [18]. Without antibiotics, death rates increase up to 80% [19]. Of the surviving patients, 5-20% experiences permanent damage, such as amputation of necrotic extremities, mental retardation or deafness [1,19]. The rapid onset of invasive meningococcal disease, the high incidence in childhood, the severity of the sequellae and the high mortality rate clearly indicate the importance of vaccine development against *Neisseria meningitidis.*

Surface structures of meningococci and vaccine candidates

Meningococci display a double-membrane structure typical of gram-negative bacteria (Fig. 1), with a peptidoglycan layer embedded between the cytoplasmic and the outer membrane [20]. A polysaccharide capsule covers the outer membrane of meningococci. The different polysaccharide structures are used to classify meningococci in thirteen serogroups (see above) [2]. The polysaccharide capsule is a virulence factor for meningococci and, although capsular-deficient strains are commonly isolated, these are non-infectious [10]. Vaccines based on polysaccharides against meningococci of groups A, C, Y and W135 are being developed or are already licensed and available worldwide [21,22,23,24,25]. Efforts to use the capsular polysaccharide of group B meningococci have been hampered by its poor immunogenicity, even when conjugated with a carrier protein [26,27]. This poor immunogenicity is probably due to immunological tolerance induced by cross-reactive polysialylated glycoproteins that are especially abundant in fetal neural tissue [28,29]. A list of antigens located in the outer membrane that have been investigated as vaccine candidates against type B meningococci is shown in Table 1.

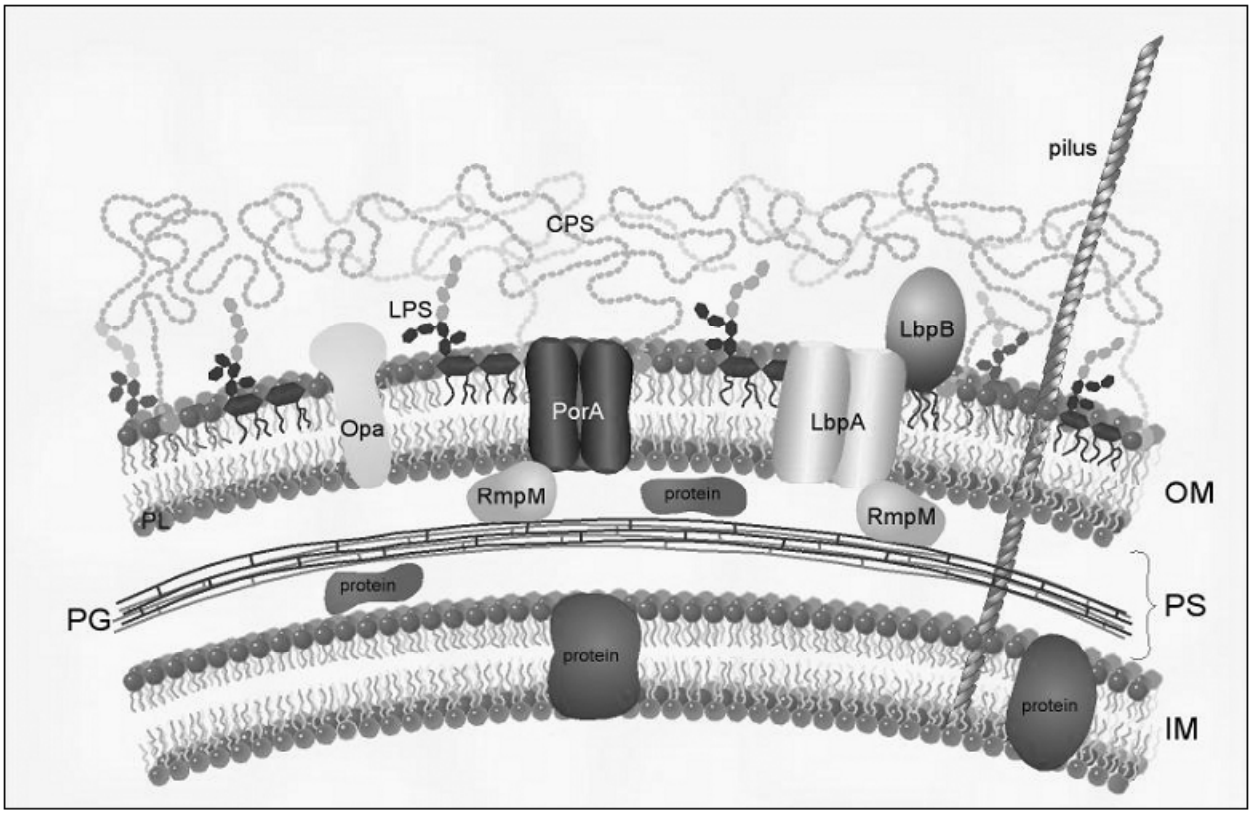

Figure 1. Surface structures of *Neisseria meningitidis.* The cell envelope is composed of an outer membrane (OM) and an inner membrane (IM), separated by a periplasmic space (PS) containing a peptidoglycan layer (PG) and proteins. The OM is an asymmetric bilayer with lipopolysaccharide (LPS) and phospholipids (PL) in the outer leaflet and PL in the inner leaflet. In the OM, LbpA and LbpB represent the integral outer membrane protein (OMP) and the cell-surface exposed protein respectively, wich together constitute the lactoferrin receptor. CPS: capsular polysaccharide; Opa: Opacity protein (class 5 OMP); PorA: porin protein A (class 1 OMP); RmpM: reduction modifiable protein M (class 4 OMP). The IM is a symmetric bilayer of PL with proteins [30].

Table 1. Major meningococcal outer membrane components.

Name	**Characteristics**	***Vaccine potential***	***Ref***
Type IV pili	Adhesion to epithelium	Low, high variability	[20,33]
LPS	Endotoxin	Only in detoxified form	[40]
PorA (class 1)	Porin	Induces protective Ab	[49,50]
PorB (class 2/3)	Porin	No protective response	[56]
RmpM (class 4)	Reduction modifiable protein	No protective response	[64]
Opa/Opc (class 5)	Opacity proteins, Involved in adhesion/invasion	Induces protective Ab	[70]
LbpA/LbpB	Lactoferrin receptor, Iron-regulated	Induces protective Ab	[71]
NspA	Neisseria surface protein A	Induces protective Ab	[74]
Various	Proteins obtained by reverse vaccinology	Induction of protective Ab	[79]

Fimbriae or type IV pili are filamentous protein projections from the meningococcal cell surface [31,32]. Type IV pili consist of at least two distinct proteins, the highly variable major subunit PilE forming the pilus fiber and the tip-associated adhesin PilC that contributes to the initial attachment of bacteria to

epithelial, or endothelial host cells and erythrocytes [33,34]. Type IV pili show a high degree of antigenic variability [20] which makes them unsuitable as vaccine candidates.

The outer membrane is composed of phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS). The outer membrane of *N. meningitidis* is typical of gram-negative bacteria and has an asymmetrical organization, with the outer leaflet primarily composed of LPS and proteins and the inner leaflet composed of phospholipids [35]. The major phospholipids of *N. meningitidis* are phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) with variable fatty acyl substituents, including C16:1, C16:0, C18:1, C14:0, C14:1 and C12:0 [36].

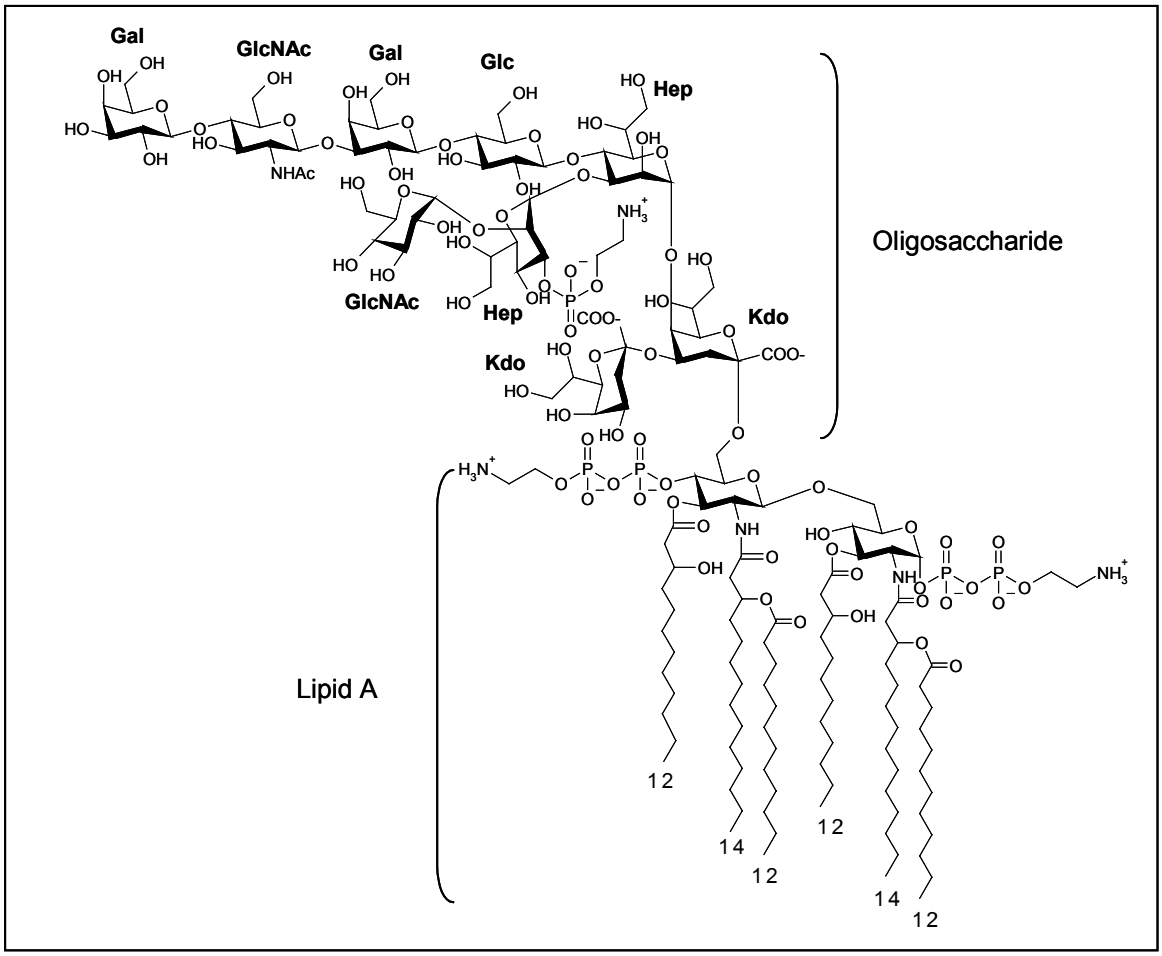

Figure 2. Schematic representation of *N. meningitidis* immunotype L3 LPS [37]. The lipid A moiety and the oligosaccharide core are indicated. Kdo: 2-keto-3-deoxyoctulonosic acid; Hep: heptose; GlcNac: N-acetyl-glucosamine; Gal: galactose; Glc: glucose.

Neisseria LPS (Fig. 2) is composed of a hydrophobic lipid A part, which anchors it in the outer membrane. Lipid A is also named endotoxin and is responsible for toxic effects, like septic shock [38]. The head group consists of a core region (oligosaccharide), and lacks the O-antigen, a polymer of repeating units, each of

them containing three to six sugar residues. Because of the absence of the O-antigen, *N. meningitidis* LPS is also referred to as lipooligosaccharide. The core region of meningococcal LPS is variable and is the basis of the classification of meningococci in twelve different immunotypes [39]. It has been shown that vaccines based on detoxified LPS or LPS-toxoid conjugates are able to induce immunotype-specific immune responses [40]. Also, conserved inner core epitopes of LPS are able to elicit protective immune responses in animals and may have potential as a cross-reactive vaccine component [41].

Bacterial OMPs are used for nutrient acquisition and protein secretion, and can play a role in the adhesion and invasion of host tissues or have an enzymatic function. The major OMPs of *N. meningitidis* have been divided into five classes on the basis of molecular weight [42].Class 1 OMP, also called PorA (44-47 kDa) and the mutually exclusive class 2 and 3, also called PorB (37-42 kDa) are porins. PorA forms cation-selective pores in the OM, whereas PorB forms anion-selective pores [43,44]. For both porins, topology models have been described [45,46], based on sequence comparisons and on the structure of PhoE, an outer membrane porin from *Escherichia coli* [47]. According to these models, each porin monomer consists of a 16-stranded anti-parallel β-barrel enclosing a transmembrane pore (Fig. 3).

The β-strands are amphipatic with the hydrophobic amino acid residues exposed to the membrane and the hydrophilic amino acid residues exposed to the interior of the channel. Short turns connect the strands at the periplasmic side and eight large loops are exposed to the external medium. In the OM, porins are assembled forming trimers. The surface-exposed loops of PorA and PorB show high sequence variability, and form the basis for sero-subtyping and subtyping of meningococci, respectively [48]. PorA is still recognized as an immunodominant antigen of *N. meningitidis* and induces strong bactericidal responses [49,50], with antibodies mainly directed against the hypervariable regions VR1 and VR2 located in loop 1 and 4, respectively [51,45]. Although it has been demonstrated that PorA undergoes high antigenic and phase variation [52], it is the basis of outer membrane vesicle (OMV) vaccines developed in various groups [49,53,54,55]. In contrast to PorA, PorB is not able to induce a protective bactericidal response [56], although it has been successfully used as carrier protein for polysaccharide conjugates [57].

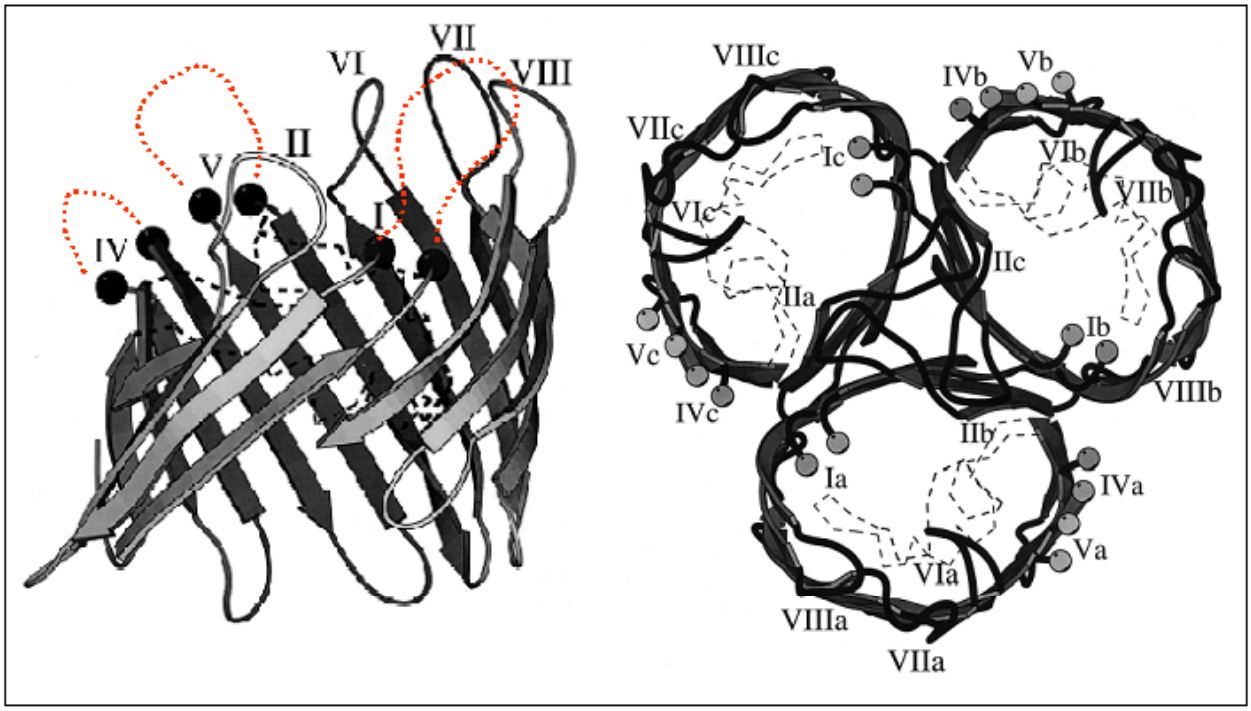

Figure 3. Structure of PorA outer membrane protein of *Neisseria meningitidis.* Left: molecular surface of a porin monomer from the PorA model. The positions of the external loop regions are indicated as I, II, and so forth, with the exception of loop III which lies inside the protein (broken line). The longer loops (I, IV and V) are not based on computer modeling and are therefore drawn as broken lines. Right: model of PorA trimer, viewed from above. The positions of the external loop regions are marked as in the monomer (left), with the subunits designated a, b, c. Adapted from [44].

The vaccine developed at the Netherlands Vaccine Institute (former part of the National Institute for Public Health and the Environment, RIVM) consists of three OMV preparations, each containing three different PorA serosubtypes (trivalent OMV) representing the majority of circulating serosubtypes in the Netherlands and other countries in Europe [58]. This vaccine has been tested in phase I and II trials and has been proven to be safe and immunogenic [50,55]. Since PorA serosubtype P1.7-2,4 is the cause of a current epidemic in New Zealand and is the most prevalent serosubtype in the Netherlands, a monovalent vaccine strain with double expression of this PorA was also constructed at the RIVM. This vaccine appeared to be safe and immunogenic in toddlers [59].

Class 4 OMP (33-34 kDa) or RmpM (**R**eduction-**m**odifiable **p**rotein **M**) is highly conserved, shows minor sequence variation among strains and shares sequence homology with the *N. gonorrhoeae* PIII protein and *E. coli* OmpA [60]. RmpM is firmly associated to porins [61] and to iron-limitation inducible proteins [62], and it is not accessible on the cell surface [63]. Antibodies raised against purified RmpM are not bactericidal and they showed to block the bactericidal activity of antibodies directed against PorA [64]. Although these results have been questioned [63], RmpM will

probably never be a vaccine candidate. On the other hand, its presence in PorA-based vaccines may improve the stability of PorA trimers [61].

Class 5 OMP, also called opacity proteins (26-32 kDa) includes two groups: Opa and Opc proteins. *Neisseria meningitidis* can carry up to four opa genes, which can be independently switched on or off [20], whereas only a subset of *N. meningitidis* expresses the Opc protein [65]. Opa proteins are predicted to form 8-stranded β-barrels in the outer membrane, with four surface-exposed loops [66], while Opc spans the membrane 10 times, forming five surface-exposed loops [67]. Both Opa and Opc play an important role in the adhesion to and invasion of host cells, but only in the absence of a polysaccharide capsule [68,69]. Although Opa and Opc proteins have been shown to elicit a strong bactericidal response [70], their phase and antigenic variabilities are a drawback for their use in meningococcal vaccines.

In addition to the major OMP, *N. meningitidis* expresses numerous other OMPs, in some cases under special growth conditions. For example, growth in an iron-limiting environment results in the expression of receptors for heme (iron-porphyrin complex) and for the host iron-binding proteins lactoferrin, transferrin, hemoglobin and haptoglobin-hemoglobin [71]. The bactericidal activity of antibodies elicited against these proteins is comparable with those against class 1 OMPs. Therefore, these iron-regulated proteins have attracted considerable attention as vaccine candidates [72].

Neisserial surface protein A (NspA) is a recently discovered, highly conserved membrane protein that is present in small amounts in the outer membrane. NspA elicits serum bactericidal antibodies that are protective in animal models of meningococcal group B bacteremia [73,74]. However, there are large differences in the accessibility of NspA on the surface of different encapsulated meningococcal strains [75,76]. Moreover, mouse polyclonal and monoclonal antibodies that have been raised against recombinant NspA are bactericidal against only about 50% of group B meningococcal strains [75]. In patients recovering from meningococcal disease NspA is a weak immunogen [77].

A novel approach in the development of vaccines against meningococci is called reverse vaccinology. This approach consists of the analysis of sequences of meningococcal unassembled DNA fragments with computer algorithms to identify open reading frames that potentially encode surface-exposed proteins. Proteins that

are thought to be surface-exposed and conserved are then cloned, expressed in *E. coli*, and tested in mice. This resulted in the finding of 28 newly discovered proteins, some of which elicited antibodies that bound to the bacterial surface or had bactericidal activity against group B meningococci [78,79,80]. However, the bactericidal titers induced by these proteins were lower as compared to those elicited by an OMV vaccine, and probably resulted from cross-reactivity with some PorA serosubtypes [81].

Antigen presentation and the immune response

A natural infection will mostly cause a specific response of the immune system. This response clears the pathogen and also causes immunity towards it. The specific immune response consists of two components: a humoral (antibody-mediated) and a cell-mediated component. The key cells in the specific immune response are antigen presenting cells (APC) and lymphocytes. For a successful vaccination the vaccine antigens have to be presented by APC that activate the effector cells of the immune system: naïve T-cells and B-cells. B-cells are responsible for antibody production and T-cells are responsible for cell-mediated reactions, like the induction of cytotoxic T-cells. Vaccination aims to stimulate a specific immune response and induce a long lasting immunological memory to protect against subsequent disease.

Induction of an immune response against protein antigens requires the partial intracellular degradation of the antigen in APC [82]. Antigens can follow two degradation pathways in APC. Endogenous antigenic peptide fragments generated in the cytoplasm of cells (e.g. after virus infection) by the proteasome are presented by the major histocompatibility complex (MHC) class I molecules and stimulate cytotoxic T lymphocytes (CTL). Specific class I restricted $CD8^+$ T-cells can recognize APC presenting MHC class I-peptide complexes on the surface and lead to lysis of the target cell. Peptide fragments of exogenous protein acquired from outside the cell are presented by MHC class II molecules and stimulate $CD4^+$ T-helper cells, that will activate B-cells to become antibody-secreting plasma cells. However, this traditional mechanism is now being reviewed due to evidence of cross-talk between these two

pathways, which allows certain types of exogenous antigens to be presented by MHC class I molecules [83,84].

There are different, specialized APC, like macrophages, dendritic cells (DC), or even B-cells. Of these cell types, only DC are efficient stimulators of primary immune responses and subsequent establishment of immunological memory [85]. Dendritic cells (Fig. 4) take up antigens in peripheral tissues, process them into peptides and load these peptides onto MHC class I and II molecules. Dendritic cells then migrate to secondary lymphoid organs and become competent (i.e. mature) to present antigens to naïve T-cells, thus initiating antigen-specific immune responses [88]. One can then hypothesize that one of the ways to improve the immune response induced by certain antigens (e.g. PorA) might be achieved by their targeting to DC.

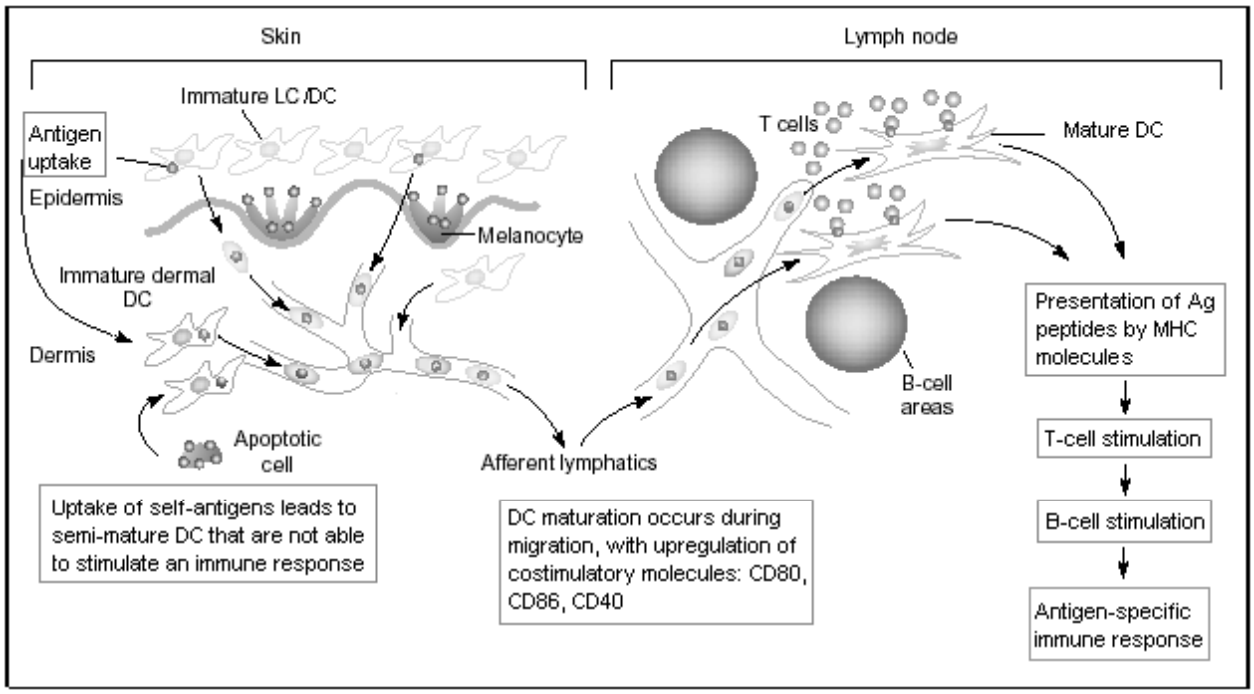

Figure 4. Maturation and migration of DC upon uptake of exogenous antigens. In the periphery, immature DC have a high phagocytic activity. Upon antigen uptake, DC migrate towards lymph nodes, and they 'mature', with up-regulation of co-stimulatory molecules such as CD80, CD86, CD40. Efficient maturation results in the production of cytokines (e.g. IL-12) by DC. The exogenous antigen is hydrolyzed in endocytic compartments, and the formed immunogenic peptides are presented in MHC class II complex to T-cells. Interaction of MHC class II-peptide with the T-cell receptor (TCR) leads to T-cell stimulation, which will stimulate B-cells and results in an antigen-specific immune response. The uptake of self-antigens leads DC to a semi-mature state that is unable to initiate an immune response. Adapted from [86].

At least two signals are required for stimulation of naïve T-cells by APC. The first signal is the presentation of antigenic peptide fragments on the surface of APC in the context of the MHC class I or class II for the recognition of the T-cell receptor

(TCR) on T-cells. The second signal is given by co-stimulatory molecules on the APC surface that are recognized by receptors on the T-cell surface [87,88]. Non-professional APC lack co-stimulatory signaling and can therefore not stimulate naïve T-cells to become effector T-cells. It is believed that in the absence of appropriate co-stimulatory signals, TCR recognition of peptides presented by MHC leads to anergy, which would constitute a mechanism for tolerization to self-antigens [89].

Two major subsets of $CD4^+$ T-cells have been described in mice and humans: T-helper 1 (Th1) and Th2 and can be characterized based on the secretion of different cytokine patterns [90,91]. Th1 responses are characterized by the induction of delayed-type hypersensitivity responses and the secretion of interferon-γ (INF-γ), interleukin-2 (IL-2) and IL-12. Th2 responses are characterized by the induction of circulating or secretory antibodies and the secretion of IL-4, IL-5, IL-6 and IL-10. The different cytokine secretion patterns are mutually antagonistic, and upregulation of one type of response normally results in downregulation of the alternative. In mice, the production of IgG2a antibody isotype is characteristic of a Th1 response, whereas a Th2 response is associated with the induction of IgG1 [92,93].

The activation of B cells is based on the recognition of native antigens through their B-cell receptor (BCR). However, the recognition event by itself does not induce full cell activation. Therefore it needs the interaction of primed T-cells with antigen-specific B-cells plus additional co-stimulatory signals provided by the T-cell (e.g. CD40/CD40L interaction). This interaction induces B-cell differentiation, germinal center development, isotype switching and memory B-cell development [94].

Protective immunity against meningococcal disease mainly relies on antibody-mediated effector functions as serum bactericidal activity and opsonophagocytosis [95]. A Th2 type immune response should be advantageous, as it results in the production of circulating or secretory antibodies. However, complement-activating antibodies are necessary in immune responses against *Neisseria meningitidis*, as they induce killing of bacteria, considered one of the correlates of protection against *N. meningitidis* [96,97]. Murine IgG of the isotypes 2a and 2b induce complement-mediated killing of meningococci. The production of IgG2a is associated with a Th1 type response [93]. Probably a combination of Th2 responses, with induction of high antibody titers and Th1 responses, with the

preferred production of IgG2a, is the preferred immune response for vaccines against *Neisseria meningitidis*. T-cells also play an important role in the regulation of the immune response, including stimulation of B-cells for antibody production. Moreover, T-cells are necessary for the establishment of immunological memory and to promote activation of phagocytic cells, thereby facilitating the uptake and destruction of meningococci. Both the measurement of antibodies in sera and T-cell stimulation assays are indirect ways to measure protection against meningococcal disease. An important barrier in the development of vaccines against type B meningococci is the lack of a good animal model for human meningococcal disease and active immunization. Without this model, the correlates of protection remain indirect measures of the vaccine potential

Antigen delivery systems: OMV, liposomes and microspheres

Antigen delivery systems are necessary for optimal presentation of many subunit vaccines (e.g. proteins). They have the advantage of being naturally targeted for uptake to APC to facilitate the induction of potent immune responses. Several particulate systems have been studied for the delivery of subunits antigens, e.g. liposomes, ISCOMs and microparticles [98,134]. These delivery systems have comparable dimensions to pathogens and allow the inclusion of immunostimulatory adjuvants.

For antigens such as PorA, localized in the outer membrane of *N. meningitidis*, the most straightforward delivery systems consist of outer membrane vesicles extracted from bacteria. Meningococcal cells contain large amounts of loosely bound outer membranes, and during normal growth meningococci release large amounts of these membranes in the form of vesicles (OMV) [99]. OMV are a natural environment for meningococcal outer membrane proteins. However, the composition of the OMV is poorly defined and is difficult to vary in a controlled way, as it is governed by the strain from which OMV are purified and the growth conditions.

Liposomes

Liposomes are artificial vesicles, with particle sizes ranging between 30 nm to several microns, consisting of one (unilamellar vesicle) or more (multilamellar vesicle)

concentric bilayers enclosing one or more aqueous compartments. Liposomes can either incorporate hydrophilic drugs in the aqueous compartment(s), hydrophobic drugs in the lipid bilayer(s) or amphipatic compound that partition between both phases (Fig. 5). The composition of the lipid bilayer consists of (natural or synthetic) phospholipids and cholesterol, and can be manipulated to influence the physicochemical characteristics of the liposomes (e.g. bilayer rigidity, surface charge, pH sensitivity) [100,101]. Liposomes have been used as membrane models, e.g. to study membrane proteins [102], to study the immune system [103,104] and as drug delivery systems [105,106,107]. A number of parenterally administered liposomal products were approved by the FDA for use in humans [108]. The idea to use liposomes as antigen presentation forms was already tested in the early 1970's [109]. Liposomes allow the study of the immunogenicity of antigens in a well-defined presentation form, as well as the investigation of particle characteristics on the antigen immunogenicity. Other advantages of the use of liposomes as antigen formulations are their low intrinsic immunogenicity [110], the fact that they can easily be prepared with varying composition [111], their ability to function as multivalent presentation forms for subunit antigens [112] and allow the co-encapsulation of other adjuvants [113]. Also, targeting moieties can be attached to the outside of the liposomal bilayer, allowing specific uptake by cell types involved in the immune response [112,114]. Furthermore, the insertion of virus fusion proteins into a lipid bilayer creates fusogenic liposomes, also known as virosomes [115]. Virosomes are used to encapsulate protein antigens for delivery to the cytosol and/or into the endosomal route of APC. Therefore, virosomes are able to load antigens both in MHC class I and II molecules, resulting in both cytotoxic and helper responses [116]. There are numerous literature reports on the use of liposomes as antigen presentation forms in vaccines against viral, bacterial and parasitic infections or against tumors [110,117,118] as well as in genetic vaccines [119]. The immune response generated by liposomal vaccines can be further enhanced by incorporation of adjuvants in the liposomes [120,121], or by incorporation of antigen-carrying liposomes in microspheres [122].

For purified antigens that are vaccine candidates against type B meningococci (e.g. PorA) liposomes are potentially suitable presentation forms, as the bilayer structure mimics their native outer membrane environment to a certain extent. It has been demonstrated that meningococcal outer membrane antigens, when

administered in a purified form, are not able to induce a protective immune response [123,124]. The humoral reaction against liposome-associated antigens from *Neisseria* species has been studied in detail [125,126,127,128]. However, it is difficult to determine the formulation parameters that result in an improved immune response for *Neisseria* antigens, as variables such as the composition of the bilayer, the type of antigen, or the animal models used for testing of liposomal vaccines are far from being standardized and no systematic studies have been done on the influence of these variables on the immunogenicity of meningococcal antigens.

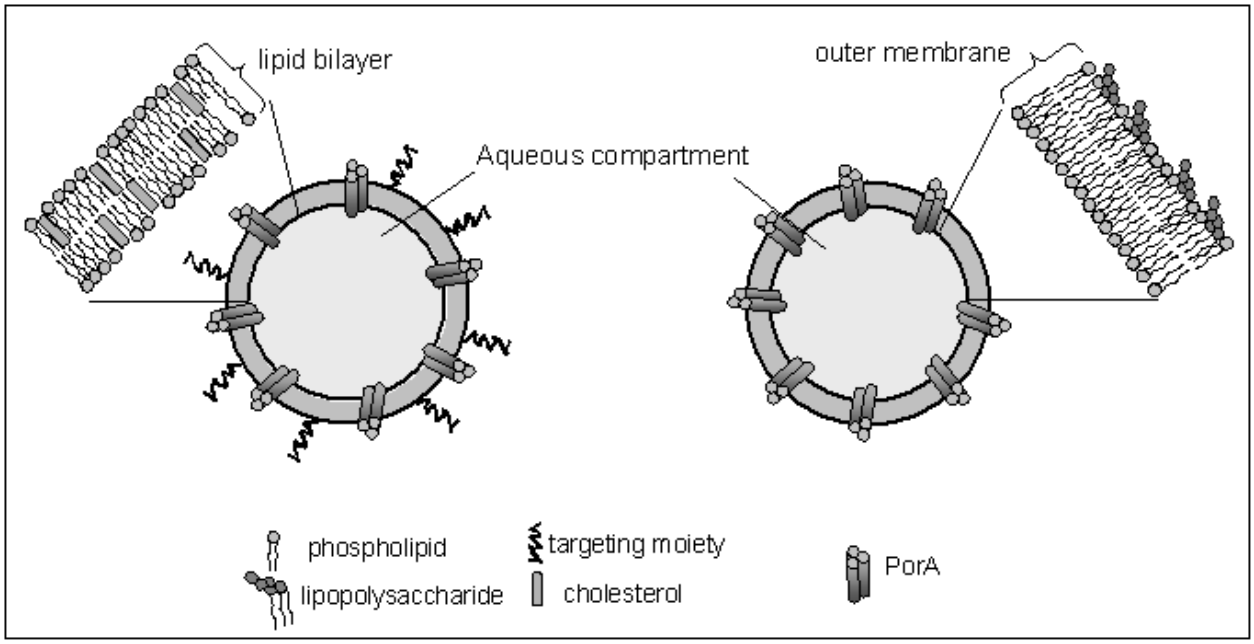

Figure 5. Schematic representation of the structure of a unilamellar liposome (left) and an outer membrane vesicle (right). The lipid bilayer of liposomes is composed of phospholipids, with their hydrophilic head group facing the aqueous phases. In the aqueous compartment, hydrophilic compounds can be encapsulated, whereas lipophilic compounds will be localized in the bilayer. By incorporation of targeting moieties on the surface of liposomes, specific cell types can be targeted. In OMV, the outer membrane is composed of a phospholipid bilayer containing lipopolysaccharide in the outer leaflet.

Microspheres

Biodegradable polymeric microspheres have been investigated in the last decade as antigen delivery systems for a variety of (model) antigens [129,130]. Two approaches have been followed: the development of pulsed release and sustained release systems. The pulsed release of vaccine antigens could be achieved by selecting polymers and encapsulation conditions yielding microspheres with different degradation rates. Microspheres with different, pulsatile antigen release rates could be mixed and the antigen would then be released at defined intervals, mimicking the

multiple doses given in conventional vaccination ("built-in booster") [131,132]. In general, however, the encapsulation of antigens in microspheres provides sustained antigen release rather than distinct pulses. Moreover, this strategy has not (yet) resulted in the development of single-shot vaccine formulations.

The second approach is the continuous release of antigen for prolonged periods. This could result in high levels of antibodies usually observed after multiple injections. Also in this approach, the characteristics of the polymer affect the release profile of antigens. The release time can last from a few days to several months [130]. Contrary to conventional thinking in immunology, continuous antigen delivery is able to produce immunity, as it results in affinity maturation, isotype switching and immune memory [133].

The rational manipulation of delivery methods is likely to result in significant improvement in the effectiveness of existing vaccines. For *Neisseria meningitidis,* neither pulsed, nor sustained antigen release systems have been described before in the literature.

Adjuvants in vaccine formulations against type B meningococci

Adjuvants are a heterogeneous group of substances used to enhance the immune response of antigens [134]. At the moment, the only adjuvants regularly used in humans are aluminum salts (a Th2-type adjuvant), which are also used in vaccines developed against type B meningococci [59]. However, more potent adjuvants than aluminum salts are needed, as three to four doses of the vaccine are required to induce an effective antibody response [55,135]. Adjuvants can improve the immune response by increasing cellular infiltration, inflammation, and trafficking to the injection site (particularly for APC), promoting the activation state of APC by upregulating costimulatory signals or MHC expression, enhancing antigen presentation, or inducing cytokine release for indirect effects [134].

The most appropriate adjuvant for a given vaccine will depend to a large extent on the type of immune response that is required for protective immunity. In vaccines against *N. meningitidis* mixed Th1/Th2 responses are probably preferred, as they result, respectively, in B cell isotype switching to IgG2a production [93] and increased production of antibodies [90]. Lipopolysaccharide (LPS) has been

recognized as a very potent Th1 adjuvant [136]. LPS present in the outer membrane of meningococci has been suggested to bind to OMP, thereby probably having a stabilizing effect. Moreover, LPS is a vaccine candidate because of its ability to induce a LPS-specific immune response in humans [137]. The endotoxic activity of LPS has limited its use in vaccines [138]. Although the toxicity induced by LPS can be strongly reduced by incorporation into liposomes [139], a lot of work has been done to develop less toxic derivatives of LPS.

An example of such a derivative is monophosphoryl lipid A (MPL) that has been widely tested in man [140,141], frequently formulated into liposomes [120] and that results in an increased function of APC [142]. More recently, lipid A biosynthesis has been genetically modified in *Neisseria meningitidis*, resulting in an *lpxL1* mutant with penta- instead of hexa-acylated lipid A. This mutant LPS possesses adjuvant activity similar to that of meningococcal wild-type LPS, but shows a reduced toxicity [143]. Therefore, this *lpxL1* mutant of neisserial LPS is a promising candidate to be used in novel type B meningococcal vaccines.

Novel adjuvants can be included in different ways in PorA-based vaccines. Adjuvants derived from meningococcal LPS can be included in the vaccine by preparing OMV derived from mutant meningococcal strains containing a less toxic derivative (e.g. the *lpxL1* mutant). Also, purified adjuvants can be included in formulations such as liposomes. This second possibility is preferred, as the characteristics of the formulation (e.g. adjuvant/antigen ratio) can be easily varied and better defined systems can be obtained.

Aims and outline of this thesis

Many efforts are being made to develop potent vaccines against *Neisseria meningitidis* serogroup B strains. Class 1 OMP or PorA formulated in OMV, is one of the most promising vaccine candidates for this purpose. Among the PorA serosubtypes, the serosubtype P1.7-2,4 is present in up to 40% of the serogroup B strains causing bacterial meningitis [6]. 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 [55,144]. Therefore we selected PorA P1.7-2,4 as a model for our studies. The primary goal of the investigations

described in this thesis was to establish the formulation parameters that result in an improved immune response against PorA.

In **Chapter 2**, the long-term stability of OMV derived from three different meningococcal strains, all containing PorA serosubtype P1.7-2,4 were studied. The aim of this study was to find out the storage conditions that provide optimal stability for the vaccine formulations, and to investigate which degradation mechanism(s) lead to loss of immunogenicity of PorA. The effects of storage of OMV formulations at different temperatures on both physico-chemical and immunological characteristics of PorA were studied, paying special attention to the thermal degradation of PorA. Both trivalent and monovalent OMV were sensitive to elevated temperatures, but fairly stable in the frozen state (-70ºC) or when stored at 4ºC in the liquid or freeze-dried state.

Chapter 3 describes the efforts to obtain a well-defined and immunogenic PorA formulation. In these studies, PorA subtype P1.7-2,4 was purified from OMV and compared for structure and immunogenicity with both OMV and outer membrane complexes (OMCs). Although a detailed characterization of the formulations did not reveal major differences in the conformation of non-purified and purified PorA, the purification of PorA resulted in a total loss of its capacity to induce a bactericidal immune response in mice. This capacity was restored by the incorporation of purified PorA into liposomes. Liposomal PorA, in the absence of other adjuvants, was as immunogenic as (LPS-containing) OMV.

As described above (*Antigen presentation and the immune response* section) DC are one of the most powerful APC. In **chapter 4**, PorA-liposomes targeted to DC were designed in order to improve the immunogenicity of liposomal PorA. The results *in vitro* indicated that targeting results in an increased uptake of liposomal PorA by DC as compared to non-targeted liposomes, and also induced effective maturation of DC. *In vivo*, targeted liposomes showed improved localization in APC in the lymph nodes. Although the bactericidal response induced by targeted liposomes was not improved with respect to that of OMV, an increased number of responding mice per group was obtained with targeted PorA-liposomes as compared to OMV and non-targeted liposomes.

In **chapter 5**, the interaction of (targeted) PorA-liposomes with human monocytes-derived DC and murine bone marrow-derived DC was studied *in vitro*. The results indicated that in both the human and the murine system, the liposome

bilayer composition had a major and comparable effect on the uptake of the formulations by DC.

In line with the previous experiments, the objective of **chapter 6** was to improve the immunogenicity of liposomal PorA. Here, the effect of co-encapsulation of LPS-derived adjuvants was studied. Various LPS-derived adjuvants were studied for their effect on the immunogenicity of liposomal PorA formulations. The use of *galE* LPS or *lpxL1* LPS mutants resulted in an improved humoral immune response in mice. PorA-specific cellular responses for *lpxL1* LPS-adjuvated liposomes were also investigated. This adjuvant induced an improved PorA-specific proliferation of cells in the lymph nodes as compared to non-adjuvated PorA-liposomes.

In **chapter 7** the possibility to encapsulate PorA-liposomes and OMV in biodegradable dextran and mannan microspheres was studied. The aim of this study was to determine whether the microsphere-encapsulated antigens were able to induce a similar or even improved immune response as non-encapsulated antigen, by attracting APC and triggering internalization. Furthermore, an adjuvant effect related to the use of mannan was investigated. Microspheres, containing PorA-liposomes and OMV, with similar release profiles were prepared from derivatized mannan or dextran. *In vitro* release studies showed partial release of antigen, within 10-11 days. The immunogenicity of the formulations was maintained upon encapsulation.

The final chapter of this thesis, **chapter 8**, provides a summary and general discussion of the results. Moreover, suggestions are made for future investigation of PorA formulations for vaccines against type B meningococcal disease.

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CHAPTER 2

Stability of mono- and trivalent meningococcal outer membrane vesicle vaccines

Carmen Arigita[a.b], Wim Jiskoot[a], Janny Westdijk[b], Cor van Ingen[b], Wim E. Hennink[a], Daan J.A. Crommelin[a], Gideon F.A. Kersten[b]

[a]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands

[b]Laboratory for Product and Process Development, Netherlands Vaccine Institute, Bilthoven, the Netherlands

(submitted)

Abstract

The stability during storage of outer membrane vesicles (OMV) of *Neisseria meningitidis* group B was studied. Three types of OMV were compared for their stability, containing either one (monovalent) or three different PorA subtypes (trivalent), the latter with and without class 4 outer membrane protein (RmpM).

Aqueous formulations were stored at -70ºC, 4ºC, 37ºC and 56ºC. Some of the observed, unexpected changes in fluorescence characteristics were studied in more detail with tryptophan and tyrosine derivatives incubated at 56ºC. Furthermore, the possibility to freeze-dry OMV was investigated.

The conformation of PorA and the physicochemical properties of the OMV were monitored during one year by electron microscopy, dynamic light scattering, fluorescence spectroscopy, and electrophoretic methods. The antigenicity of the PorA subtype P1.7-2,4, present in all formulations, was studied by ELISA with a bactericidal anti-P1.4 monoclonal antibody. The immunogenicity of OMV (i.e. bactericidal response) was determined in Balb/c mice.

When stored at -70ºC or 4ºC, the structure and immunogenicity of OMV was preserved. In contrast, storage of OMV at higher temperatures (37ºC or 56ºC) induced destruction of the OMV structure and denaturation of PorA, followed by chemical degradation, as well as a decrease or complete loss of immunogenicity. Changes observed in the fluorescence spectra of degraded OMV were also seen in tryptophan and tyrosine derivatives incubated at 56ºC, indicating the occurrence of chemical degradation of tryptophan and tyrosine residues in PorA. Trivalent OMV were slightly more stable at 37ºC than monovalent OMV as assessed by *in vitro* methods, but these observations did not result in differences in the measured immunogenicity. The stability of trivalent OMV was not affected by the presence of RmpM. Both trivalent and monovalent OMV could be freeze-dried with preservation of their immunogenicity.

In conclusion, OMV are sensitive to degradation at elevated temperatures, but are fairly stable in the frozen state (-70ºC) or when stored at 4ºC in the liquid or freeze-dried state.

Introduction

Neisseria meningitidis is a human pathogen and one of the major causes of bacterial meningitis and sepsis [1]. Vaccines against meningococci of serogroups A, C, Y and W-135 are based on capsular polysaccharides. However, the polysaccharide of group B meningococci is poorly immunogenic and cross-reactive with human tissues [2]. Vaccines containing outer membrane proteins (OMP) have been developed and have shown to induce protective immune reponses [3,4]. The vaccines developed at the Netherlands Vaccine Institute (NVI, formerly a division of the National Institute of Public Health and the Environment, RIVM) consist of outer membrane vesicles (OMV) purified from genetically modified meningococcal strains expressing either one (monovalent) or three (trivalent) subtypes of class 1 OMP (PorA). Two different trivalent OMV are mixed to form a hexavalent vaccine that represents the majority of circulating subtypes in Western Europe. This vaccine has been proven to be safe and immunogenic [5]. The monovalent OMV vaccine contains PorA P1.7-2,4, the most prevalent subtype in The Netherlands and the subtype causing an epidemic in New Zeeland [1,9]. This vaccine is also safe and immunogenic in toddlers [6]. The trivalent strains contain class 4 outer membrane protein (RmpM). RmpM is firmly associated to PorA in the bacterial outer membrane, which is probably essential for the localization and the stability of the PorA trimers in the outer membrane [15].

In previous studies, we have shown that both the conformation and the presentation form determine the quality of the immune response against PorA [7]. Thus, both characteristics have to be maintained upon storage in order to preserve the vaccine functionality.

The composition of OMV is complex. The main constituents are: PorA, phospholipids, lipopolysaccharide (LPS), residual DNA, RmpM, and residual detergent [12]. The interaction of these compounds with each other through various non-covalent forces determine the physicochemical properties and stability of OMV. Diverse degradation processes may occur simultaneously during storage of OMV vaccines. Not only can the protein undergo chemical and conformational changes, but the OMV structure may also change or even be destroyed. However, it is not clear if changes in OMV affect the potency and efficacy of meningococcal vaccines.

The bactericidal activity of sera from humans after vaccination with OMV is accepted as correlate for protection [8].

To our knowledge, no studies on long-term stability of protein-containing vesicular systems such as PorA-OMV have been reported. In this study, trivalent OMV containing PorA subtypes P1.7-2,4, P1.5-2,10 and P1.12,13 were compared to monovalent OMV containing PorA subtype P1.7-2,4. The possible stabilizing effect of RmpM on trivalent OMV was also investigated. OMV containing subtype P1.7-2,4 were chosen, since 43% of all strains causing bacterial meningitis in The Netherlands belongs to this subtype [9]. At the same time, P1.7-2,4 is one of the weakest immunogens in multivalent OMV vaccines [5].

The stability of liquid (stored 4ºC, 37ºC and 56ºC) and frozen (-70ºC) PorA-OMV has been assessed during 12 months of storage. The possibility to freeze-dry OMV preserving its physicochemical and immunogenic characteristics was also studied. The stability of the presentation form was assessed with electron microscopy and dynamic light scattering measurements. The physicochemical stability of PorA was monitored by fluorescence spectroscopy, SDS-PAGE and ELISA. The functional immunogenicity (i.e. bactericidal response) of the vaccines was evaluated *in vivo*. The storage conditions for functionally stable OMV were determined.

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. Strains JB10124 (P1.5-2,10, P1.12,13, P1.7-2,4, PorB$^-$, RmpM$^-$, low expression Opa/Opc) and HP10124 (P1.5-2,10, P1.12,13, P1.7-2,4, PorB$^-$, RmpM$^+$, low expression Opa/Opc) were used for the production of trivalent PorA-OMV. Bacteria were grown in a defined medium, with as main components L-glutamic acid, glucose, L-cysteine and ammonium chloride, at 35ºC for 15 h in a 40 L stirred fermentor under pH and pO_2 control.

Preparation of OMV formulations

PorA-OMV were prepared as previously described [7]. Briefly, PorA-OMV were extracted from bacteria with EDTA. DNA was partially removed by DNase

treatment. Lipopolysaccharide (LPS) was partly depleted by gel permeation chromatography (GPC) in the presence of deoxycholate (DOC). In order to remove DOC, a second GPC step was performed, which also served to formulate the OMV in 10 mM Tris-HCl, 3% sucrose pH 7.4.

OMV derived from the three strains were stored at –70, 4, 37 and 56ºC in 2 ml plastic tubes with screw caps (Greiner, Alphen a/d Rijn, The Netherlands). For storage at 37ºC and 56ºC, 0.01% sodium azide was added. For fast freezing of OMV, 2 ml samples were submerged in an ethanol/dry ice mixture and immediately stored at –70ºC.

Freshly prepared OMV were freeze-dried in aliquots of 2 ml in 5-ml glass vials. The rubber stoppers were dried at 85ºC for 2 days prior to use. The vials containing the OMV formulations were placed directly on the shelf of a Leybold GT 4/6 freeze-dryer with a temperature of -35ºC. In the first step of the freeze-drying process, the temperature of the shelf was maintained at –35ºC and the chamber pressure set at 20 Pa. After 1-3 hours, the shelf temperature was raised to –25ºC and the pressure set to 5-6 Pa. These conditions were maintained for 65 hours, followed by additional secondary drying steps at plate temperatures of –20ºC, -5ºC, 10ºC, 25ºC and 35ºC, each for at least 5 hours at a pressure of 4-5 Pa. At the end of the freeze-drying process the vials were closed under vacuum with the dried rubber stoppers and capped with aluminium seals.

Freeze-dried OMV were stored at 4ºC until reconstitution. Analysis of frozen and liquid formulations was performed before storage (t=0) and after 3, 6 and 12 months. Freeze dried OMV were reconstituted and analyzed after 3 months storage.

Composition of OMV

The protein content of the OMV was measured according to Peterson [10] with BSA as standard. The PorA content (relative to total protein content) was determined by SDS-PAGE (see below). The presence of LPS was analyzed by gas chromatography (GC). In short, LPS was hydrolyzed in 3.45 M NaOH in 50% (v/v) methanol. Then, the fatty acids were methylated in 5.5 M HCl in 27% (v/v) methanol and extracted with a hexane/methyl tert-butyl ether mixture (1/1, v/v). Methylated fatty acids were separated and quantified by GC on an HP Ultra column (25 m x 0.2 mm, Agilent). The different fatty acids were identified based on their retention time using a calibration mixture of known fatty acids (Calmix, Midi, Newark, DE). The

amount of C14:3OH fatty acids was calculated with a calibration curve of *Serratia marcensens* LPS. As internal standard, C14:2OH was used. DNA content was determined by an ethidium bromide assay [11] with salmon sperm DNA (ICN, Rotterdam, The Netherlands) as standard.

The water content of freeze-dried OMV was measured with the Karl-Fisher method using a Mitshubishi Moisture analyzer. Differential scanning calorimetry was performed to determine the glass-transition temperature of the freeze-dried OMV directly after drying and after 12 months of storage at 4ºC.

Physical characteristics of OMV

To check the OMV structure after freeze-drying and storage, electron microscopy (EM) was performed as described by Claassen et al. [12]. The particle size of monovalent and trivalent OMV 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 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 a heterodisperse dispersion. The zeta potential of the OMV was measured with a Malvern Zetasizer 2000 with an aqueous dip-in cell and a computer with PCS software (version 1.35, Malvern).

Fluorescence measurements and guanidine denaturation assay

The local environment of tryptophan (Trp) 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 at 150 nm/min, with excitation and emission band widths of 2.5 and 5 nm, respectively. Samples contained either 25 µg/ml protein (F91, JB10124) or 20 µg/ml (HP10124). Sets of 5 spectra were averaged and corrected for buffer background.

Upon incubation with increasing concentrations of guanidinium hydrochloride (Gnd-HCl), PorA in OMV denatures, causing increased exposure of fluorescent

groups (Trp), which results in a shift of the fluorescence maximum towards higher wavelengths [13]. Samples were diluted with Gnd-HCl to final Gnd-HCl concentrations ranging from 1 M to 5 M, and incubated for 2 hours at room temperature before measurement. Fluorescence spectra of these samples were measured as described above. Sets of 5 spectra were averaged and corrected for buffer/Gnd-HCl background. The emission maximum was plotted against the Gnd-HCl concentration. The curves obtained were fitted, assuming a two-state equilibrium between the native and the denatured protein [14]. The concentration of Gnd-HCl necessary to obtain 50% denaturation of PorA was determined based on the midpoint of the denaturation curve.

Analysis of the denaturation of model compounds

N-acetyl tryptophan amide (NATrA), N-acetyl tyrosine amide (NATyA) were dissolved in 10 mM Tris-HCl, 3% sucrose pH 7.4 plus 0.01% sodium azide and incubated at 56ºC. Spectral properties of the samples were monitored during 6 months. UV spectra (200-400 nm) of samples were taken with a Perkin-Elmer spectrophotometer and normalized (Abs_{280} = 1). Samples used for fluorescence measurements had an absorbance maximum $\leq$ 0.1. Fluorescence emission spectra after excitation at 295 (for tryptophan) and 280 nm (tyrosine) were recorded as described above.

SDS-PAGE and Western blotting

The trimeric folding of PorA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 'native' gels as described by Jansen et al. [15]. Prior to electrophoresis, samples were incubated for 10 min at room temperature or 100ºC in sample buffer [16] containing 0.05% SDS. Polyacrylamide gels were prepared as described [16], 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 (4ºC) to prevent denaturation of the formulations containing folded PorA. Gels were stained with Coomassie Brilliant Blue.

Relative PorA content was measured by SDS-PAGE under denaturing conditions [16]. Gels were scanned after running and staining in an Agfa Fotolook 3.5

scanner and analyzed with Phoretix 1D Quantifier software (version 5.10, Biozym, Landgraaf, The Netherlands) for determination of the relative PorA content.

Western blotting was performed as described [17] after SDS-PAGE under denaturing conditions. The monoclonal antibodies (mAb) used to detect the different PorA subtypes were MN20B9.34 (anti-P1.4); MN20F4.17 (anti-P1.10); MN20A7.10 (anti-P1.12) and MN3B9F (anti-RmpM).

PorA inhibition ELISA

Antigenicity of the P1.4 epitope of PorA P1.7-2,4 in OMV was tested by an inhibition ELISA as previously described using the mAb MN20B9.34 [7]. Briefly, a dilution series of the sample was incubated with a fixed concentration of mAb. Free mAb was quantified with an OMV-coated ELISA plate. The coating OMV batch contained at least 90% PorA, as determined by SDS-PAGE. The results are shown as the ratio between the PorA concentration determined by ELISA and the protein concentration determined by Peterson (total protein) corrected for protein impurities. As a reference, the antigenicity of freshly produced monovalent OMV was arbitrarily set at 1.

Immunization experiments

Balb/c mice, 6 to 8 weeks old (10 animals in each group) were immunized subcutaneously on day 0, 14 and 28. PorA (1.5 µg/subtype) in OMV were administered adjuvated with 1.5 mg $AlPO_4$ (NVI) (0.25 ml/mouse) (see Table 3). The mice were bled on day 42, and sera were collected and stored at -20ºC until analysis.

Anti-PorA whole-cell ELISA

The antibody titer (total IgG) of each individual mouse serum was determined by whole cell ELISA as described [18]. *Neisseria meningitidis* isogenic H44/76-based strains (B:15P1.7,16:L3,7,9) expressing P1.7-2,4 (H44/76 7-2,4), P1.12,13 (H44/76 12,13) or P1.5-2,10 (H44/76 5-2,10), and the H44/76-derived mutant strain HI5 (lacking PorA) were used. Titers were determined with goat anti-mouse (GAM) total IgG conjugates, labeled with horseradish peroxidase (HRP) (Southern Technology Associates; dilution 1/5000). The titer is defined as the dilution of the serum where 50% of the OD_{max} in the assay is reached.

Serum bactericidal assay

The serum bactericidal activity was measured as previously described [19] against the four *N. meningitidis* strains: H44/76 7-2,4; H44/76 12,13; H44/76 5-2,10 and HI5 (PorA$^-$). From each group of 10 mice, 2 pools were made (5 mice/pool) and every pool was tested at least twice. Pooled sera were heat inactivated for 30 min at 56ºC 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) rabbit serum was used as complement source. 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 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. IgG titers are expressed as the mean $\log_{10}$ titer of ten independent observations. Bactericidal titers are expressed as the mean $\log_{10}$ titer of at least four 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 and bactericidal titers was determined by the least-significant-difference (LSD) test at a confidence level of 95%.

Results

Characterization of OMV

The composition of the OMV derived from the three strains (F91, JB10124 and HP10124) is shown in Table 1. The protein present in all OMV was mainly PorA. Most impurities were found in OMV from strain JB10124. OMV had a negative surface charge (zeta potential) ranging between -40 and -50 mV.

The average particle size of freshly prepared OMV ranged between 150-350 nm, with a rather high polydispersity around 0.4, typical of OMV (Table 1). Both particle size and polydispersity were smaller for OMV derived from the trivalent strain JB10124, indicating a more homogenous particle size distribution for these OMV. The presence of partly aggregated vesicles with a diameter around 80 nm for all

OMV was shown by electron microscopy (see Fig. 2 A for a representative picture). Both freezing and freeze-drying of OMV induced the formation of large aggregates, accompanied by an increase of the polydispersity index (see Fig. 1 A-D). The size of the aggregates was smaller in freeze-dried OMV than in freeze-thawed OMV. A representative picture of the aggregates present after freeze and thawing can be observed in Fig. 2 B.

Table 1. Physicochemical characteristics of the OMV used in this study

Strain	% PorA[a,b]	PorA subtypes	Class 4 protein[c]	LPS content (%)[b]	DNA content (%)[b]	Particle size (nm)	PD[d]	Zeta potential (mV)
F91 (monovalent)	94	P1.7-2,4	-	9.4	7.7	362	0.42	-49.4
JB10124 (trivalent)	76	P1. 7-2,4 P1.12,13 P1.5-2,10	-	10.6	19.2	138	0.22	-41.8
HP10124 (trivalent)	87	P1.7-2,4 P1.12,13 P1.5-2,10	+	3.7	8.2	304	0.45	-47.5

[a] Determined by scanning the SDS-PAGE profile.
[b] Relative to the total amount of protein present in the formulation.
[c] The presence of class 4 protein was demonstrated by Western blotting with monoclonal antibody MN3B9F.
[d] Polydispersity: indication of the size distribution of the OMV; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.

The water content of freeze-dried OMV was below 3% for all formulations. Freeze-dried OMV showed a glass transition temperature of ca. 50ºC. These characteristics were maintained over one year of storage at 4ºC.

The fluorescence emission spectrum of PorA was similar in OMV derived from the three different strains studied. The emission maximum was around 338 nm, indicating a relatively hydrophobic environment of the tryptophan residues [13]. Upon titration with Gnd-HCl, a shift in the fluorescence maximum was observed from ca. 338 nm to ca. 350 nm. This shift was similar in magnitude for OMV from the three strains. The denaturation midpoint was at 3.8 M Gnd-HCl for monovalent OMV and slightly higher (4.1 M) for both trivalent OMV.

The trimeric structure typical of native PorA was monitored by 'native' SDS-PAGE (Fig. 3) [15]. Freshly prepared PorA-containing OMV showed the presence of a high molecular weight band (~130 kDa) corresponding to PorA trimers (Fig. 3 A, lanes a); no monomers were visible. In samples incubated at 100ºC prior to electrophoresis the trimers were converted to monomers (41 kDa) (Fig. 3 A, lanes b).

Freeze-drying of OMV did not induce changes in the electrophoretic characteristics of PorA in the three OMV studied (not shown).

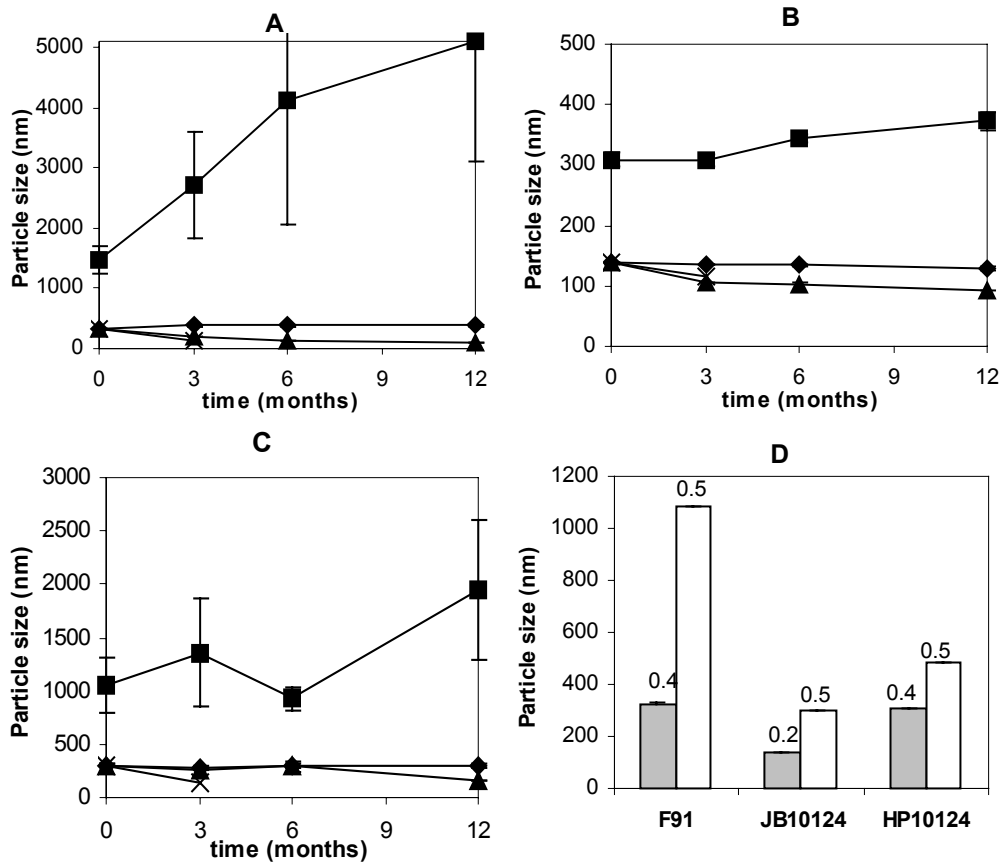

Figure 1. Panels A, B, C: effect of storage on the particle size of OMV of strains F91 (A), JB10124 (B) and HP10124 (C). Frozen samples were stored at -70ºC (squares); liquid samples at 4ºC (diamonds), 37ºC (triangles) or 56ºC (cross). Bars indicate SD (n=3). **Panel D:** effect of freeze drying on the particle size of OMV. The bars indicate the particle size ± SD (n=3) of OMV before (grey) and after (white) freeze-drying and reconstitution after 3 months storage at 4ºC. The numbers above the bars indicates the polydispersity of the samples.

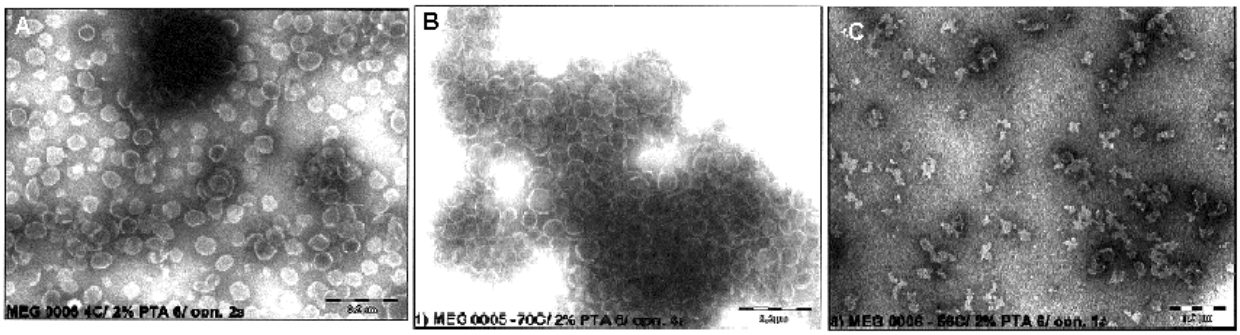

Figure 2. Electron micrographs of OMV stored at 4ºC (A: strain JB10124), -70ºC (B: strain F91) and 56ºC (C: strain HP10124) during 6 months. The bar indicates 0.2 µm. Micrographs are representative for all three OMV stored at the indicated temperature.

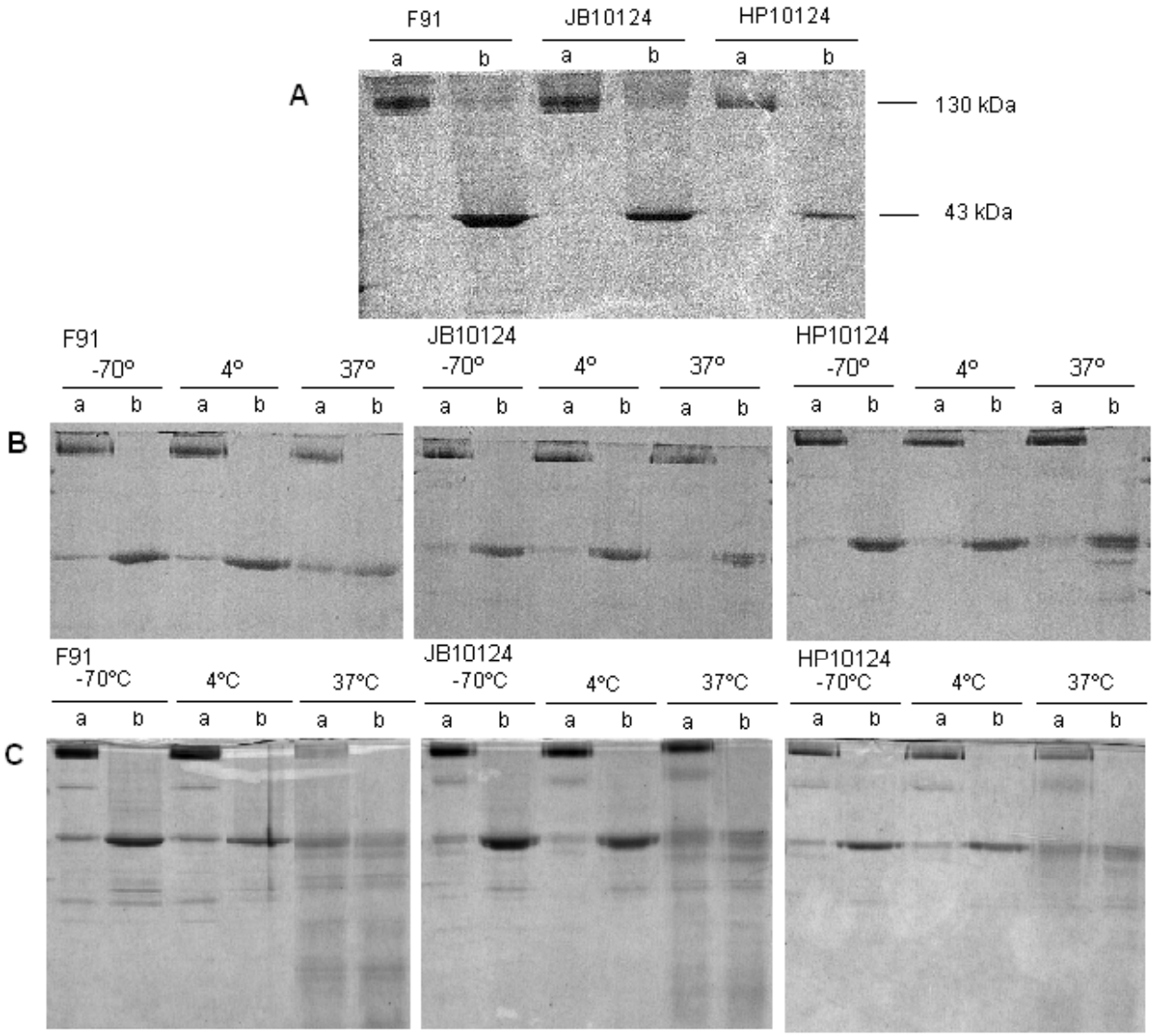

Figure 3. SDS-PAGE under mild conditions (see materials and methods) of OMV after production (A), after 6 months storage (B) or after 12 months storage (C). In panel A is indicated the molecular weight of PorA monomers (43 kDa) and trimers (~130 kDa). The OMV strains and the storage temperature are indicated above the gels. Each sample was mixed with sample buffer containing 0.05% SDS and incubated either at room temperature (lanes a) or at 100ºC (lanes b).

The interaction of PorA P1.7-2,4 with the bactericidal mAb MN10B9.34 (directed against loop 4 of this PorA subtype) was studied by ELISA (Fig. 4). When assuming that the ratio of the three PorA subtypes in trivalent OMV is 1:1:1, the antigenicity of PorA P1.7-2,4 in trivalent OMV is expected to be one third of that of monovalent OMV. However, the results show that the antigenicity of PorA P1.7-2,4 in trivalent OMV was only 1/8 and 1/4 for strains JB10124 and HP10124, respectively, as compared with monovalent OMV. Freeze-drying and reconstitution of OMV did not significantly reduce the antigenicity of PorA P1.7-2,4 in any of the OMV (Fig. 4 D).

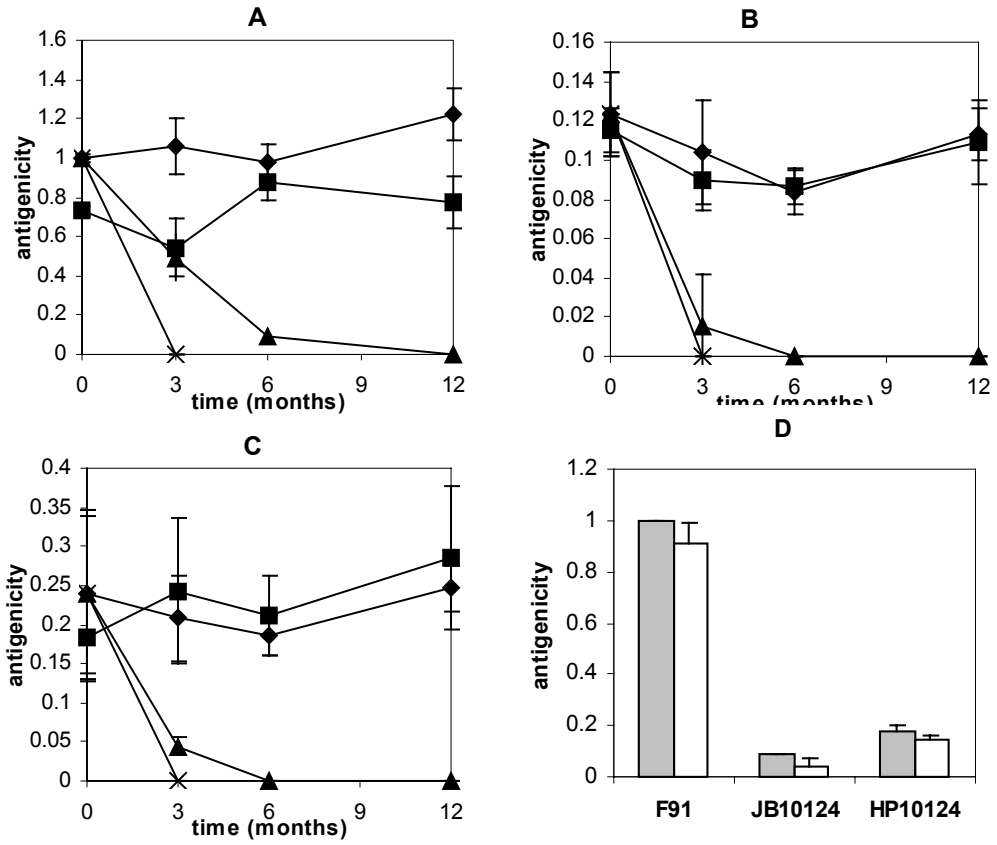

Figure 4. Panels A, B, C: Effect of storage on the antigenicity of PorA P1.7-2,4 in OMV of the strains F91 (A), JB10124 (B) and HP10124 (C). Samples were stored at -70ºC (squares), 4ºC (diamonds), 37ºC (triangles) or 56ºC (cross). **Panel D:** effect of freeze drying and storage on the antigenicity of PorA P1.7-2,4 in OMV. The bars indicate the antigenicity of PorA P1.7-2,4 in OMV before (grey) and after (white) freeze-drying in samples stored for 3 months at 4ºC. The results are shown as mean values (±SD) of at least 3 measurements.

Effect of storage on the physicochemical characteristics of OMV

During 12 months, the pH of the preparations increased from 7.4 to 8.1 for almost all the formulations studied, except for trivalent OMV incubated at 56ºC, where the solution acidified, reaching pH values around 6. The zeta potential of OMV did not change upon storage under different conditions (not shown).

OMV stored at –70ºC and thawed (at 37ºC, 10 min) showed aggregation, which tended to increase with storage time (Fig. 1), parallel to an increase in the polydispersity index. The size of OMV in liquid formulations did not change during storage at 4ºC. At higher temperatures (37ºC, 56ºC), the average particle size of OMV derived from the three strains gradually decreased (Fig. 1 A, B, C). After 6 months, the particle size and zeta potential of samples incubated at 56ºC could not be measured anymore, as the scatter intensity had become too low. This indicates total destruction of the OMV, as confirmed by the presence of amorphous granular material in electron micrographs (Fig. 2 C).

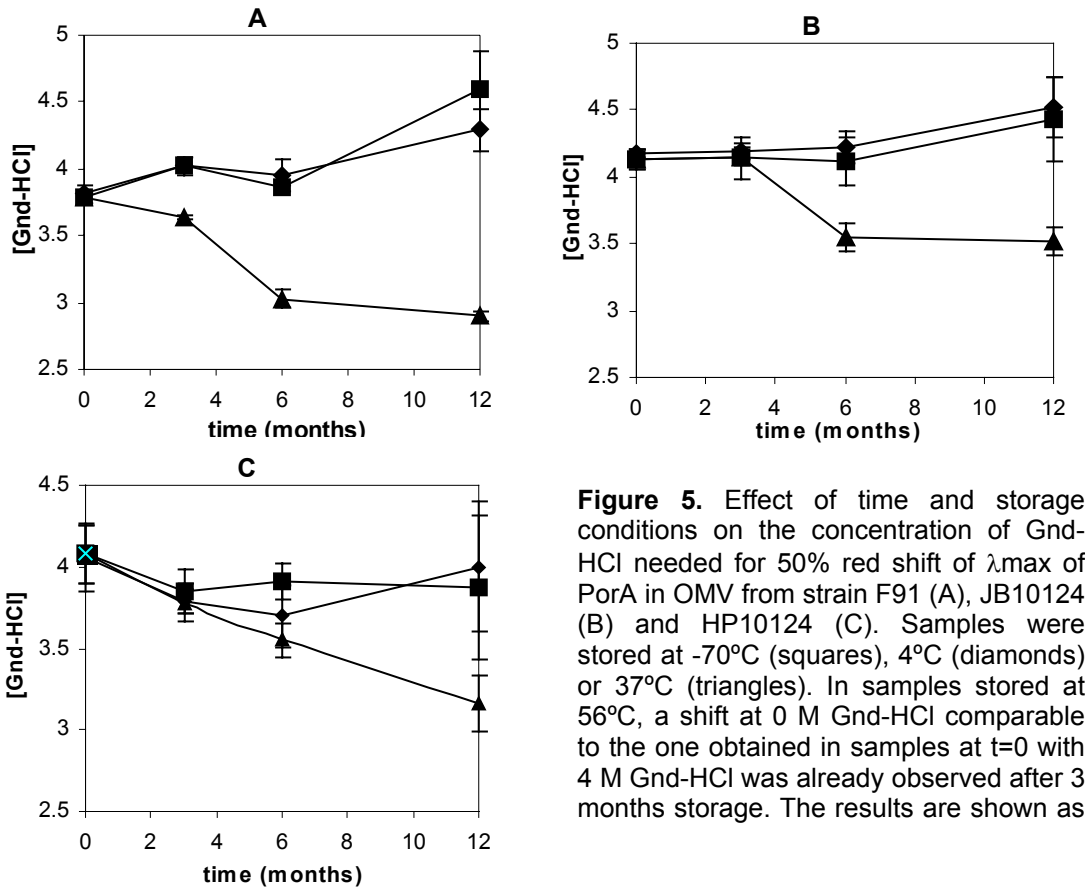

Figure 5. Effect of time and storage conditions on the concentration of Gnd-HCl needed for 50% red shift of λmax of PorA in OMV from strain F91 (A), JB10124 (B) and HP10124 (C). Samples were stored at -70ºC (squares), 4ºC (diamonds) or 37ºC (triangles). In samples stored at 56ºC, a shift at 0 M Gnd-HCl comparable to the one obtained in samples at t=0 with 4 M Gnd-HCl was already observed after 3 months storage. The results are shown as

The concentration of Gnd-HCl necessary to induce a shift in the fluorescence emission maximum decreased in liquid samples of all three strains incubated at 37ºC, as compared to non-treated OMV. This indicates a decreased stability of these samples. The red shift of samples stored at 56ºC could not be measured, as in the absence of Gnd-HCl they showed a broadened emission spectrum with a maximum above 350 nm after 3 months. After 6 and 12 months totally different fluorescence spectra were observed: the tryptophan emission peak disappeared and a new spectrum was formed instead with an emission maximum of ca 405 nm (Fig. 6). In these degraded samples, the fluorescence spectra were similar when measured at an excitation wavelength of 280 or 295 (not shown). The newly formed spectra, which cannot originate from natural aromatic amino acids, indicate the formation of (fluorescent) chemical degradation products due to storage at high temperatures. To study these observations in more detail, the fluorescent amino acids N-acetyl tryptophan (NATrA) and N-acetyl tyrosamine (NATyA) were used as model compounds. Solutions of these amino acids in the same buffer as OMV were stored for 6 months at 56ºC, and their fluorescence (λ_{exc} 295 and 280 nm, respectively) and

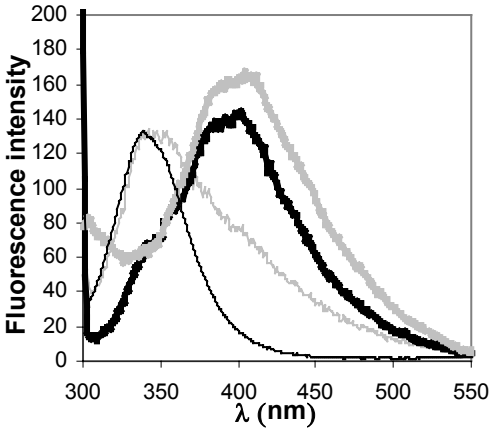

Figure 6: Fluorescence emission spectra of monovalent OMV stored at 56ºC. Thin black line: before storage; thin gray line: after 3 months storage; thick black line: after 6 months storage; thick gray line: after 12 months storage.

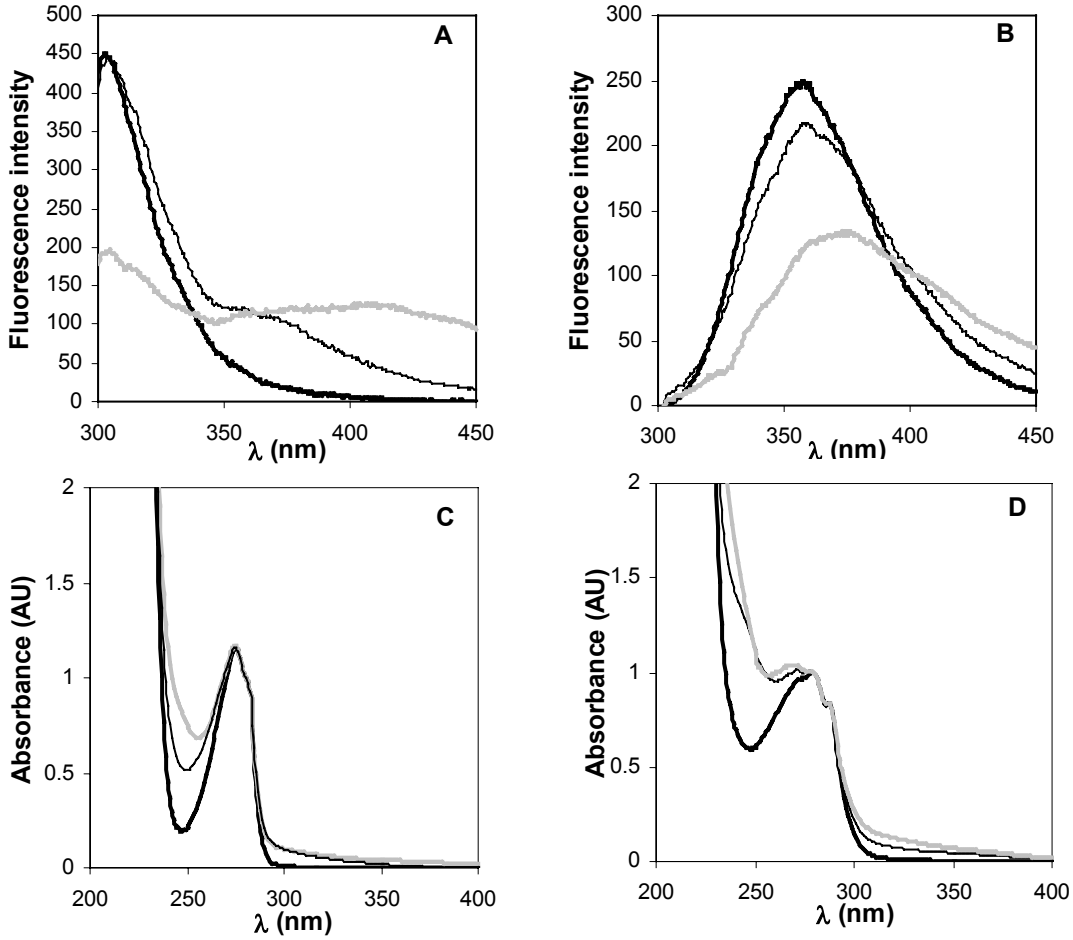

Figure 7. Panels A and B. Fluorescence spectra of NATyA (A, λ_{exc}: 280 nm) and NATrA (B, λ_{exc}: 295 nm) freshly prepared (thick black line) and after 3 (thin black line) and 6 months (thick gray line) incubation at 56ºC. See materials and methods for details. **Panels C and D**: normalized UV spectra of NATyA (C) and NATrA (D) freshly prepared (thick black line) and after 3 (thin black line) and 6 months (thick gray line) incubation at 56ºC.

UV spectra were monitored (Fig. 7). The intensity of the maximum fluorescence signal decreased in all model compounds and a new fluorescence emission peak was formed at higher wavelengths (ca. 405 nm for NATyA, 375 nm for NATrA). As

expected (because NATyA does not absorb at 295 nm), when freshly prepared NATyA was excited at 295 nm, no emission spectra could be observed. After 3 and 6 months of incubation at 56ºC, however, the NATyA sample did absorb at 295 nm (Fig. 7A, inset) and showed an emission spectrum with a maximum around 405 nm upon excitation at 295 nm (not shown), indicating the formation of a fluorescent degradation product.

The trimeric structure of PorA was still present in frozen and liquid OMV stored at 4 and 37ºC after 3 months (not shown). At this time point no protein bands were observed for samples incubated at 56ºC. Samples incubated at 56ºC were not studied further by SDS-PAGE. After 6 months, part of the trimeric PorA was converted to monomers for all OMV stored at –70, 4 and 37ºC (Fig. 3B). In trivalent OMV (JB10124 and HP10124) incubated at 37ºC, extra bands with lower molecular weights appeared below the monomer band (43 kDa) and this band was separated in two bands (Fig. 3 B, lanes b). The structural changes of PorA were clearer after 12 months of incubation at all temperatures (Fig. 3 C). In native samples (lanes a), trimers and monomers were still observed, and an extra band appeared at ~ 80 kDa, indicating the presence of PorA dimers. Degradation bands at lower molecular weight were also observed. This degradation pattern was most apparent for samples incubated at 37ºC, especially for monovalent OMV, where the intensity of the trimer band had strongly decreased (Fig. 3 C, F91). For all samples incubated at 37ºC, a smear could be observed. The presence of P1.4, P1.5-2 and the P1.12 epitopes in degradation bands was checked by Western blotting. No degradation bands were observed for PorA subtypes P1.7-2, 4 and P1.5-2,10. Only in the case of the P1.12,13 subtype one minor degradation band in addition to the original band was observed containing the P1.12 epitope (not shown).

Effect of storage on the immunochemical characteristics of OMV

The antigenicity of liquid samples stored at 4ºC was preserved in time. Frozen monovalent OMV showed a slight decrease in the antigenicity, which was not observed for trivalent samples stored at –70ºC. When OMV were incubated at 37ºC the antigenicity decreased to (almost) zero within 6 months of incubation. When samples were incubated at 56ºC the antigenicity disappeared within 3 months (Fig. 4 A, B, C).

Table 2. IgG titers induced by OMV incubated at different temperatures

OMV strain	*Storage*	*IgG titers*[a]															
		7-2,4[b]				*5-2,10*[b]				*12,13*[b]				*HI.5*[c]			
		t=0[d]	*t=3*	*t=6*	*t=12*	*t=0*	*t=3*	*t=6*	*t=12*	*t=0*	*t=3*	*t=6*	*t=12*	*t=0*	*t=3*	*t=6*	*t=12*
F91	-70ºC	3.4	3.9	4.2	3.8	-[e]	-	-	-	-	-	-	-	<1	<1	2.2	1.2
	4ºC	3.9	4.5	3.8	3.6	-	-	-	-	-	-	-	-	0.6	0.2	2.0	0.8
	37ºC	3.9	4.2	4.3	3.5	-	-	-	-	-	-	-	-	0.6	0.2	1.4	0.3
	56ºC	3.9	3.6	-	0.8	-	-	-	-	-	-	-	-	0.6	<1	-	0.8
JB10124	-70ºC	3.7	4.0	3.8	3.5	4.3	4.3	4.7	4.3	3.8	4.2	4.3	3.9	3.4	2.9	3.5	3.7
	4ºC	3.8	3.9	3.8	3.5	4.4	4.4	4.7	4.4	3.9	4.3	4.2	3.9	3.5	2.5	3.5	3.7
	37ºC	3.8	3.7	3.5	3.5	4.4	4.4	4.6	4.2	3.9	4.1	4.2	3.9	3.5	0.2	0.8	<1
	56ºC	3.8	3.3	-	1.3	4.4	4.2	-	0.5	3.9	3.2	-	1.3	3.5	<1	-	1.4
HP10124	-70ºC	3.9	3.9	3.9	4.2	4.2	4.4	4.5	4.5	4.0	3.9	4.1	4.0	1.4	<1	1.6	2.8
	4ºC	3.9	3.7	3.8	3.7	4.3	4.4	4.6	4.4	4.0	3.7	4.2	3.7	1.8	<1	1.2	2.6
	37ºC	3.9	3.9	3.7	3.7	4.3	4.3	4.3	4.1	4.0	3.8	3.8	3.7	1.8	<1	1.1	1.4
	56ºC	3.9	3.1	-	1.3	4.3	3.7	-	<1.0	4.0	1.9	-	<1.0	1.8	<1	-	<1

[a] The total IgG titer of each mouse serum was determined by whole-cell ELISA and is expressed as the mean $\log_{10}$ titer. Plates were coated with *Neisseria meningitidis* strains: H44/76 7-2, H44/76 5-2,10, H44/76 12,13 and HI.5 (see materials and methods). The means were compared by the LSD test with a confidence level of 95%: $LSD_{0.05}$ ($7\text{-}2,4_{t=0}$) = 0.4; $LSD_{0.05}$ ($7\text{-}2,4_{t=3}$) = 0.4; $LSD_{0.05}$ ($7\text{-}2,4_{t=6}$) = 0.4; $LSD_{0.05}$ ($7\text{-}2,4_{t=12}$) = 0.6; $LSD_{0.05}$ ($5\text{-}2,10_{t=0}$) = 0.3; $LSD_{0.05}$ ($5\text{-}2,10_{t=3}$) = 0.3; $LSD_{0.05}$ ($5\text{-}2,10_{t=6}$) = 0.3; $LSD_{0.05}$ ($5\text{-}2,10_{t=12}$) = 0.4; $LSD_{0.05}$ ($12,13_{t=0}$) = 0.4; $LSD_{0.05}$ ($12,13_{t=3}$) = 0.7; $LSD_{0.05}$ ($12,13_{t=6}$) = 0.3; $LSD_{0.05}$ ($12,13_{t=12}$) = 0.5; $LSD_{0.05}$ ($HI.5_{t=0}$) = 0.9; $LSD_{0.05}$ ($HI.5_{t=3}$) = 0.5; $LSD_{0.05}$ ($HI.5_{t=6}$) = 1.2; $LSD_{0.05}$ ($HI.5_{t=12}$) = 0.9.

[b] PorA subtype against which IgG titers are specific.

[c] IgG titers specific for other structures than PorA.

[d] months.

[e] -: not determined.

Table 3. Bactericidal titers induced by OMV incubated at different temperatures

OMV strain	Storage	Bactericidal titers[a,b]											
		7-2,4[c]				5-2,10[c]				12,13[c]			
		t=0[d]	t=3	t=6	t=12	t=0	t=3	t=6	t=12	t=0	t=3	t=6	t=12
F91	-70°C	1.8	2.6	2.1	3.1	-[e]	-	-	-	-	-	-	-
	4°C	2.5	2.8	2.7	3.1	-	-	-	-	-	-	-	-
	37°C	2.5	2.9	2.5	1.2	-	-	-	-	-	-	-	-
	56°C	2.5	<1	-	<1	-	-	-	-	-	-	-	-
JB10124	-70°C	1.2	<1	1.0	1.6	2.5	2.9	3.3	3.3	2.4	2.5	2.2	3.1
	4°C	<1	1.2	<1	1.5	3.1	2.5	3.3	3.3	2.2	2.1	1.9	3.1
	37°C	<1	<1	<1	<1	3.1	3.1	3.3	3.4	2.2	1.5	2.8	2.7
	56°C	<1	<1	-	<1	3.1	2.2	-	<1	2.2	<1	-	<1
HP10124	-70°C	2.1	2.1	1.5	2.1	3.0	3.1	3.2	3.4	2.1	1.2	2.8	3.1
	4°C	2.0	<1	<1	2.7	2.8	3.1	3.0	3.3	1.8	1.6	2.5	2.7
	37°C	2.0	<1	1.2	1.6	2.8	3.1	3.4	3.3	1.8	1.4	1.6	2.9
	56°C	2.0	<1	-	<1	2.8	1.8	-	<1	1.8	1.1	-	<1

[a] The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of H44/76-based strains expressing the indicated PorA subtype (see material and methods) and is expressed as the mean $\log_{10}$ titer. Serum of each groups was pooled in two samples. Each pool was tested at least four times. The means were compared by the LSD test with a confidence level of 95%: $LSD_{0.05}$ (7-2,$4_{t=0}$) = 0.5; $LSD_{0.05}$ (7-2,$4_{t=3}$) = 0.4; $LSD_{0.05}$ (7-2,$4_{t=6}$) = 0.4; $LSD_{0.05}$ (7-2,$4_{t=12}$) = 0.3; $LSD_{0.05}$ (5-2,$10_{t=0}$) = 0.5; $LSD_{0.05}$ (5-2,$10_{t=3}$) = 0.4; $LSD_{0.05}$ (5-2,$10_{t=6}$) = 0.2; $LSD_{0.05}$ (5-2,$10_{t=12}$) = 0.1; $LSD_{0.05}$ (12,$13_{t=0}$) = 0.6; $LSD_{0.05}$ (12,$13_{t=3}$) = 0.6; $LSD_{0.05}$ (12,$13_{t=6}$) = 0.4; $LSD_{0.05}$ (12,$13_{t=12}$) = 0.2.

[b] Sera were tested for bactericidal activity against the PorB-containing *N. meningitidis* strain HI.5. None of the pools tested induced killing of cells.

[c] PorA subtype against which bactericidal titers are specific.

[d] months.

[e] -.: not determined

Table 4. Effect of freeze-drying on the immune response induced by OMV. Freeze-dried OMV were reconstituted after 3 months storage at 4°C and measured in parallel to liquid OMV stored under the same conditions.

		IgG titers[a]				*Bactericidal titers*[b]		
OMV strain	*Storage conditions*	*7-2,4*[c]	*5-2,10*[c]	*12,13*[c]	*HI.5*[d]	*7-2,4*[c]	*5-2,10*[c]	*12,13*[c]
F91	Liquid, 4°C	3.9	-[e]	-	0.6	2.3	-	-
	F-D[f], 4°C	4.2	-	-	0.6	2.2	-	-
JB10124	Liquid, 4°C	3.8	4.4	4.0	3.6	0.0	2.9	2.8
	F-D, 4°C	3.9	4.2	4.1	3.3	0.0	2.6	2.8
HP10124	Liquid, 4°C	3.7	4.4	4.1	1.7	2.0	3.1	2.4
	F-D, 4°C	3.6	4.0	3.8	1.4	0.9	2.9	0.8

[a] The total IgG titer of each mouse serum was determined by whole-cell ELISA and is expressed as the mean $\log_{10}$ titer. Plates were coated with *Neisseria meningitidis* strains: H44/76 7-2, H44/76 5-2,10, H44/76 12,13 and HI.5 (see materials and methods). The means were compared by the LSD test with a confidence level of 95%: $LSD_{0.05}$ (7-2,4) = 0.3; $LSD_{0.05}$ (5-2,10) = 0.4; $LSD_{0.05}$ (12,13) = 0.3; $LSD_{0.05}$ ($HI.5_{4°C}$) = 0.9.

[b] The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of H44/76-based strains expressing the indicated PorA subtype (see material and methods) and is expressed as the mean $\log_{10}$ titer. Serum of each group was divided in two pools. Each pool was tested at least four times. The means of these tests were compared by the LSD test with a confidence level of 95%: $LSD_{0.05}$ (7-2,4) = 0.5; $LSD_{0.05}$ (5-2,10) = 0.4; $LSD_{0.05}$ (12,13) = 0.8.

[c] PorA subtype against which IgG/bactericidal titers are specific.

[d] IgG titers specific for other structures than PorA. Sera were tested for bactericidal activity against the strain HI.5. None of the pools tested induced killing of cells.

[e] -.: not determined,

[f] F-D: Freeze-dried.

Effect of storage on the immunogenicity of OMV

The ability of monovalent and trivalent OMV stored at –70ºC, 4ºC, and 37ºC to induce high IgG titers against the different PorA subtypes did not decrease with time (Table 2). A pronounced decrease in the ability to elicit an IgG response was only observed with OMV stored at 56ºC. Antibodies induced by monovalent OMV and trivalent OMV from strain HP10124 (containing RmpM) were mainly PorA-specific, as demonstrated by the low titers against the PorA-lacking strain HI.5. OMV from strain JB10124 induced the formation of antibodies against other structures than PorA, probably LPS or impurities (cf. Table 1). The antibody titers against other structures than PorA were fairly constant for samples stored during 12 months at –70ºC and 4ºC, but the titers were lower in sera of mice immunized with OMV stored at 37ºC and 56ºC.

Sera of animals were also tested for the presence of bactericidal antibodies (Table 3). Only antibodies directed against PorA were bactericidal, as determined by the lack of bactericidal serum activity against meningococci of strain HI.5 (not shown). The capability of monovalent OMV to induce a bactericidal response against the PorA P1.7-2,4 was unchanged in samples stored at –70 or 4ºC, and significantly reduced in OMV stored at 37ºC for 12 months. OMV of strain F91 stored for 3 months at 56ºC did not induce a bactericidal response at all. The P1.7-2,4 specific bactericidal activity induced by OMV from strain JB10124 was weak or absent. OMV from strain HP10124 induced anti-P1.7-2,4 bactericidal antibodies only in samples stored at –70ºC. OMV from this strain stored at 4ºC did not induce bactericidal anti-P1.7-2,4 antibodies after 3 or 6 months storage. Surprisingly, after 12 months storage at 4ºC this formulation induced again significant bactericidal antibodies. This is probably due to the variability in the test and the fact that the titers against this subtype are low [5]. Although samples incubated at 37ºC induced detectable bactericidal titers after 6 and 12 months, these titers were not significantly above the background (LSD of 0.4 and 0.6, respectively). The PorA P1.5-2,10 induced the highest bactericidal titers of all three subtypes in both trivalent strains. Also, the anti-P1.5-2,10 bactericidal titers were maintained for OMV of both strains (JB10124 and HP10124) upon storage at all temperatures except 56ºC, where bactericidal activity of these formulations dropped to undetectable levels after 3 months storage. A similar trend was observed for the P1.12,13-specific bactericidal response. The anti-P1.12,13 bactericidal titers induced by trivalent OMV after 12 months were relatively

high (i.e. ≥ 2.7); the OMV from strain HP10124 induced a bactericidal response that was even higher than the one induced by the same OMV freshly prepared.

Freeze-drying and reconstitution did not affect the immunogenicity of monovalent OMV and trivalent OMV of strain JB10124 (without RmpM) (Table 4). Surprisingly, in trivalent OMV containing RmpM, only PorA subtype P1.5-2,10 preserved the capacity to induce a bactericidal response after freeze-drying.

Discussion

The stability of three types of outer membrane vesicle vaccines from *Neisseria meningitidis* was studied, with a detailed *in vitro* and *in vivo* characterization of these OMV. The preservation of the native PorA conformation as well as the structure of the presentation form were monitored, as both are crucial for the quality of the immune response [7]. PorA is a porin, belonging to a class of membrane proteins that appear to be exceptionally stable [20]. The physical stability of purified recombinant PorA has been previously studied, showing its ability to maintain its trimeric membrane arrangement in the presence of low SDS concentrations [15].

As expected, the physicochemical characteristics of PorA-OMV were best preserved by storage at 4ºC. However, partial unfolding of PorA was observed in all three OMV formulations stored at this temperature after 12 months (Fig. 3). Although this unfolding did not measurably affect the antigenicity or the protective immunogenicity generated by OMV, it may be a first step in the degradation leading to decreased immunogenicity of PorA during storage.

Only the incubation of OMV at high temperatures (56ºC) resulted in the total destruction of both the OMV structure and the protein conformation (DLS, EM, SDS-PAGE, ELISA), accompanied by change of the pH. The modification of the PorA structure was accompanied by a dramatic change in the fluorescence emission maximum towards higher wavelengths (from 338 nm to ca. 405 nm), reflecting chemical modifications of PorA, with formation of new fluorescent degradation product(s). This was confirmed using NATrA and NATyA as model compounds. Similar changes in the fluorescence spectrum have been reported for tetanus toxoid incubated at pH 2.5 and 37ºC for 14-28 days [21] and tetanus and diphtheria toxoids stored at 60ºC for 21 days [22]. The modifications of Trp and Tyr described in literature that lead to fluorescence shift are oxidation of Trp or dimerization of Tyr

[23]. Other oxidation reactions (e.g. methionine, histidine) as well as other chemical degradation reactions (e.g. deamidation, chain scission) may also have occurred [24,25], but this was not investigated in this study.

Unfolding of PorA and degradation of the OMV structures was also observed at 37ºC but obviously, much more slowly than at 56ºC. At this temperature, the change in the conformation of PorA could also be observed by SDS-PAGE, with monomerization of PorA (Fig. 3). This was also translated in a drastic drop in antigenicity (Fig. 4). However, a decrease in functional immunogenicity could not be detected, indicating that the epitope investigated in our ELISA system is not the only epitope important for the development of a protective immune response. The unfolding of PorA was accompanied by a reduced stability of the OMV structure, as their particle size decreased (Fig. 1).

Freezing or freeze-drying of OMV resulted in aggregation (Fig. 1). Probably, the freezing step is responsible for this aggregation. During freezing, ice crystal formation may partially rupture the vesicle structure, resulting in formation of larger vesicles and/or aggregates after thawing or reconstitution. The presence of 3% sucrose that could act as cryoprotectant or lyoprotectant was not enough to prevent this aggregation. However, the conformation of PorA did not change upon freeze-thawing and freeze-drying/reconstitution, which may explain the fact that the immunogenicity of aggregated OMV was not decreased. Although the functional immunogenicity of monovalent OMV and trivalent OMV from strain JB10124 was maintained when freeze-dried, lyophilized OMV from strain HP10124 induced a decreased P1.7-2,4- and P1.12,13-specific bactericidal response upon reconstitution. This difference can be related to the strain or the batch. No attempts were made to improve the freeze-drying process by adding other lyoprotectants or cryoprotectants than sucrose.

The trimeric PorA association was more stable in trivalent OMV than in monovalent OMV. This is shown by the relatively higher amounts of trimeric PorA in trivalent OMV incubated at 37ºC (Fig. 3 B, C) and the higher concentration of Gnd-HCl necessary to unfold PorA in trivalent OMV (Fig. 5). As the composition of both trivalent and monovalent OMV is similar, i.e. most of the protein is PorA, this increased physicochemical stability of trivalent OMV could be due to differences in the stability among PorA serosubtypes or differences among strains in the quality of the membrane association of PorA.

It is difficult to correlate physicochemical characteristics of trivalent and monovalent OMV with immunogenicity, as the PorA subtype present in monovalent OMV (P1.7-2,4) is the least immunogenic subtype in trivalent OMV [6]. Antigenicity could not help to predict the bactericidal response induced by OMV stored at high temperatures. Whereas the antigenicity of the P1.4 epitope in all OMV was decreased or completely absent in OMV stored at 37ºC, these OMV were still able to induce polyclonal bactericidal antibodies in mice. Possibly, the immune response was directed against other epitopes of PorA subtype P1.7-2,4 than the epitope recognized by the monoclonal antibody used for the antigenicity test. Also, as previously shown in man [27], the presence of IgG in mice serum was not always accompanied by a bactericidal activity.

The ELISA data (relative to epitope P1.4 of PorA P1.7-2, 4) show that the antigenicity of this epitope was lower than expected in trivalent OMV than in monovalent OMV. This may be due to a lower P1.7-2,4 expression in trivalent OMV, or to a decreased accessibility of the P1.4 epitope in these OMV for the monoclonal antibodies used in the assay. Moreover, the P1.7-2,4-mediated bactericidal serum activity induced by trivalent OMV was reduced as compared to the bactericidal activity induced by monovalent OMV. It was previously shown that bactericidal activity induced by the P1.7-2,4 subtype in trivalent OMV and mixtures of three monovalent OMV was decreased in both cases [26]. This indicates that the exposure of this PorA subtype is similar in trivalent and monovalent OMV, but that P1.7-2,4 – when simultaneously administered with other subtypes– is less efficiently processed and/or that P1.7-2,4 epitopes generate antibodies with low affinity. From the other PorA subtypes in trivalent OMV, P1.5-2,10 induced the highest bactericidal serum activity. This subtype was also previously found to induce the highest bactericidal response in toddlers and school children [27]. Even after 3 months incubation at 56ºC, still bactericidal activity was detected against this subtype. This is very surprising, as no PorA could be seen by SDS-PAGE and samples incubated at 56ºC showed no antigenicity. This bactericidal activity could be due to the presence of a non-detectable fraction of intact, denatured or fragmented PorA in the formulations.

The low molecular weight bands observed by Western blotting, indicating the presence of degradation products containing epitopes located in the variable regions 1 and 2 (VR1 and VR2, respectively) [28], was observed for PorA subtype P1.12,13 in trivalent OMV stored for 12 months at –70ºC, 4ºC or 37ºC. The fragmentation of

PorA did not affect the immunogenicity of this PorA subtype in trivalent OMV, probably because the fragmentation was incomplete.

The presence of RmpM in the OMV from strain HP10124 did not improve their long-term stability. On the contrary, the immunogenicity induced by these OMV was decreased upon freeze-drying (Table 4). It was not expected that these OMV were less stable (after freeze-drying) than trivalent OMV that do not contain RmpM, as this protein has an important role in the localization and stability of PorA trimers in the outer membrane [15]. The yield (by means of the amount of total protein) of OMV purified from HP10124 strain ($RmpM^+$), was also 3-fold lower than the yield in $RmpM^-$ strains (not shown), and the amount of RmpM present in OMV was very low, only detectable by Western blotting. A reason for these relatively low yields may be the strong effect of RmpM on the structure of the bacterial outer membrane. $RmpM^+$ bacteria form a well fitted, tight outer membrane, whereas the outer membrane of $RmpM^-$ stains has an 'over-sized' appearance [29]. The RmpM seems to influence the attachment of the outer membrane to the underlying peptidoglycan layer. The result is that the OMV yield of the RmpM- strains is higher, at least in the case of detergent-free extraction.

In conclusion, trivalent OMV appeared slightly more stable than monovalent OMV, although these differences are not reflected in the *in vivo* activity. All OMV are stable for at least one year if stored at 4ºC or frozen. Freeze-drying provides a good alternative to storage in the liquid state and may be advantageous for long-term (> 1 year) storage.

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CHAPTER 3

Restored functional immunogenicity of purified meningococcal PorA by incorporation into liposomes

Carmen Arigita[a,b], Gideon F.A. Kersten[b], Ton Hazendonk[b], Wim E. Hennink[a], Daan J.A. Crommelin[a], Wim Jiskoot[a]

[a]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands

[b]Laboratory for Product and Process Development, Netherlands Vaccine Institute, Bilthoven, the Netherlands

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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

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 pO_2 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

(PC, Rhône-Poulenc Rorer, Köln, Germany), dimyristoylphosphatidylglycerol (PG, a gift from Lipoïd 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 (4º 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 µg/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 µg/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: $F_0/\Delta F=1/f_a+1/(f_a*K_{SV}*[Q])$, where F_0 is the fluorescence intensity in the absence of

the quencher (acrylamide) and ΔF 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(B: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 56ºC 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 1. Composition of the formulations used in this study.

Formulation	*LOS content (%)*[a]	*DNA content(%)*[a]	*Particle size (nm)*	*PD*[b]	*Antigenicity*[c]
OMV	4.6	1.3	270	0.43	1.00 ± 0.00
OMC	0.5	0.4	612	0.58	0.77 ± 0.17
Purified PorA	0.3	n.d.[d]	-[e]	-[e]	1.64 ± 0.31
Liposomes	0.3	n.d.[d]	215	0.30	1.01 ± 0.18
Denatured OMV	5.3	4.6	105	0.83	0.09 ± 0.02

[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).

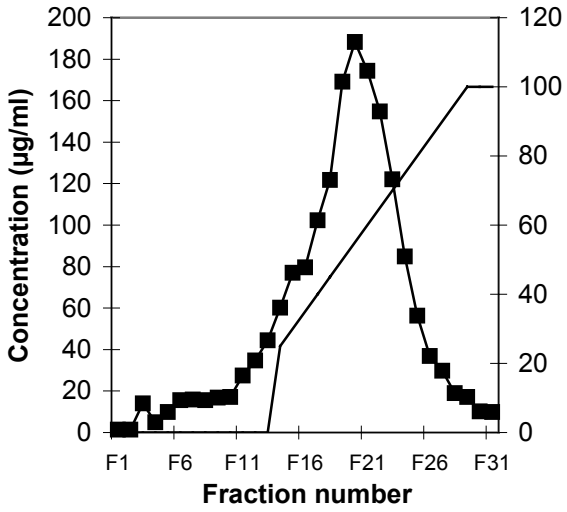

Figure 1. Typical anion-exchange elution chromatogram of disrupted and DNase-treated OMV containing PorA P1.7-2,4. Squares: protein elution pattern, solid line: NaCl gradient, expressed as the percentage of elution buffer containing 1 M NaCl. Fractions 16 to 24 were pooled and dialyzed overnight against elution buffer without NaCl.

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 100ºC (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.

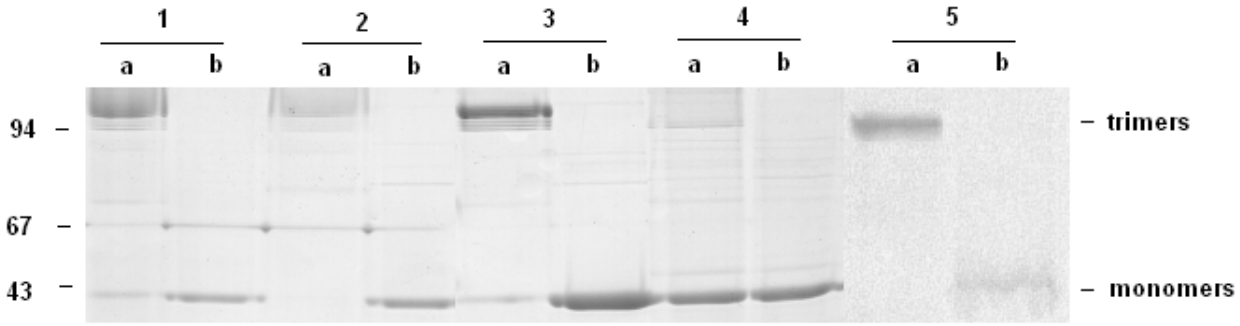

Figure 2. SDS-PAGE of PorA P1.7-2,4 in different formulations. The samples were dissolved in sample buffer containing 0.05% SDS and incubated either at room temperature (a) or 100ºC (b). The molecular weight of reference proteins are indicated at the left (kDa). Lane 1: PorA-OMV, lane 2: PorA-OMC, lane 3: purified PorA, lane 4: denatured PorA-OMV, lane 5: liposomes.

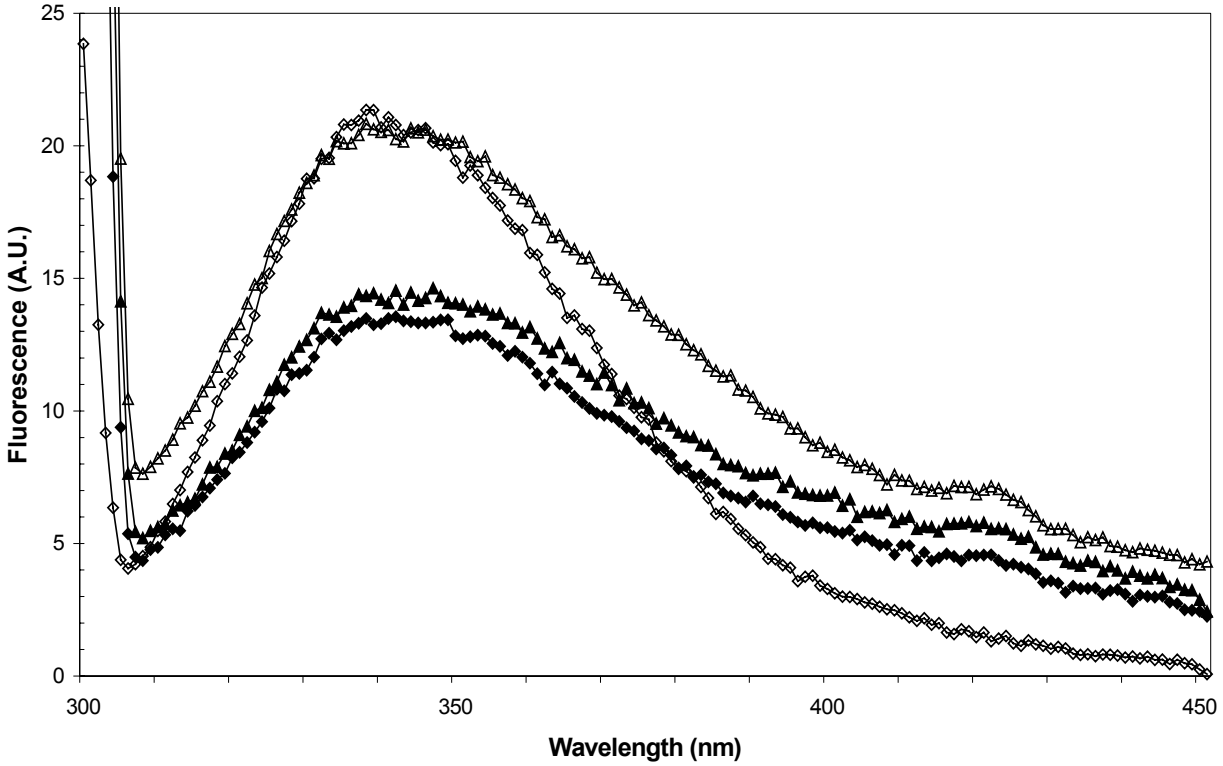

Figure 3. Fluorescence emission spectra of PorA P1.7-2,4 formulations, excited at 295 nm. Closed diamonds: PorA-OMV, open diamonds: purified PorA, closed triangles: denatured PorA-OMV, open triangles: PorA-OMC.

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 (λ_{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.

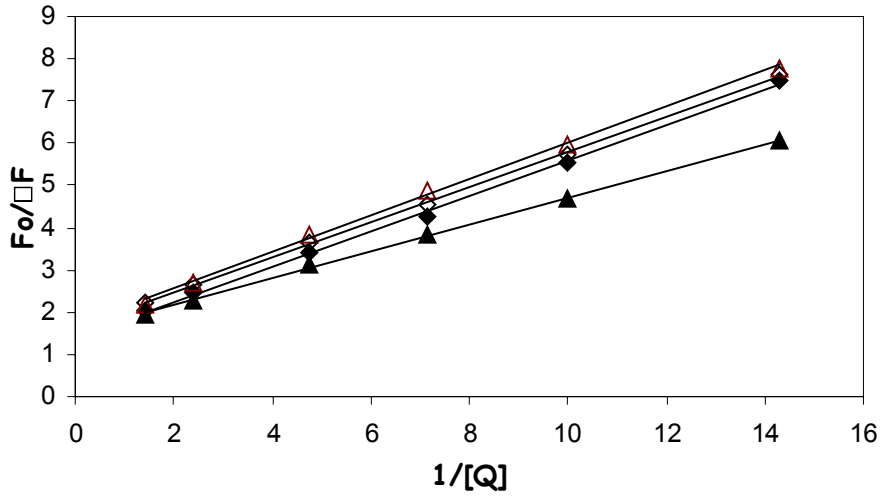

Figure 4. Modified Stern-Volmer plots of the quenching of PorA fluorescence by acrylamide. Quenching experiments were performed by adding aliquots of acrylamide to PorA formulations (ca. 100 µg/ml). The data were fitted by linear regression of 4 sets of measurements and analyzed with a modification of the Stern-Volmer equation: $F_0/\Delta F=1/f_a+1/(f_a*K_{SV}*[Q])$. 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[1)]

Formulation	K_{sv} (M^{-1})	f_a	r^2
OMV	3.47 ± 0.84	0.72 ± 0.06	0.9985
Purified P1.4	3.95 ± 0.45	0.62 ± 0.07	0.9995
OMC	3.95 ± 0.48	0.59 ± 0.01	0.9981
Denatured OMV	4.91 ± 0.81	0.65 ± 0.05	0.9989

[1)] Data were fitted by linear regression. From the fitted line and according to $F_0/\Delta F=1/f_a+1/(f_a*K_{SV}*[Q]$, f_a 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.

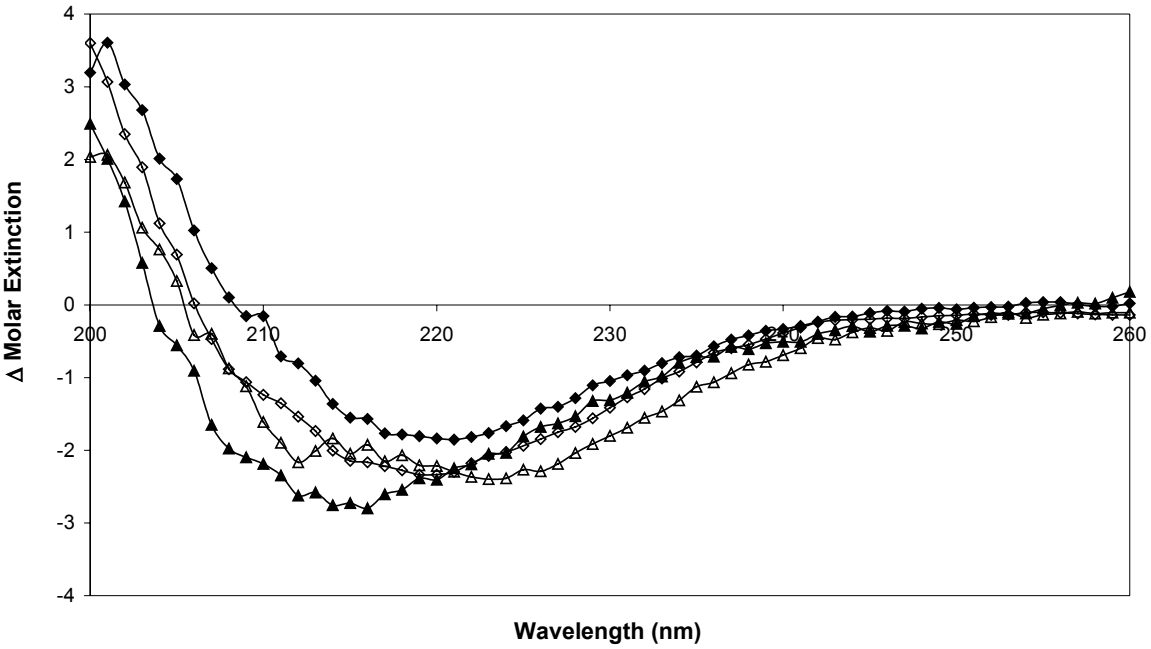

Figure 5. Far-UV CD spectra of PorA P1.7-2,4. Closed diamonds: PorA-OMV, open diamonds: purified PorA, closed triangles: denatured PorA-OMV, open triangles: PorA-OMC.

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 ß-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 compostion 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. $AlPO_4$ 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

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 $AlPO_4$: effect of the presentation form.

Antigen[a]	*Dose (µg)*	*Whole-cell ELISA titer*[b]					*Bactericidal titer*[c]	*Responders*
		IgG	*IgG1*	*IgG2a*	*IgG2b*	*IgG3*		
OMV	1.5	4.5	3.7	4.2	4.4	0.8	2.40 ± 0.49	6/8
OMC	1.5	4.0	2.3	3.6	4.0	1.7	2.07 ± 0.48	5/8
Pure PorA	1.5	3.3	3.3	2.5	3.0	0.3	<1.0	0/8
Pure PorA	10	4.2	4.3	3.7	4.1	0.4	<1.0	0/8
Denatured OMV	1.5	4.3	4.0	3.6	4.4	0.9	1.35 ± 0.25	4/8

[a] Group 5 was measured in a separate experiment. As bridging group OMV were used, giving similar results as in the previous experiment.
[b] 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) = 0.8; $LSD_{0.05}$ (IgG2a) = 0.5; $LSD_{0.05}$ (IgG2b) = 0.6; $LSD_{0.05}$ (IgG3) = 1.5.
[c] 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 $\log_{10}$ titer of the responders $\pm$ 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 $AlPO_4$ 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).

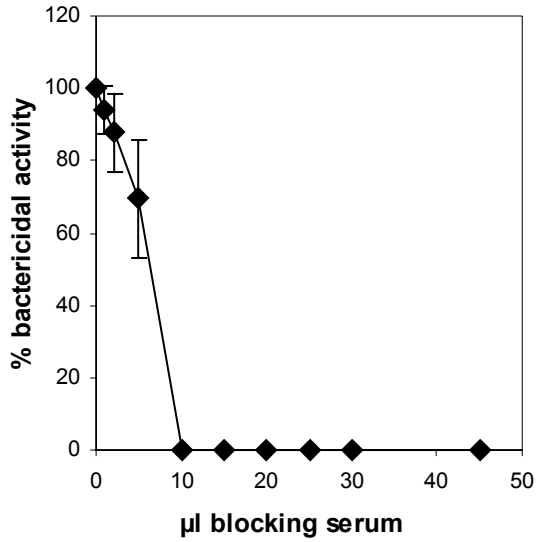

Figure 6: Effect of blocking antibodies elicited with purified PorA (table 3, group 4 pooled sera) in bactericidal response of anti-OMV antiserum (table 3, group 1 pooled sera). Bactericidal antiserum (5µl) was diluted 10-fold in buffer containing increasing volumes of the blocking antiserum. The results are shown as mean values (±SD) of 3 measurements.

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 $AlPO_4$, immunizations were performed without adjuvant (Table 4). In the absence of $AlPO_4$, OMV still induced substantial

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 the immunogenicity of purified PorA by incorporation into liposomes

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

[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 $AlPO_4$ 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 $AlPO_4$ 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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CHAPTER 4

Liposomal meningococcal B vaccination: role of dendritic cell targeting in the development of a protective immune response

Carmen Arigita[a,b], Lisette Bevaart[c], Linda A. Everse[a], Gerben A. Koning[a], Wim E. Hennink[a], Daan J. A. Crommelin[a], Jan G. J. van de Winkel[c], Martine J. van Vugt[c], Gideon F. A. Kersten[b], Wim Jiskoot[a]

[a]*Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands*

[b]*Laboratory for Product and Process Development, Netherlands Vaccine Institute, Bilthoven, the Netherlands*

[c]*Immunotherapy Laboratory, University Medical Center Utrecht, Utrecht, the Netherlands*

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Abstract

The effect of targeting strategies for improving the interaction of liposomal PorA with dendritic cells (DC) on the immunogenicity of PorA was investigated. PorA, a major antigen of *Neisseria meningitidis,* was purified and reconstituted in several types of (targeted) liposomes, i.e. by use of mannose or phosphatidylserine (PS) as targeting moieties or with positively charged liposomes. We studied the efficiency of liposome uptake and its effect on the maturation of and IL-12 production by murine DC. Moreover, mice were immunized subcutaneously to study the localization and immunogenicity of PorA-liposomes. Uptake of liposomes by DC was significantly increased for targeted liposomes and resulted in the maturation of DC, but to varying degrees. Maturation markers (CD80, CD86, MHC class II, CD40) showed enhanced expression on DC incubated with targeted PorA-liposomes relative to those incubated with non-targeted PorA-liposomes. Moreover, only the uptake of targeted PorA-liposomes induced production of IL-12 by DC, with levels similar to those produced by LPS-pulsed DC. Targeted PorA-liposomes administered subcutaneously had an increased localization in draining lymph nodes with respect to non-targeted PorA-liposomes. Liposomes in draining lymph nodes interacted preferentially with antigen presenting cells (APC), and this was enhanced with targeted PorA-liposomes. Immunization studies showed an improvement of the bactericidal antibody response (i.e. increased number of responders) generated by targeted PorA-liposomes compared to non-targeted ones or LPS-containing outer membrane vesicles (OMV). In conclusion, the use of targeted PorA-liposomes results in an improved uptake by and activation of DC and an increased localization in draining lymph nodes. These effects correlate with an increased percentage of mice showing serum bactericidal activity.

Introduction

Infections caused by *Neisseria meningitidis* are a serious threat to children and young adults. Vaccines based on capsular polysaccharides are available against serogroups A, C and W135 [1]. A polysaccharide-based vaccine is not available for serogroup B meningococci, due to the low immunogenicity of their polysaccharides and the risk of induction of autoantibodies that cross-react with glycosylated host antigens. Still, it remains important to develop new vaccines for serogroup B meningococci that make use of other antigenic epitopes in the outer membrane, as respectively 63% and 32% of the reported cases of meningococcal disease in Europe and the USA are attributable to serogroup B [2,3].

Class 1 porin protein (PorA) is a good vaccine candidate because is a major antigen and induces a strong bactericidal immune responses [4]. Serogroup B meningoccal vaccines are being developed by different groups, based on PorA formulated in outer membrane vesicles (OMV) [4,5,6]. However, despite purification, OMV still contain small amounts of toxic LPS. Furthermore, their composition is difficult to manipulate because it is governed by the strain from which the OMV are derived. As an alternative for OMV, liposomes are an attractive presentation form for purified membrane proteins like PorA, as the liposomal bilayer mimics the membrane environment Moreover, liposomes are well-defined structures allowing improvement of the formulation, e.g. through attachment of targeting ligands or variations in the (membrane) composition.

A rational approach for making better vaccines is to improve the delivery of (liposomal) antigens to dendritic cells (DC). Dendritic cells are the most efficient APC, being able to initiate and modulate immune responses both *in vitro* and *in vivo* [7]. Several strategies can be conceived to target liposomes to DC. On the surface of DC, various receptors of the C-type lectin family are expressed, including the mannose receptor (MR) and DEC-205 [8]. Receptors of this family have in common the recognition of bacterial structures, and antigen capture by these receptors has been shown to result in an efficient antigen presentation [9,10]. Another efficient uptake mechanism by DC and other APC is the endocytosis of apoptotic bodies. This is probably mediated by specific recognition of phosphatidylserine (PS) at the surface of apoptotic cells [11].

The aim of this study was to design a well-defined PorA-based vaccine with improved immunogenicity compared to OMV by formulating PorA into liposomes targeted to DC. Three strategies were used to improve the uptake of PorA-liposomes by DC: liposomes with mannose ligands on the surface for targeting C-type lectin receptors; liposomes containing PS for targeting to the PS receptor; and positively charged liposomes to induce an efficient uptake mediated by non-specific electrostatic interactions with (negatively charged) DC membranes. As control, non-targeted negatively charged PorA-liposomes and OMV were used. *In vitro*, the uptake of targeted and non-targeted PorA-liposomes by bone marrow-derived murine DC was studied, as well as their ability to induce functional maturation of DC. *In vivo*, the localization of targeted liposomes (Man-liposomes) and non-targeted liposomes was compared in draining lymph nodes after subcutaneous immunization of mice. Finally, the immune response of all liposomal PorA-formulations was studied in mice and compared with the immune response induced by (standard) OMV. Our data suggest that an increased antigen loading of DC through the use of targeted liposomes results in an improved immune response. Therefore, targeted liposomes are attractive presentation forms for future development of type B meningococcal vaccines.

Materials and methods

Materials

All phospholipids used were synthetic. Dimyristoyl phosphatidylcholine (PC) was purchased at Rhône-Poulenc Rorer (Köln, Germany). Dimyristoyl phosphatidylglycerol (PG) was a gift from Lipoïd GmbH (Ludwigshafen, Germany). Cholesterol (Chol) was obtained from Sigma (Zwijndrecht, The Netherlands). Dimyristoyl phosphatidylserine (PS), dimyristoyl phosphatidylethanolamine (PE) and dimyristoyl trimethylammonium propane (TAP) were purchased from Avanti Polar Lipids (Alabaster, AL). Mannosylated PE (Man-PE) was prepared by ethyldiisopropylamine-promoted coupling of PEA and mannopyranosylphenyl isothiocyanate (Sigma) in chloroform/methanol (7:1 v/v). The compound was purified by preparative TLC, and its structure was confirmed by nuclear magnetic resonance (^{1}H-NMR), liquid chromatography and mass spectrometry (LC-MS).

PorA was purified from outer membrane vesicles obtained from *Neisseria meningitidis* strain F91 (P1.7-2,4, $PorB^-$, $RmpM^-$, low expression of Opa/Opc) as previously described [12]. Prior to incorporation of PorA into liposomes, the protein was precipitated with 80% (v/v) ethanol at -20ºC and solubilized in 150 mM n-octyl β-d-glucopyranoside (Sigma) in Tris-buffered saline (TBS, containing 50 mM Tris-HCl, 150 mM NaCl pH 7.4).

Monoclonal antibodies against CD80 (clone 1G10), CD86 (clone GL1), FITC-labeled mAb against CD11b (clone M1/70), phycoerythrin-labeled mAb against Gr-1 (clone RB6-8C5) and biotinylated mAb against CD11c (clone HL3) and CD45R/B220 (clone RA3-6B2) were obtained from BD PharMingen (San Diego, CA). Streptavidin-phycoerythrin was purchased from Becton Dickinson (San Jose, CA). Biotinylated mAb against F4/80 (clone Cl: A3-1) was obtained from Serotec (Oxford, UK). PE-labeled antiCD40 mAb (clone 3.23) was purchased from Immunotech (Marseille, France), and mouse anti-rat IgG (H+L) $F(ab')_2$ fragment was purchased from Jackson ImmunoResearch (West Grace, PA). M5/114 anti-MHC class II [13] was kindly provided by Dr. Georg Kraal (Vrije Universiteit, Amsterdam, The Netherlands).

Preparation and characterization of PorA- liposomes

Liposomes were made of PC, PG, Chol and Man-PE in a 8:2:2:0.6 mol ratio (Man-liposomes); PC, PS and Chol in a 8:2:2 ratio (PS-liposomes) or PC, TAP, Chol in a 8:2:2 ratio (TAP-liposomes). Control liposomes consisted of PC, PG and Chol in a 8:2:2 mol ratio (PG-liposomes). Liposomes were prepared by detergent dilution and characterized as previously described [12]. The initial protein/lipid ratio used was 25 µg protein/µmol lipid. As fluorescent marker, 0.1 mol % 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD; Molecular Probes, Leiden, The Netherlands) was included in the bilayer when indicated. Liposomes were filtered through sterile 0.45-µm filters. The zeta potential of the liposomes in 3% sucrose, 10 mM Tris pH 7.4 was measured with a Zetasizer 2000 with an aqueous dip-in cell and a computer with PCS software version 1.35 (Malvern Ltd., Malvern, UK). Protein content was determined according to Peterson [14] with BSA (Pierce, Rockford, IL) as standard. The phospholipid content was determined according to Rouser [15] with sodium phosphate (Merck, Darmstadt, Germany) as standard. The presence of mannose on the outside of Man-liposomes was studied by

monitoring liposome aggregation in time in the presence of 8 µg/ml of concanavalin A (ConA, Sigma) 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), a remote interface controller and PCS software.

The correct folding of PorA into liposomes was analyzed by SDS-PAGE in 'native' gels as previously described [16]. 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 [12].

Mice

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

Generation of DC and characterization following incubation with PorA-liposomes

Bone-marrow derived DC were obtained as described by Inaba et al. [17]. Briefly, bone marrow was flushed from mouse femora, erythrocytes were lysed, and cells were grown at 1×10^6/ml in filtered culture medium consisting of RPMI 1640 medium with 10% FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin in the presence of 10 ng/ml murine GM-CSF (Immunex, Seattle, WA) and 50 ng/ml murine IL-4 (R&D Systems, Minneapolis, MN). Non-adherent cells were replated on day 1, and cells still non-adherent at days 2 and 4 were removed from the cultures, with concomitant refreshment of culture medium. On day 7, non-adherent and loosely adherent cells were harvested. These cells were $MHCII^+$, $CD11c^+$and $CD11b^+$. From these, 3×10^6 cells per well were plated with fresh culture medium together with PorA-liposomes (80 nmol phospholipid) and further incubated for 48 hours at 37ºC. As controls, cells were incubated in medium, with non-labeled liposomes or with LPS (1 µg/ml). After incubation, cells were harvested and analyzed. The supernatants were kept at -70ºC until IL-12 quantitation. IL-12 was quantified in 48-hour supernatants of DC cultures incubated with liposomes or controls using an OptEIA ELISA kit (BD PharMingen) according to the manufacturer's protocol.

Flow cytometry

Cells (1×10^5 in 50 µl medium) were blocked with 5% heat-inactivated mouse serum in FACS buffer (PBS, 0.1% azide, 1% BSA) for 30 min at room temperature. Blocked cells were then washed with FACS buffer and incubated with relevant antibodies for 20 min at room temperature. After this time, cells were washed and, if required, incubated with a specific fluorescently labeled secondary antibody. Cells (1×10^4) were analyzed by flow cytometry using a FACS Calibur and Cell Quest software (BD Biosciences). Win MDI 2.8 software was kindly provided by Joseph Trotter and was used for further analysis. Negative controls included unstained cells, cells stained with isotype control Ab, and cells stained with only the secondary antibody.

Confocal laser scanning microscopy

Cells (1×10^5), incubated with PorA-liposomes under similar conditions as for flow cytometry studies, were washed with 2 ml PBS and subsequently fixed in 4% formaldehyde in PBS for 20 min at room temperature. After fixation, cells were washed twice with PBS and mounted on glass slides with FluorSave reagent (Calbiochem, San Diego, CA). Slides were examined by using a confocal laser scanning microscope equiped with a 488 nm Argon, 568 nm Krypton and 633 nm HeNe laser (Leica TCS-SP, Leica Microsystems, Rijswijk, the Netherlands). Images were analyzed using Leica TCS-SP Power Scan software.

In vivo studies

Localization study: mice (3 animals per group) were immunized subcutaneously in the groin (0.25 ml, 1000 nmol phospholipid/mouse) with DiD-labeled PorA-liposomes. After 48 h, popliteal and inguinal lymph nodes located near the injection site were removed and pooled for each mouse. Lymph nodes were treated with 160 U/ml collagenase type 3 (Worthington Biochemicals, Lakewood, NJ) and 180 U/ml DNase I (Sigma) for 45 min at 37ºC and forced through a 70-µm filter. The resultant cells were washed with FACS buffer and analyzed by flow cytometry as described above.

Immunization study: mice (8 animals per group) were immunized subcutaneously (0.25 ml/mouse) on day 0, 14 and 28 with 1.5 µg liposomal PorA or

PorA-containing OMV. Sera were collected at day 42 and stored at -20ºC until analysis.

Serum analysis

The antibody titers (total IgG and individual isotypes) in mouse sera were determined in an ELISA as previously described [18]. *Neisseira meningitidis* strain H44/76 (B:15P1.7-2,4:L3,7,9) and the H44/76-derived mutant strain HI.5 (lacking PorA) were used. 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), using tetramethyl benzidine (TMB) as substrate. The titer is defined as the dilution of the serum where 50% of the OD_{max} (λ=450 nm) in the assay is reached. OD_{max} is the absorbance obtained with 2-fold diluted serum.

The serum bactericidal activity was measured as previously described [19] against the *N. meningitidis* strains: H44/76 (B:15P1.7-2,4:L3,7,9) and HI5 ($PorA^-$). Sera from mice were heat inactivated for 30 min at 56ºC prior to use. Bacteria were incubated for 10 to 15 min at room temperature with the serum samples before the addition of complement. As complement source, 80% (v/v) rabbit serum 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

Antibody and bactericidal titers were log_{10} transformed in order to obtain a gaussian distribution. ANOVA 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 groups included both responders and non-responders. Individual values are given together with the mean of the indicated responders.

Results

Liposomal PorA formulations

All liposomes had particle sizes around 200 nm (Table 1). As expected, PG-, PS- and Man-liposomes were negatively charged, whereas TAP-liposomes were positively charged, as indicated by their zeta potential. The initial protein/lipid ratio (25 µg protein/µmol lipid) was preserved in the resulting liposomes. The presence of accessible mannose residues in the surface of Man-liposomes was confirmed by their aggregation in the presence of the carbohydrate binding lectin ConA (Fig. 1).

Table 1. Characteristics of PorA-liposomes[a]

Liposome formulation	*Composition*	*Particle size (nm)*	*PD*[b]	*Zeta potential*[c] *(mV)*	*Antigenicity*[d]
PG-liposomes	PC:PG:Chol	191	0.27	-54	0.87
Man-liposomes	PC:PG:Chol:Man-PE	201	0.34	-37	0.51*
PS-liposomes	PC:PS:Chol	228	0.35	-50	0.73
TAP-liposomes	PC:TAP:Chol	175	0.40	+44	0.57*

[a] Data in this table are representative for PorA-liposomes (with or without DiD) used in the experiments described.
[b] Polydispersity: indication of the size distribution of the liposomes; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse suspension.
[c] Surface charge of liposomes.
[d] Ratio between the protein concentration determined by ELISA and the protein concentration determined according to Peterson [21]. As a reference, the antigenicity of PorA in the outer membrane (OMV) was arbitrarily set at 1. * $p < 0.01$.

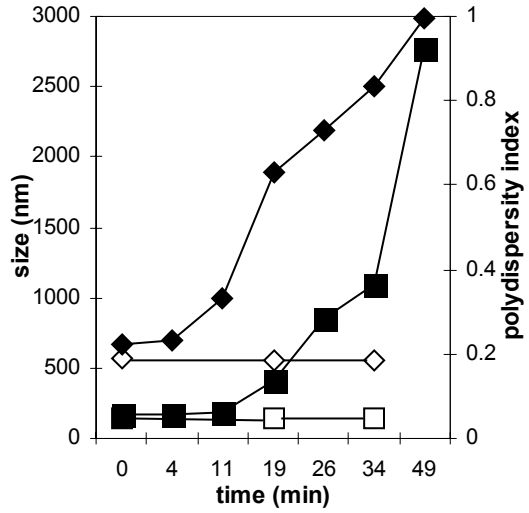

Figure 1. ConA mediated aggregation of Man-liposomes measured by DLS. Squares: particle size of Man-liposomes (closed symbols) and (control) PG-liposomes (open symbols). Diamonds: polydispersity index of Man-liposomes (closed symbols) and (control) PG-liposomes (open symbols).

A correct PorA conformation is necessary in order to induce an optimal B-cell response that will result in the production of protective, i.e. bactericidal, antibodies. In order to investigate the PorA conformation, SDS-PAGE under mild conditions was

performed. In all formulations, PorA was present as trimers (Fig. 2). Even in samples incubated for 5 min at 100ºC, some PorA-trimers were still visible, suggesting that the incorporation of PorA into a lipid bilayer partially protects the protein from heat-denaturation.

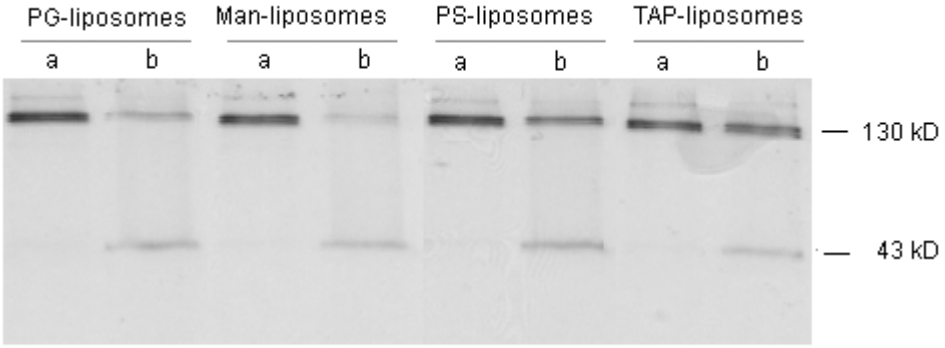

Figure 2. SDS-PAGE of PorA-liposomes. Each sample was dissolved in sample buffer containing 0.05% SDS and incubated either at room temperature (lanes a) or at 100ºC (lanes b). The number on the right indicates the molecular weight of PorA monomers (43 kD) and trimers (130 kD).

The ability of PorA-liposomes to interact with specific bactericidal mAb (i.e. antigenicity) was measured by ELISA. The antigenicity of PorA in PG- and PS-liposomes was only slightly lower than the antigenicity of the same PorA in outer membrane vesicles (OMV, Table 1), indicating that PorA is preferentially embedded in the liposomal bilayer with relevant epitopes directed to the outside. In Man- and TAP-liposomes the antigenicity of PorA was significantly reduced ($p < 0.01$), possibly caused by steric hindrance due to mannose molecules located on the surface and the positive charge, respectively.

Interaction and uptake of PorA liposomes by bone-marrow derived DC

We studied the interaction of DiD-labeled PorA-liposomes with DC. After 4 h, only the incubation of DC with TAP-liposomes resulted in high fluorescence levels of the cells (not shown). After 48 h incubation with all PorA-liposome types, almost all cells showed fluorescence, regardless of the targeting moiety (Fig. 3, inset). However, differences among mean fluorescence intensity (MFI) of cells were found. The use of targeted PorA-liposomes resulted in an increased fluorescence of DC as compared to non-targeted liposomes. The highest MFI was observed for DC incubated with TAP liposomes. Incubation with PS- and Man-liposomes induced

intermediate MFI, and cells incubated with (non-targeted) PG-liposomes showed the lowest MFI (Fig. 3).

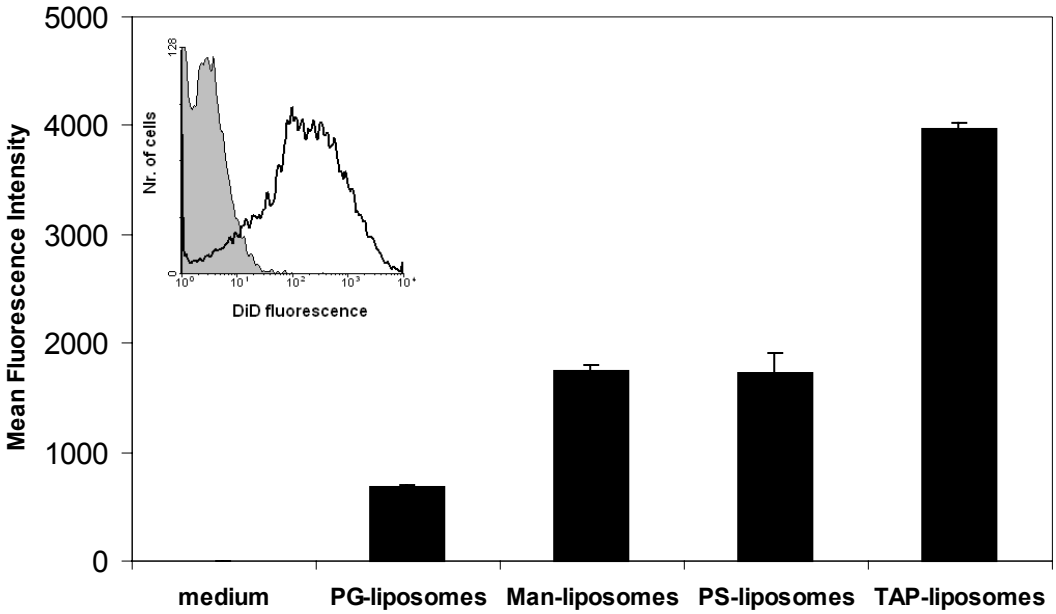

Figure 3. Interaction of DiD-labeled liposomes with DC after 48 h incubation. The results are indicated as mean fluorescence intensity (MFI), and error bars represent SEM of 10-fold measurements. Inset: typical example of the shift observed in the fluorescence of DC cultured for 48 h in the presence of fluorescent PorA-liposomes (shown: PG-liposomes).

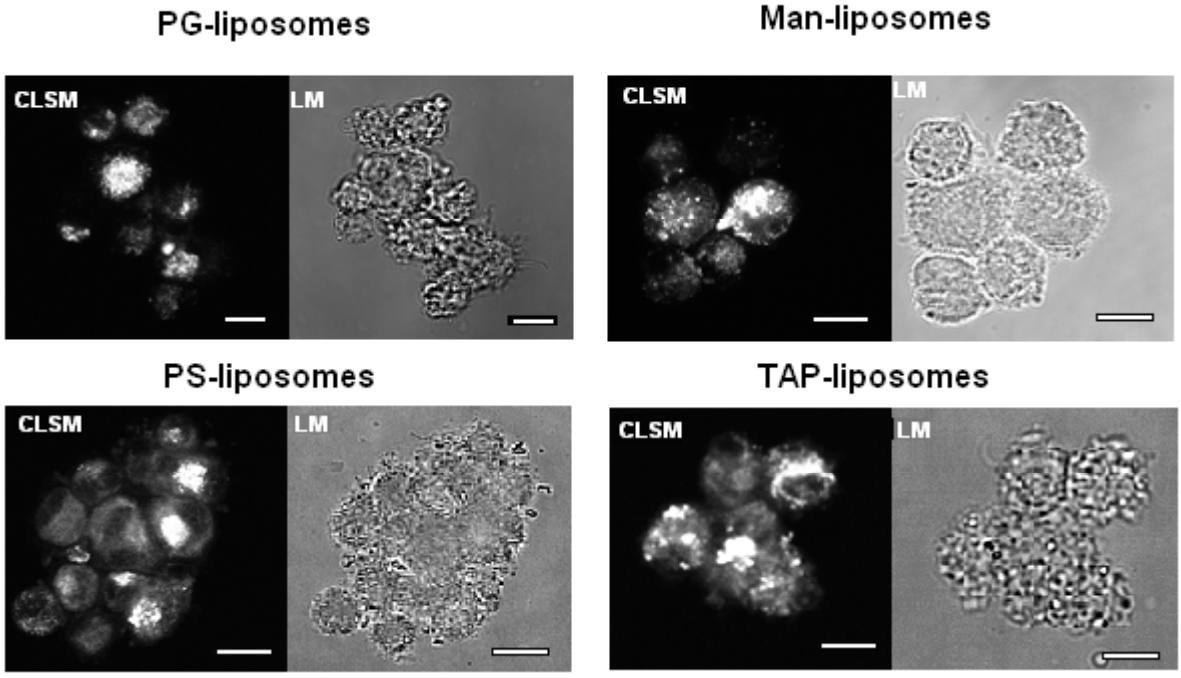

Figure 4. Internalization of PorA-liposomes by DC. Typical examples of confocal laser scanning micrographs (CLSM) and light microscopy images (LM) of DC after 48h incubation with PG-liposomes, Man-liposomes, PS-liposomes and TAP-liposomes, as indicated. Bars: 10 µm.

Cells were also visualized by confocal microscopy (Fig. 4). After incubation for 48 h, bright punctate fluorescence could be observed intracellularly, indicating internalization of the liposomes. In agreement with the flow cytometry data, the fluorescence intensity was increased in cells incubated with targeted liposomes (Man-, PS- and TAP-liposomes). Moreover, when incubated with positively charged TAP-liposomes, all cells showed fluorescence. That was not the case with other liposome types, indicating that the positive charge on the surface of PorA-liposomes induced an (unspecific) electrostatic interaction with the negative surface of cultured cells.

Maturation and IL-12 production by liposome-pulsed DC

Bone marrow-derived DC were characterized with specific monoclonal antibodies (MHC Class II, CD80, CD86 and CD40) by flow cytometry (Fig. 5A). Day 7 DC (t=0) showed moderate levels of MHC Class II and low levels of CD80, CD86 and CD40 expression. After 48 h incubation (day 9 DC), the levels of these markers were increased. LPS-pulsed DC showed a clear maturation, with increased expression of MHC Class II, CD80, CD86 and CD40.

All liposomal formulations induced increased levels of maturation markers on DC after 48 h incubation (Fig. 5B). A substantial increase in MHC Class II (comparable to MHCII expression in LPS-pulsed DC), CD80, CD86 and CD40 expression was observed with all targeted PorA-liposomes (Man-, PS- and TAP-liposomes). Much less pronounced effects were observed for non-targeted (PG) liposomes, suggesting that enhanced uptake of PorA-liposomes through targeting leads to increased maturation.

The IL-12 concentration was measured in supernatants of DC pulsed with liposomes and LPS (Fig. 6). After 48 h incubation, only targeted PorA-liposomes (Man-, PS- and TAP-liposomes) had induced IL-12 production to the same extent as LPS. Non-targeted liposomes failed to induce IL-12 production by cultured DC. Differences were also found in the concentration of IL-12 in supernatants of the different types of targeted liposomes. For DC incubated with Man-liposomes, the IL-12 production was significantly higher than that of DC incubated with PS- or TAP-liposomes ($p < 0.01$). These findings suggest that, besides increased maturation, targeted PorA-liposomes induce functional maturation of DC.

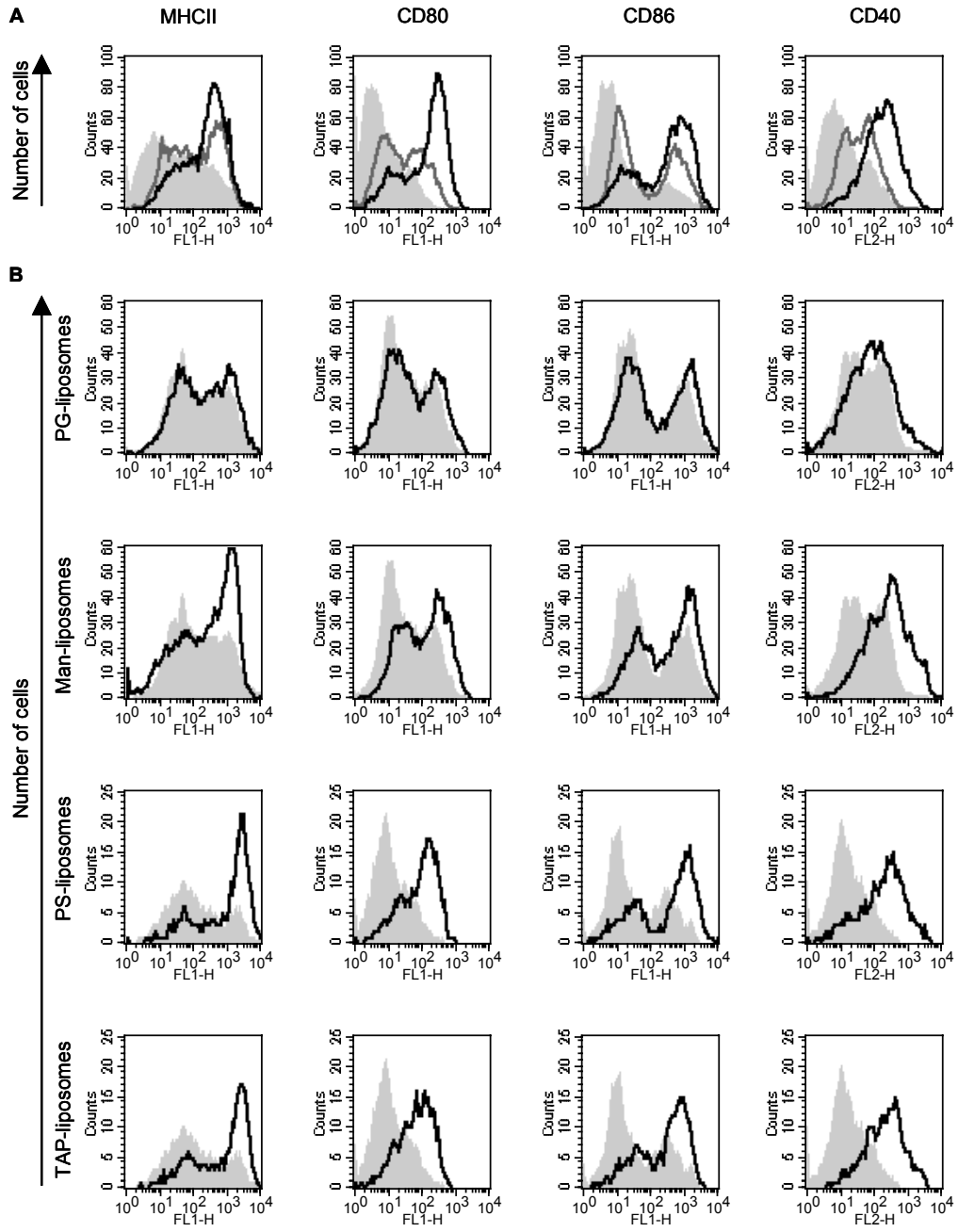

Figure 5. Panel A. Characterization of cultured DC. Histograms illustrate the expression of specific cell surface markers on cells at day 7 (t=0, filled histograms), day 9 (t=48 h, gray line) and in DC pulsed for 48 h with LPS (black line). Data are representative of 4 independent experiments. **Panel B:** Effect of uptake of PorA-liposomes on DC maturation. Histograms illustrate the expression of specific cell surface markers on DC pulsed for 48 h with the medium only (filled histograms) or with the indicated liposomes (black line). Data are representative of at least two experiments.

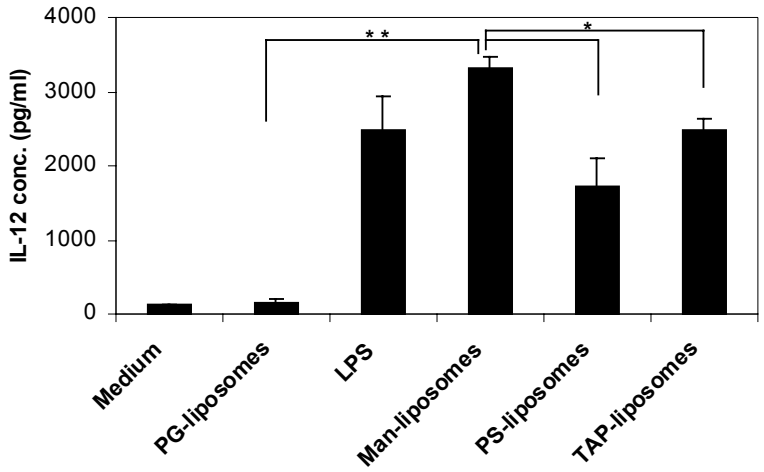

Figure 6. IL-12 quantitation in supernatants of DC cultured for 48 h. The results are shown as mean (± SEM) of at least 4 measurements. * $p < 0.01$; ** $p < 0.001$.

In vivo localization of PorA liposomes

The localization of fluorescently-labeled PG- and Man-liposomes after immunization was studied in draining (popliteal and inguinal) lymph nodes. The choice of these formulations was based on the results obtained with bone marrow-derived DC, where targeted liposomes showed an improved binding, uptake and subsequent DC maturation with respect to control PG-liposomes. From the three types of targeted liposomes, Man-liposomes induced the highest IL-12 production and therefore were chosen for the localization study.

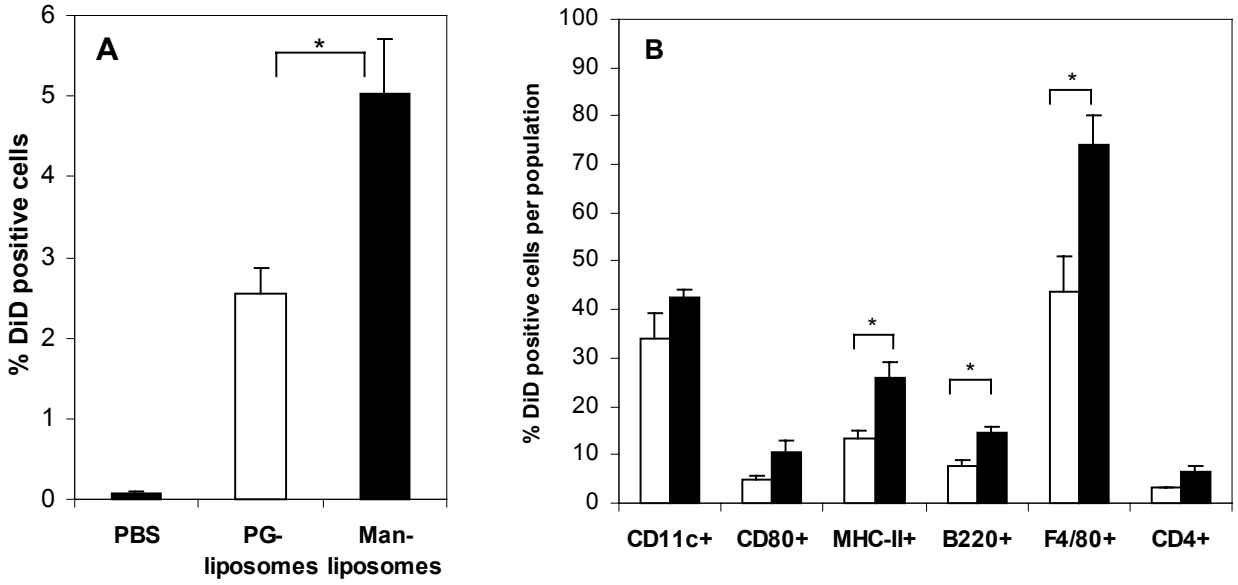

Figure 7. Localization of PorA-liposomes in draining lymph nodes. Panel A. Percentage of fluorescent cells isolated from draining lymph nodes of control mice and mice immunized with PG-liposomes and Man-liposomes. B. Percentage of fluorescent cells found in various cell populations. White bars: mice immunized with PG-liposomes; black bars: mice immunized with Man-liposomes. The results are shown as mean ± SEM of three mice.* $p < 0.05$.

The number of cells isolated from draining lymph nodes increased with 50% in mice immunized with both PG-liposomes and Man-liposomes as compared to control mice (injected PBS). No differences were found in the number of cells isolated from animals immunized with the two types of PorA-liposomes (PG- and Man-liposomes). The percentage of fluorescent cells found in animals immunized with Man-liposomes was significantly higher than in animals immunized with PG-liposomes ($p = 0.031$; Fig. 7, panel A). This indicates that Man-liposomes effectively target the draining lymph nodes *in vivo*. PorA-liposomes were preferentially associated in draining lymph nodes with APC. Around 30% of $CD11c^+$ cells, 15-25% of MHC class II^+, 10-15% of $B220^+$ and 50-70% of $F4/80^+$ cells showed DiD fluorescence, whereas only 5% of $CD4^+$ cells were fluorescent. Interestingly, targeted liposomes (i.e. Man-liposomes) showed a significantly increased interaction with MHC class II^+, $B220^+$ and $F4/80^+$ cells as compared with non-targeted liposomes ($p < 0.05$; Fig. 7, panel B), indicating that the use of mannosylated liposomes results in preferential uptake by APC.

Immune responses generated by PorA liposomes

The immune response generated by PorA-liposomes and OMV was studied *in vivo*. All targeted liposomes (Man-, PS- and TAP-liposomes) were tested, together with non-targeted liposomes and OMV as control. This choice was based on *in vitro* data, where the antigenicity of PorA in PorA-liposomes was variable (lower for Man- and TAP-liposomes than for PS- and PG-liposomes; Table 1). On the other hand, all targeted PorA-liposomes were able to improve the interaction with DC and induce their functional maturation (i.e. IL-12 production).

Sera (day 42) were analyzed not only for the presence of IgG and the IgG isotype distribution, but also for their functionality, i.e. complement-mediated bactericidal serum activity. Despite the variable antigenicity, all formulations induced similarly high IgG titers (Table 2). No differences were found in the isotype distribution of IgG in sera of mice immunized with the different liposomal PorA formulations and OMV. In contrast with what was expected from the *in vitro* data, the mean bactericidal titers of the responding mice were similar in all groups (Table 2). However, the percentage of responding mice increased in the groups immunized with targeted liposomes with respect to PG-liposomes and OMV (both non-targeted; Table 2).

Table 2. Humoral immune response induced by PorA-liposomes and OMV.

Antigen	*Whole-cell ELISA titer*[a]					*Bactericidal assay*	
	IgG	*IgG1*	*IgG2a*	*IgG2b*	*IgG3*	% responders	titer[b]
OMV	3.40	3.16	3.21	3.52	2.87	62.5	2.38 ± 0.29
PG-liposomes	3.34	3.19	3.15	3.49	2.77	50	2.25 ± 0.52
Man-liposomes	3.26	2.98	3.15	3.30	2.84	87.5	2.30 ± 0.49
PS-liposomes	3.40	3.01	3.20	3.33	2.78	100	1.99 ± 0.47
TAP-liposomes	3.34	3.09	3.51	3.56	2.71	87.5	1.88 ± 0.54

[a] The titer of each anti-PorA 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)=0.23; $LSD_{0.05}$ (IgG1)=0.35; $LSD_{0.05}$ (IgG2a)=0.29; $LSD_{0.05}$ (IgG2b)=0.3; $LSD_{0.05}$ (IgG3)=0.35.

[b] The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of strain H44/76 7-24 and is expressed as the averaged mean $\log_{10}$ titer of the responders ± SD.

Discussion

In this study, we designed well-defined liposomal PorA formulations, aiming to improve the immunogenicity of PorA. DC in the periphery capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate immune responses [7]. The question is then: does an enhancement of antigen loading of DC result in an improved immune response? In order to address this question, diverse strategies were chosen to increase the uptake of PorA-liposomes by DC: first, receptor-mediated targeting towards a) receptors with specificity for mannose (Man-liposomes) [9,10] or b) the PS receptor (PS-liposomes) [11]; second, increasing the uptake of PorA-liposomes by non-specific electrostatic interaction of positively charged liposomes (TAP-liposomes) with (negatively charged) DC.

The formulation of PorA in both Man-liposomes and TAP-liposomes resulted in a decreased ability of PorA to interact with specific mAb (Table 1), despite the preservation of the trimeric protein conformation (Fig. 2). This was probably due to steric hindrance caused by mannose molecules at the outside of the liposomes and the positive charge of TAP-liposomes, respectively, which hindered the interaction of PorA and its specific mAb.

Targeted PorA-liposomes showed an increased uptake by DC *in vitro*, as compared with non-targeted liposomes (Fig. 3). Increased uptake has been ascribed to the multivalent character of liposomal antigens as compared with soluble antigens [20]. More efficient antigen presentation by DC was obtained by targeting liposomes

towards Fc receptor or Class I/Class II molecules [20]. Also, mannosylated protein antigen and peptides showed enhanced potency to stimulate specific T cell clones as compared to non-mannosylated peptides [21]. More recently, the same has been proven with mannosylated liposomes [22]. However, these systems have only been tested *in vitro* and with model antigens. In our study, *in vivo* experiments were included as promising *in vitro* results are not always predictive for improved vaccine efficacy *in vivo*.

Our results confirmed that PorA-liposomes are efficiently internalized by cultured DC (Fig. 4). The uptake of PorA-liposomes induced maturation of DC (Fig. 5). However, only targeted liposomes induced IL-12 production by DC (Fig. 6). The improved uptake of Man-liposomes can be explained by the interaction of mannose moieties with receptors of the C-type lectin family (MR, DEC-205). For PS-liposomes, their increased internalization with respect to non-targeted liposomes can be explained by an uptake mechanism similar to that of apoptotic cells. Phagocytosis of apoptotic cells is related to the recognition of PS exposed externally due to the loss of asymmetry of plasma membrane phospholipids during apoptosis [23,24], by the PS receptor [25]. On the other hand, the uptake of positively charged TAP-liposomes is probably not receptor mediated, but caused by nonspecific electrostatic interactions with negatively charged DC. This mechanism is just as efficient mediating the uptake of PorA-liposomes as receptor-mediated endocytosis. Moreover, the confocal micrographs indicate that electrostatic interactions of TAP-liposomes with the surface of DC resulted in similar punctuate fluorescence inside the cells as with other types of PorA-liposomes. This suggests internalization of all types of PorA-liposomes via an endocytotic pathway.

The *in vivo* localization of targeted (i.e. Man-liposomes) and non-targeted liposomes was studied in afferent lymph nodes after subcutaneous administration. The findings of these experiments confirm some of the results obtained *in vitro*. Targeted liposomes (Man-liposomes) had an increased localization in draining lymph nodes with respect to non-targeted PG-liposomes (Fig. 7, panel A). Both types of liposomes interacted preferably with APC. However, targeted liposomes (i.e. Man-liposomes) showed a significantly increased interaction with MHC class II^+, $B220^+$ and $F4/80^+$ cells as compared with non-targeted liposomes. (Fig. 7, panel B). These results indicate a good correlation between the *in vitro* and *in vivo* results, as the use

of mannosylated liposomes resulted in both cases in an improved loading of APC as compared to non-targeted liposomes.

The cytokines generated by DC are critically important for subsequent T-cell differentiation. IL-12 produced by DC is pivotal for the development of Th1 responses [26]. A Th1-type immune response can switch B-cells to IgG2a production [27]. This is preferable as IgG of the isotypes 2a and 2b are able to interact with complement and induce complement-mediated killing of bacteria [19]. Bactericidal antibody titers in human sera are considered one of the correlates of protection in type B meningococcal vaccination [28,29]. It has been demonstrated that IL-12 is required for development of an effective cellular and/or humoral antimicrobial defense against bacteria [30], parasites [31] and viruses [32]. Targeting of PorA-liposomes resulted in induction of IL-12 *in vitro*, in contrast to non-targeted PorA-liposomes. Surprisingly, all types of liposomes induced similarly high IgG titers *in vivo*. These IgG titers were comparable to those induced by (LPS containing) OMV. Moreover, the production of IL-12 *in vitro* induced by targeted PorA-liposomes was not translated *in vivo* into an IgG istotype switching (Table 2). Also the bactericidal titers found in serum of mice immunized with targeted liposomes were not improved with respect to those of mice immunized with non-targeted PorA-liposomes and OMV. However, the number of responding mice per group increased from 50-60% with non-targeted PorA-liposomes and OMV, to almost 100% (Fig. 9) in mice immunized with targeted PorA-liposomes. We can conclude that targeting of PorA-liposomes to DC resulted *in vivo* in a more homogenous immune response as compared to other vaccine formulations such as OMV, despite the absence of a measurable isotype switching.

Previous studies have shown that targeting of DC results in an improved *in vitro* immune response (i.e. T-cell activation) [20,21,22]. The very different conditions *in vivo*, with complex interactions between various cell types and tissues complicates the extrapolation of *in vitro* data to the *in vivo* situation. This is confirmed by our observation that the clear improvement on the liposomal antigen uptake and subsequent DC maturation *in vitro* was only translated in a marginal improvement of the immune response *in vivo* (i.e. increase of the percentage of responders).

Liposomes are well-defined systems composed of purified materials. In our experiments, liposomes contained LPS-depleted PorA and therefore offer an important safety advantage over LPS-containing OMV, which are the only PorA vaccines tested in the clinic so far [4,5,6]. LPS is a potent activator of DC [9], as

confirmed in this study (Fig. 5 and 6), but is also a toxic compound, which should preferably be absent in vaccine formulations [33].

In summary, targeted PorA-liposomes showed efficient loading and activation of cultured DC as compared to non-targeted PorA-liposomes. In line with these findings, improved uptake in draining lymph nodes as well as preferential uptake by APC was observed *in vivo*. Importantly, immunization with targeted PorA-liposomes resulted in a partial improvement of the immune response in comparison with non-targeted liposomes or OMV. However, the improvement *in vivo* is less pronounced that the strong effects seen *in vitro.* Our data encourage the development of meningococcal immunization strategies using antigens encapsulated in liposomes targeted towards APC.

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CHAPTER 5

Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition

Camilla Foged[a,b], Carmen Arigita[c], Anne Sundblad[b], Gerben A. Koning[c], Wim Jiskoot[c], Gert Storm[c], Sven Frokjaer[a]

[a]Department of Pharmaceutics, The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

[b]Division of Hematology, Center for Molecular Medicine, Karolinska Hospital and Institute, Stockholm, Sweden

[c]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciencens, Utrecht, the Netherlands

(*submitted)*

Abstract

Vaccine efficacy might be improved by exploiting the potent antigen presenting properties of dendritic cells (DC), since their ability to stimulate specific major histocompatibility complex-restricted immune responses has been well documented during the recent years. In that light, we investigated how the interaction of antigen-containing liposomes with DC was affected by the bilayer composition. Monocyte-derived human DC and murine bone marrow-derived DC were analyzed and compared upon *in vitro* incubation with liposomes by flow cytometry and confocal microscopy. Anionic liposomes with a bilayer composition of phosphatidylcholine, cholesterol and phosphatidylglycerol or phosphatidylserine interacted with a limited fraction of the total DC population in case of both DC types. Inclusion of mannosylated phosphatidylethanolamine for targeting to the mannose receptor increased the interaction of negatively charged liposomes with both human and murine DC. This increase could be blocked in human DC by addition of the polysaccharide mannan indicating that uptake might be mediated by the mannose receptor. Cationic liposomes containing trimethyl ammonium propane interacted with a very high percentage of both DC types and could be detected in high amounts intracellularly. In conclusion, liposome bilayer composition has an important effect on interaction with DC and might be critical for the vaccination outcome.

Introduction

Dendritic cells (DC) are highly specialized cells of the immune system with antigen presenting properties important for initiating adaptive immune responses [1]. In the developmental state referred to as immature cells, DC patrol and take up antigens in peripheral tissue, and process them into peptides, which are loaded into MHC class I and II molecules. Upon encounter with pathogens and/or inflammatory stimuli, DC maturation is initiated. The cells migrate to secondary lymphoid organs and are turned into efficient presenters of antigens to T-cells resulting in activation of naïve T-cells and thereby in initiation of antigen specific immune responses or immunological tolerance.

Recent advances in the research area of DC biology combined with the urgent need for improved vaccines regarding antigen delivery capacity and adjuvant potency has led to the hypothesis that exploitation of DC properties might be a rational way of improving vaccine efficacy [2,3]. Modulating DC features with appropriately designed vaccines could possibly result in the generation of enhanced specific T-cell responses and thereby more efficient combat of pathogens. One area of DC biology that is of interest when investigating the adjuvant role of DC in vaccine design is antigen uptake and presentation by DC. Enhancement of the interaction between the vaccine formulation and DC could be a potential method for increasing vaccine immunogenicity. In the present study we aim at investigating the interaction of liposomal antigen formulations containing the *Neisseria meningitidis* type B antigen PorA with human monocyte-derived DC (MoDC) and murine bone marrow-derived DC (BMDC) *in vitro*. To our knowledge, no studies so far have reported on the effect of liposomal lipid composition on the interaction with human MoDC and murine BMDC.

Liposomes have potential as efficient presentation form for PorA in the design of vaccines against type B meningococcal infections [4]. Liposomes not only mimic the natural outer membrane environment of PorA but they also allow the manipulation of the formulation, by e.g. the attachment of targeting ligands or the variation of the (membrane) composition.

We analyzed the interaction of different liposomal formulations of meningococcal PorA (see chapter 4 of this thesis) with human Mo-DC and murine BMDC by flow cytometry and confocal laser scanning microscopy (CLSM).

Negatively charged liposomes with phosphatidylglycerol (PG) or phosphatidylserine (PS) and cationic liposomes containing trimethyl ammonium propane (TAP) as charged phospholipids were used. Also, liposomes with mannosylated phosphatidylethanolamine (Man-PE), which is supposed to be recognized by the DC surface molecule mannose receptor (MR), were studied. The possibility to block the interaction of mannosylated liposomes with DC by addition to the culture medium of mannan, a polysaccharide of mannose, was investigated. The data obtained in our studies, and its relevance for the development of efficient vaccine formulations, are discussed.

Material and methods

Materials

Dimyristoyl phosphatidylcholine (PC) was purchased at Rhône-Poulenc Rorer (Köln, Germany). Dimyristoyl phosphatidylglycerol (PG) was a gift from Lipoïd GmbH (Ludwigshafen, Germany). Dimyristoyl phosphatidylserine (PS), dimyristoyl trimethylammoniumpropane (TAP) and dimyristoyl phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Texas Red® 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine and DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbo-cyanine, 4-chlorobenzenesulfonate salt, were purchased from Molecular Probes (Leiden, the Netherlands). α-D-mannopyranosyl phenyl isothiocyanate, cholesterol (Chol), n-octyl β-d-glucospyranoside (OG) and Molybdenum Blue spray reagent were obtained from Sigma (Zwijndrecht, the Netherlands). analytical and preparative silica gel 60 F_{254} TLC plates were obtained from Merck, Darmstadt, Germany. Water purified through reversed osmosis was applied throughout the study. All other chemicals were of analytical grade or the best available.

Synthesis of mannosylated-PE

N-(3-(4-(α-D-Mannopyranosyl)phenylthiureido)-L-1,2-dimyristoyl-3-phosphatidylethanolamine (Man-PE) was prepared by ethyldiisopropylamine-promoted coupling of PE and α-D-mannopyranosylphenyl isothiocyanate in

chloroform/methanol (7:1 v/v). The compound was purified by preparative thin layer chromatography. Its nature was confirmed by ^{1}H- NMR and LC-MS.

Preparation of PorA-liposomes

PorA was purified from outer membrane vesicles obtained from *Neisseria meningitidis* strain F91 (P1.7-2,4, PorB^{-}, RmpM^{-}, low expression of Opa/Opc) as previously described [4]. Prior to incorporation of PorA into liposomes, the protein was precipitated with 80% (v/v) ethanol at -20ºC and dissolved in 150 mM OG in Tris-buffered saline (TBS, containing 50 mM Tris-HCl, 150 mM NaCl pH 7.4). Appropriate amounts of each lipid were dissolved in chloroform/methanol (2:1) in a round bottom flask and a lipid film was obtained by solvent evaporation in a rotavapor under reduced pressure. The film was re-suspended in the PorA solution and the resulting mixed micelles were rapidly diluted in TBS, allowing the formation of unilamellar vesicles [5]. Subsequently, the liposomes were pelleted by ultracentrifugation (160,000 g, 1 hour) and resuspended in TBS, obtaining liposomes with an epitope density of approximately 20 µg protein/µmol lipid. PorA liposomes were made with four different lipid bilayer compositions; PC:PG:Chol (8:2:2 molar ratio) (PG-liposomes); PC:PG:Chol:Man-PE (8:2:2:0.6 molar ratio) (Man-liposomes); PC:PS:Chol (8:2:2 molar ratio) (PS-liposomes); PC:TAP:Chol (8:2:2 molar ratio) (TAP-liposomes). All formulations included 0.2 mol% Texas Red-DMPH or DiD as fluorescent marker in the bilayer.

Characterization of PorA-liposomes

The average particle size and polydispersity index of PorA-liposomes were determined by dynamic light scattering at 25ºC with a Malvern 4700 system using a computer with PCS software (version 1.35, Malvern). For viscosity and refractive index the values of pure water were used. The particle size distribution was reflected in the polydispersity index, which ranges from 0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion. The zeta potential of the liposomes in 3% sucrose was measured with a Malvern Zetasizer 2000 with an aqueous dip-in cell and a computer with PCS software (version 1.35, Malvern). The phospholipid content of the liposomes was determined by the phosphate determination according to Rouser [6] The protein content was determined according to Peterson [7].

Culture of human DC from peripherial blood

DC were generated from human peripheral blood monocytes according to previous reports [8,9]. The study was approved by the ethics committee at the Karolinska Hospital, Stockholm, Sweden, and by the local ethics committee in Copenhagen, Denmark, (KF01-020/00). Briefly, peripheral blood mononuclear cells were isolated from healthy volunteer blood donors, by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The cells were resuspended to 5×10^6 cells/ml in complete medium and allowed to adhere for 35 min. at 37°C in tissue culture flasks. Non-adherent cells were removed by PBS washes. Adherent cells were cultured for 6 days in complete medium (RPMI 1640) supplemented with 2 mM L-glutamine (GibcoBRL, Life Technologies, Stockholm, Sweden), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 10% heat inactivated fetal calf serum (Sigma-Aldrich, Stockholm, Sweden), in the presence of 60 ng/ml human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Leucomax, Sandoz, Basel, Switzerland) and 50 ng/ml human recombinant interleukin 4 (IL-4) (Schering-Plough, Kenilworth, NJ, USA). For liposome uptake experiments, immature DC were harvested at day 6 of culture and seeded in 24-well tissue culture plates in 0.5 ml complete medium, 2×10^5 cells/well. For the mannan-inhibition experiment, 3 mg/ml mannan (*Saccharomyces cerevisiae,* Sigma-Aldrich A/S, Denmark) was added to the medium. Liposomes were added to the wells (20, 80 or 200 nmol lipid per well) and the mixture was incubated 2 or 4 hours at 37°C. As controls for endocytosis, cells were incubated in medium alone or with non-labeled liposomes (20 or 200 nmol lipid per well) or with Alexa-488-labeled, heat inactivated *E. coli* (Molecular Probes, Leiden, the Netherlands).

Culture of murine DC

Balb/cOlaHsd mice were obtained from Harlan (Horst, the Netherlands) and maintained under conventional conditions at the Central Animal Laboratory (Utrecht University, the Netherlands). All experiments were done with 8-12 weeks old animals and were approved by the Utrecht University Animal Ethics Committee. BMDC were obtained as described by Inaba *et al.* [10]. Briefly, bone marrow was flushed from mouse femurs, erythrocytes were lysed and cells were grown at 1×10^6/ml in filtered

culture medium consisting of RPMI 1640 medium with 10% fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin in the presence of 10 ng/ml murine GM-CSF (Immunex, Seattle, WA) and 50 ng/ml murine IL-4 (R&D Systems, Minneapolis, MN). Non-adherent cells were re-plated on day 1 and cells still non-adherent at days 2 and 4 were removed from the cultures, with concomitant refreshment of culture medium. On day 7, non-adherent and loosely adherent cells were harvested. These cells were MHC class II^+, $CD11c^+$and $CD11b^+$. From these, $3x10^6$ cells per well were plated with fresh culture medium together with 80 nmol phospholipid in PorA-liposomes and further incubated for 4 hours at 37ºC. As controls, cells were incubated in medium alone or with non-labeled liposomes. After incubation, cells were harvested and analyzed.

Flow cytometry analysis

For FACS analyses of human DC surface markers, cells were incubated with mouse serum (DAKO, Denmark) for blocking of unspecific binding sites and subsequently stained with fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against human leukocyte antigen (HLA) DR (clone L243, Becton Dickinson, San Jose, CA, USA). Mouse IgG2a served as isotype control (clone X39, Becton Dickinson). Fixation was performed with 1% paraformaldehyde. The cells were analyzed by a FACS Calibur flow cytometer (Becton Dickinson) using the CellQuest software (Becton Dickinson). Dead cells were gated out based on their light scattering properties. For the studies with murine DC, the following was used: FITC-labeled monoclonal antibody against CD11b (clone M1/70) and biotinylated mAb against CD11c (clone HL3) were obtained from BD PharMingen (San Diego, CA). Streptavidine-phycoerytrin was purchased from Becton Dickinson (San Jose, CA). M5/114 anti-class II [11] was kindly provided by Dr. Georg Kraal (Free University, Amsterdam, the Netherlands). Murine DC ($1x10^5$) were blocked with 5% heat-inactivated mouse serum in PBS for 30 min at room temperature (RT). Blocked cells were then washed with FACS buffer (PBS, 0.1% azide, 1% BSA) and incubated with relevant antibodies for 20 min at room temperature. After this time, cells were washed and, if required, incubated with a specific secondary antibody. Negative controls consisted of unstained cells, cells stained with isotype control antibody, or cells stained with only the secondary antibody. Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and Cell Quest software (BD

Biosciences). The Win MDI 2.8 program was kindly provided by Joseph Trotter (available on http://facs.scripps.edu/) and was used for further analysis of FACS-data.

Confocal laser scanning microscopy

For staining of CD1a the human cells were harvested, washed with ice-cold PBS and fixed for 15 min. in 1% paraformaldehyde on ice. Staining was performed with a primary monoclonal mouse anti human CD1a antibody (clone BL6, Immunotech, Beckman Coulter AB, Sweden) followed by a FITC-conjugated donkey anti-mouse IgG (AffiniPure, Jackson Immunoresearch Laboratories, Inc., West Grace, PA, USA) in PBS with 4% BSA. The cell preparations were washed with PBS and mounted for CLSM. Visualization of fluorescent signals and transmitted light was performed with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, GmBH, Germany) equipped with an argon laser (458 and 488 nm) and a HeNe laser (543 nm) using the LSM 510 software. Murine cells ($1x10^5$), incubated with PorA-liposomes under similar conditions as for flow cytometry studies, were washed with 2 ml PBS and subsequently fixed in 4% formaldehyde in PBS for 20 min at room temperature. After fixation, cells were washed twice with PBS and mounted on glass slides with FluorSave reagent (Calbiochem, San Diego, CA). Fluorescent and transmitted light microscope images of cells were taken simultaneously using a Leica TCS-SP microscope equipped with an 488 nm Argon, 568 nm Krypton and 633 nm HeNe laser and analyzed using Leica TCS-SP Power Scan software (Leica Microsystems, Rijswijk, the Netherlands).

Results

Characterization of liposomes

The composition and characteristics of the liposome formulations are summarized in Table 1. The PorA-containing liposomes had a mean particle size of around 200 nm. Incorporation of mannosylated phospholipid resulted in formation of liposomes with less negative surface charge and larger size as compared to PG-liposomes. TAP-liposomes exhibited, as expected, a high positive surface charge.

Table 1. Characteristics of PorA liposomes.

Liposomes	*Composition*	*PorA:lipid ratio (µg/µmol)*	*Size*[a] *(nm)*	*PD*[b]	*Zeta potential*[a,c] *(mV)*
PG	PC:PG:Chol (8:2:2)	15.0	180 ± 0.4	0.2	-54.2 ± 1.6
Man	PC:PG:Chol:Man-PE (8:2:2:0.6)	26.0	279 ± 1.2	0.3	-37.3 ± 2.5
PS	PC:PS:Chol (8:2:2)	21.3	203 ± 2.3	0.3	-50.0 ± 0.1
TAP	PC:TAP:Chol (8:2:2)	29.5	204 ± 1.9	0.3	44.2 ± 1.4

[a]Values represent mean ± S.D., n = 3.
[b]Polydispersity: indication of the size distribution of the liposomes; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse suspension.
[c]Zeta potential was measured in a 3% solution of sucrose.

FACS analysis of liposome association with DC

FACS analysis allows assessment of cell-associated fluorescence and was used to examine interaction of Texas Red- or DiD-labeled liposomes with DC. Immature DC were generated from human peripheral blood mononuclear cells and from murine bone marrow cells. We have previously reported the characteristic surface marker expression of normal human DC upon culturing under the described conditions (see Material and Methods). The human DC lack lineage-specific markers expressed on other leukocyte populations (CD3, CD14, CD19 and CD56) and express CD1a, CD40, MHC class I and II [12]. The level of CD83 expression is low on immature DC. The murine cultures were MHC class II^+, $CD11c^+$and $CD11b^+$ and had a purity of approximately 90% (results not shown) comparable to DC described by Inaba et al. [10]. The human DC were incubated with the fluorescent liposome formulations at 37°C for 2 or 4 hours, harvested and stained with a FITC-labeled monoclonal mouse-anti-human HLA-DR antibody. By FACS-analysis, the percentage of double-positive cells ($HLA\text{-}DR^+$, liposome-Texas Red^+) of the gated cells was determined. For comparison, we also studied the interaction of liposomes with murine BMDC. Incubation was done with DiD-labeled liposomes for 4 hours and the percentage of DiD-positive cells was determined after gating of the DC similarly to the human DC.

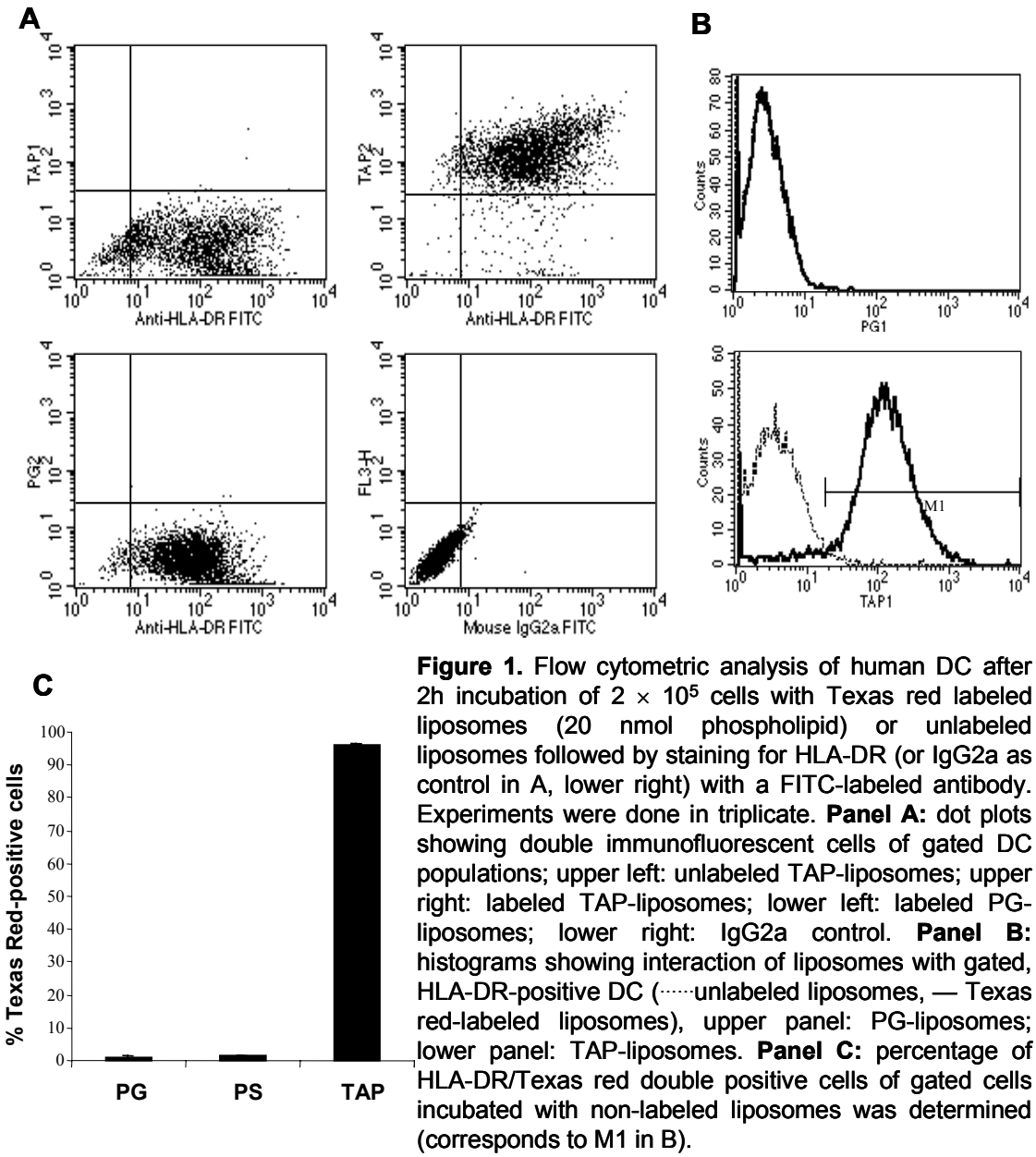

Figure 1. Flow cytometric analysis of human DC after 2h incubation of 2 × 10^5 cells with Texas red labeled liposomes (20 nmol phospholipid) or unlabeled liposomes followed by staining for HLA-DR (or IgG2a as control in A, lower right) with a FITC-labeled antibody. Experiments were done in triplicate. **Panel A:** dot plots showing double immunofluorescent cells of gated DC populations; upper left: unlabeled TAP-liposomes; upper right: labeled TAP-liposomes; lower left: labeled PG-liposomes; lower right: IgG2a control. **Panel B:** histograms showing interaction of liposomes with gated, HLA-DR-positive DC (·····unlabeled liposomes, — Texas red-labeled liposomes), upper panel: PG-liposomes; lower panel: TAP-liposomes. **Panel C:** percentage of HLA-DR/Texas red double positive cells of gated cells incubated with non-labeled liposomes was determined (corresponds to M1 in B).

In case of human DC, a striking difference in the percentage of double positive cells was observed after 2 hours incubation between the negatively charged PG- or PS-liposomes and the positively charged TAP-liposomes. While almost the entire DC population incubated with TAP-liposomes had high levels of the fluorescent marker associated, only less than 2% of the cell population had slightly increased levels of fluorescent marker associated when incubated with PS/PG-liposomes (Fig. 1). Increasing the incubation time to 4 hours did not change the number of double positive cells significantly. The same pattern was seen with murine bone marrow-derived DC (Fig. 2) although the frequency of fluorescence positive cells

varied. These results indicate that the charge of the liposome formulation plays an important role for the level of association of the liposomes with DC.

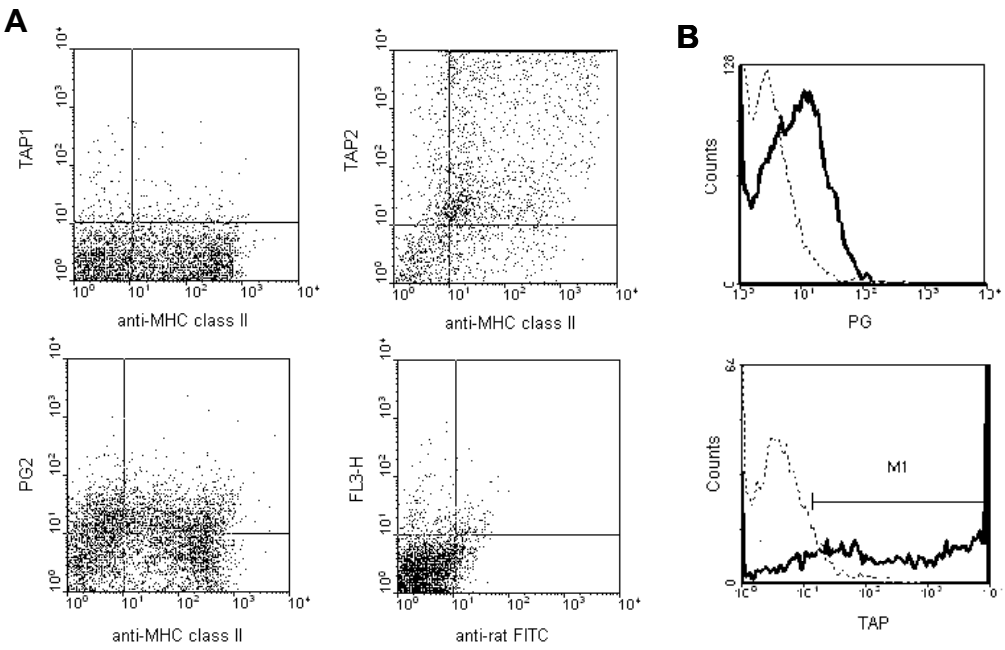

Figure 2. FACS analysis of murine DC after 4h incubation of 3 × 10^6 cells with DiD labeled or unlabeled liposomes (80 nmol phospholipid) followed by staining for MHCII (or second antibody alone as control in A, lower right) with a FITC-labeled antibody. Experiments were repeated at least twice. **Panel A:** dot plots showing double immunofluorescent cells of gated DC populations; upper left: unlabeled TAP-liposomes; upper right: labeled TAP-liposomes; lower left: labeled PG-liposomes; lower right: lsecondary antibody control. **Panel B:** histograms showing interaction of liposomes with gated, HLA-DR-positive DC (·····unlabeled liposomes, — DiD-labeled liposomes), upper panel: PG-liposomes; lower panel: TAP-liposomes. **Panel C:** percentage of DiD positive cells of gated cells incubated with non-labeled liposomes was determined (corresponds to M1 in B)

Effect of liposome mannosylation on their binding with and uptake by DC

In order to study whether mannose receptor (MR)-targeting could enhance liposome binding to and uptake by DC, a mannosylated lipid was incorporated into the liposomes. A concentration-dependent increase in the percentage of double positive cells was observed in human and murine DC cultures (Fig. 3). Moreover, a time dependent increase in the percentage of double positive cells was seen after 4 hours (results not shown). To test whether the MR could mediate the enhanced interaction, uptake of Man-liposomes was measured in the presence or absence of 3

mg/ml of mannan in the medium, a bacterial polysaccharide that binds with high affinity to MR. At this mannan-concentration, maximum inhibition is achieved [13]. As shown in Fig. 3, mannan reduced the percentage of double-positive human DC to background levels when adding 200 nmol Man-liposomes to $2x10^5$ DC. Addition of mannan to the medium of human DC cultures pulsed with TAP-liposomes had no effect on the percentage of double positive cells, while uptake of fluorescently labeled *E. coli* (i.e. whole bacteria with sugar residues in the surface) is completely inhibited by inclusion of mannan in the medium (data not shown). These results obtained with human DC indicate that uptake is mediated by MR. However, the same effect was not observed in the murine BMDC cultures (Fig. 3).

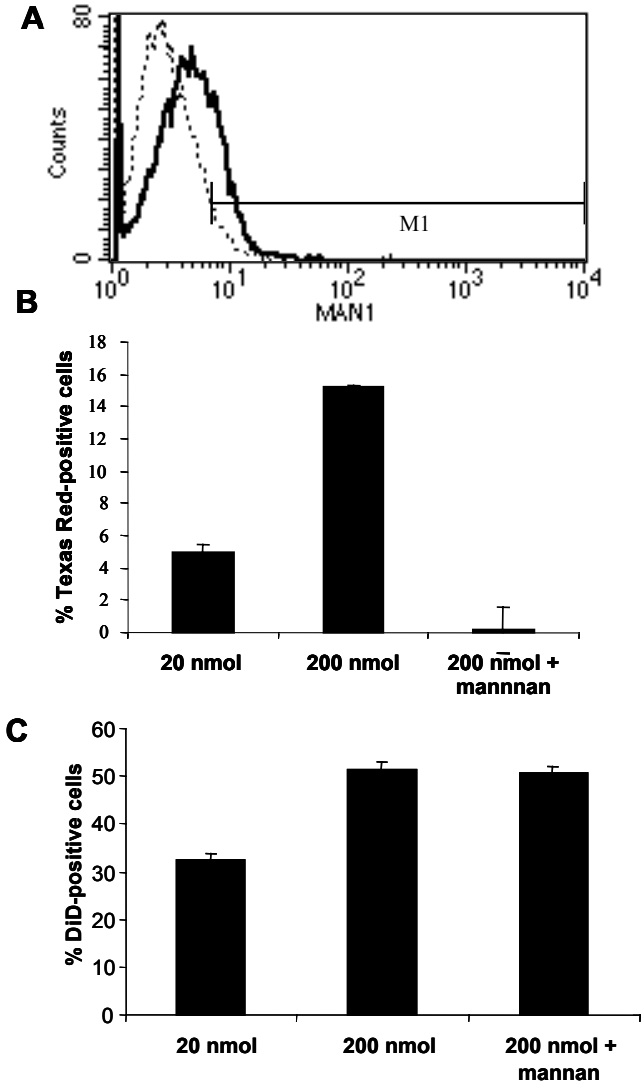

Figure 3: FACS analysis of human and murine DC after 2 hours incubation of $2x10^5$ cells with Texas-red labeled Man-liposomes (20 or 200 nmol phospholipid) or unlabeled Man-liposomes (200 nmol phospholipid) or Man-liposomes (200 nmol phospholipid) where 3 mg/ml mannan was included in the medium, followed by staining for HLA-DR or MHCII with a FITC-labeled antibody. Experiments were done in triplicate.
Panel A: Histogram showing interaction of liposomes with gated, HLA-DR-positive DC (···· unlabeled Man-liposomes, —— Texas-red labeled Man-liposomes).
Panel B: Percentage of HLA-DR/Texas red positive cells of gated cells incubated with non-labeled liposomes (corresponds to M1 in A).
Panel C: FACS analysis of murine DC after 4 hours incubation of $3x10^6$ cells with DiD-labeled Man-liposomes (concentrations as indicated) with or without 3 mg/ml mannan in the culture medium.

Intracellular localization of liposomes by CLSM

To obtain information about the cellular localization of DC-associated liposomes, CLSM was used. In order to study the localization of liposomes in DC, either intracellularly or on the DC membrane surface, human cells were incubated with fluorescent liposomes, then fixed and stained for surface CD1a expression. CD1a is considered as a human DC-specific marker under the used culture conditions. While incubation of DC with Texas red-labeled PG-, PS- and Man-liposomes gave no detectable signal in CD1a-positive cells by CLSM, incubation with TAP-liposomes heavily stained the intracellular region of the CD1a-positive cells (Fig. 4). The nuclei were free of fluorescence. This shows that at least the fluorescent label is present intracellularly. The localization was examined in murine DC as well (Fig. 5). After 4h incubation of negatively charged (PG and PS) liposomes with murine DC, low levels of cell-associated fluorescence were observed. Incubation with mannosylated liposomes resulted in increased cell-associated fluorescence in contrast to what was observed in the human MoDC. In murine BMDC, the fluorescence was punctated and located intracellularly (Fig. 5). When positively charged liposomes were used, high levels of fluorescence associated with the cells were observed, both in the intracellular compartment and on the cell surface (Fig. 5).

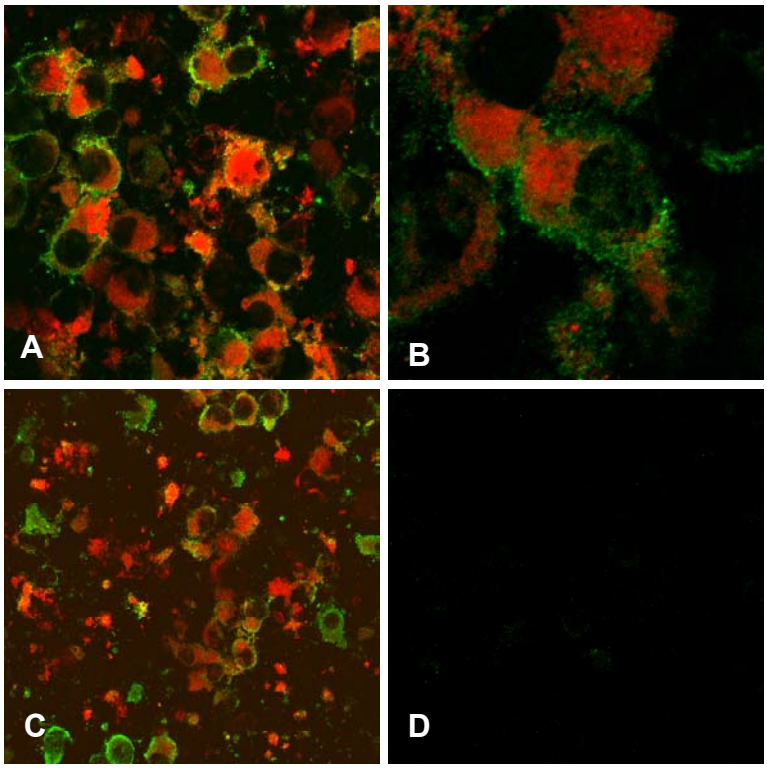

Figure 4 Two-color immuno-fluorescence of the association of TAP-liposomes with DC. Human DC were incubated 2 hours with Texas-red labeled liposomes **(Panels A-C)** (red) or unlabeled liposomes as control **(Panel D)**, fixed and stained with a primary antibody to CD1a **(Panels A-C)** or control IgG **(Panel D)** and a secondary FITC-labeled anti-CD1a antibody (green). Visualization was done by CLSM.

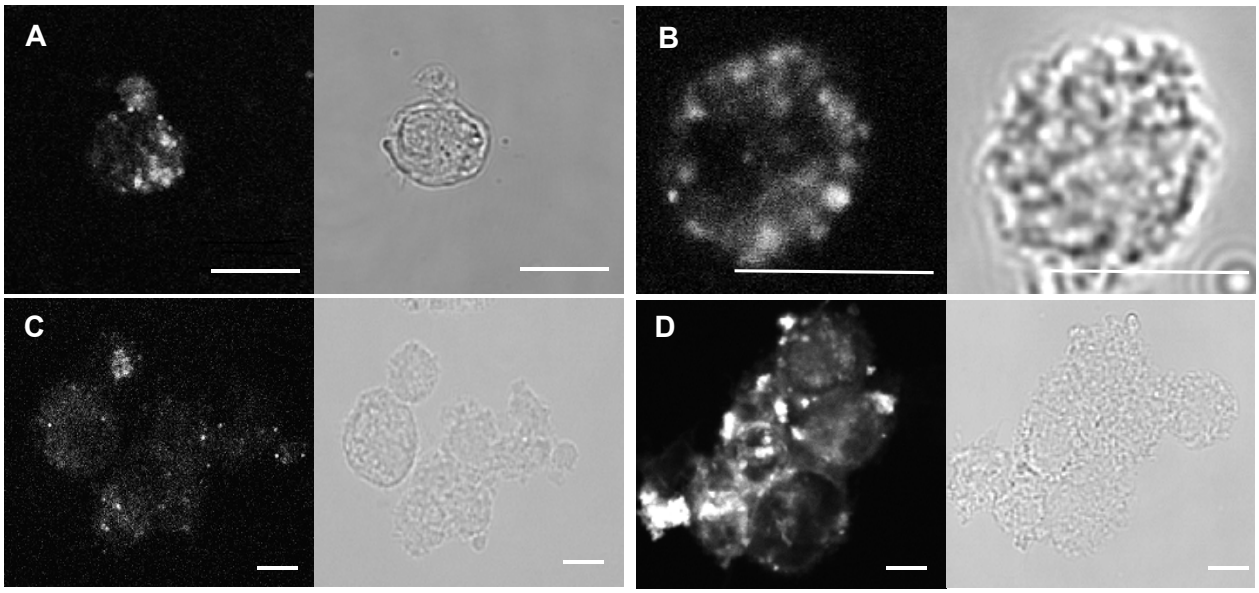

Figure 5: Confocal analysis of interaction between DiD-labeled liposomes and murine DC; left: CLSM micrographs; right: phase contrast pictures. **A:** PG-liposomes, **B:** Man-liposomes, **C:** PS-liposomes, **D:** TAP-liposomes. Bars: 10 µm.

Discussion

Liposomes have the ability to enhance immune responses against incorporated peptide and protein antigens making liposomes interesting adjuvants for vaccine delivery [14,15,16]. Liposomal vaccine formulations are usually given as a limited number of small doses, either by intramuscular or subcutaneous injection. A part of their adjuvant effect is believed to be caused by the formation of an antigen depot at the injection site and/or by the facilitated delivery of antigen to antigen presenting cells [17].

A prerequisite for antigen presentation is the introduction of exogenous antigen into the cytosol or the endosomes of antigen presenting cells. Exogenous material including liposomal antigens is usually taken up by endocytosis and presented by the MHC class II presentation pathway. In addition, liposomes (especially pH-sensitive and fusogenic) have the ability to deliver antigens to the cell cytoplasm, and liposome-associated antigens are thus presented by the MHC class I [18]. The main research focus has until now been on macrophage-liposome interaction. The powerful antigen presenting function of DC, which is considered as the most important antigen presenting cell, led us to investigate how two types of DC cultures (murine bone-marrow derived and human monocyte-derived) interact with liposomes with different liposome-bilayer composition.

Our results establish that bilayer composition has a pronounced impact on the level of interaction and uptake of liposomes by human monocyte-derived and murine bone marrow-derived DC. While anionic PG- and PS-liposomes hardly interacted with human DC at all, we observed an intensive interaction of cationic TAP-liposomes with both human and murine DC, resulting in dense intracellular localization of fluorescent label. This is in accordance with the published observation that cationic microparticles are phagocytosed by DC to a much greater extent than anionic particles [19]. The mechanism behind this is suggested to be an electrostatic interaction between the negatively charged cell surface and the net positively charged liposomal surface.

This charge-effect is observed despite the presence of serum proteins in the medium that may adsorb onto microparticulate surfaces and modify their surface characteristics [20,21]. Specific serum proteins such as IgG, complement factors and fibronectin facilitate recognition and phagocytosis by antigen presenting cells of microparticles by adsorption to the surface [22]. Thiele *et al.* [21] hypothesize that the positive surface charge provides an initial strong trigger mediating recognition and uptake by DC, which is even superior to the presence of opsonins adsorbed to the particle surface.

The differences we observe between human MoDC and murine BMDC in degree of liposome-DC interaction might be caused by differential interaction between the two cell types with serum proteins adsorbed onto the liposomal surface. Bias of results could be due to the presence of species-different serum components or serum-interacting components on the cell surfaces. Other factors that should be considered are the differences on the labels used, source of cells, culture conditions and differentiation patterns.

The MR is a surface 175 kD C-type lectin with 8 carbohydrate recognition domains and has broad substrate specificity for sugars [23]. The MR is expressed on several different cell types, among them monocyte-derived human DC [13] and human DC derived from cord blood $CD34^+$ hemopoietic progenitors [24], where it mediates efficient antigen presentation as well. The MR mediates phagocytosis of mannose-coated particles such as bacteria (i.e. *E. coli*) and yeast and endocytosis of mannosylated proteins that are taken up into vesicular structures where the ligand is released, and the MR is transported back to the cell surface [25]. The MR is thereby constitutively recycled between endosomes and the plasma membrane resulting in a

sustained capacity for antigen capture, concentrating large amounts of antigen intracellularly. Uptake of glycosylated antigen via the MR can enhance 100-10.000 fold the uptake of soluble antigen *in vitro* [25,26]. After release from the MR, antigens are transported to MHC class II compartments and delivered to the MHC class II presentation pathway. We attempted to take advantage of this uptake mechanism by actively targeting the PorA liposome formulation to the DC mannose receptor by attaching a mannose residue to the phospholipid head-group. We found a time- and dose-dependent increase in uptake of Man-liposomes compared to conventional PG-liposomes and in agreement with the results of Copland *et al.* [27]. Yet the interaction between DC and Man-liposomes was much weaker that in case of the cationic TAP-liposomes.

Whether the enhancement of the interaction by mannosylated liposomes is sufficient to affect the immunological outcome remains to be tested. *In vivo*, Man-liposomes are not expected to show strong interactions with other, irrelevant cells, whereas TAP-liposomes may bind to any cell in a non-specific way. Thus, the net effect of mannosylation and the use of negatively charged liposomes might still be advantageous over the use of positively charged liposomes. Our results demonstrate that the interaction of a liposomal vaccine formulation with DC can be tuned *in vitro* by variation of the surface charge of the liposome and by the addition of targeting moieties. Other parameters such as liposome size and antigen/liposome ratio might be important for DC interaction as well. Another important question is how liposome-mediated antigen delivery to DC influences the ability of DC to stimulate T-cells. Overloading of DC with antigen could induce tolerant T-cells [1]. Also, the liposomal antigen formulation may interfere in different ways (positively or negatively) with processes such as intracellular processing pathways, DC-maturation and antigen presentation. Human co-culture systems of antigen-loaded DC and peripheral blood mononuclear cells are currently investigated for a functional T-cell stimulation read-out in response to antigen.

Addition of mannan to the medium reduced the interaction with human monocyte derived DC to background level suggesting that uptake was specifically mediated by the MR. However, no blocking by mannan was observed in case of murine BMDC. The question arises whether the MR is involved in uptake of mannosylated entities by the latter DC type. Reis e Sousa *et al.* could not detect MR in lysates of purified murine Langerhans cells by Western blotting, although a

mannose specific uptake by the cells was identified [28]. The mannose-associated binding activity observed by us and by others in murine DC might thus be due to another (yet unidentified) receptor. Linehan *et al.* found no expression of MR on DC *in vivo* in thymus, lymph node, spleen and Peyer's patch of normal mice by *in situ* hybridization and immunocytochemistry [29]. This brings up the question to which extent cultured DC reflect the properties of DC *in situ*. Differences in MR-expression patterns in cultures of DC and by DC *in situ* are important to notice when investigating targeting potentials. A prerequisite for benefiting from a MR-targeting strategy to reach DC more efficiently is that the receptor is expressed by DC at the vaccine administration site and that this expression is sustained for a time period sufficient for antigen uptake.

Though liposomes can be efficient in *in vitro* delivery of antigens to DC, it should be realized that additional co-stimulatory signals are necessary to successfully induce an immune response *in vivo*. Liposomes by themselves act as adjuvants. Furthermore, other immunostimulatory adjuvants can be incorporated into the lipid structures to further increase the adjuvant activity of liposomes. The concept of active targeting immunostimulatory drug delivery systems to antigen presenting cells is of evident interest for vaccinology, where an urgent need for the development of more effective vaccines exists [30].

In conclusion, liposome bilayer composition has an important effect on interaction with DC and might be critical for the vaccination outcome.

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CHAPTER 6

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

Carmen Arigita[a,b], Thomas Luijkx[c], Wim Jiskoot[a], Martien Poelen[c], Wim E. Hennink[a], Daan J.A. Crommelin[a], Peter van der Ley[c], Cecile van Els[c], Gideon F.A. Kersten[b]

[a]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands

[b]Laboratory for Product and Process Development and [c]Laboratory for Vaccine Research, Netherlands Vaccine Institute, Bilthoven, the Netherlands

(*submitted)*

Abstract

Potent liposomal PorA formulations containing various lipopolysaccharide (LPS) derivatives were developed. The following adjuvants were compared: the commonly used aluminum phosphate ($AlPO_4$), 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 $AlPO_4$ 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.

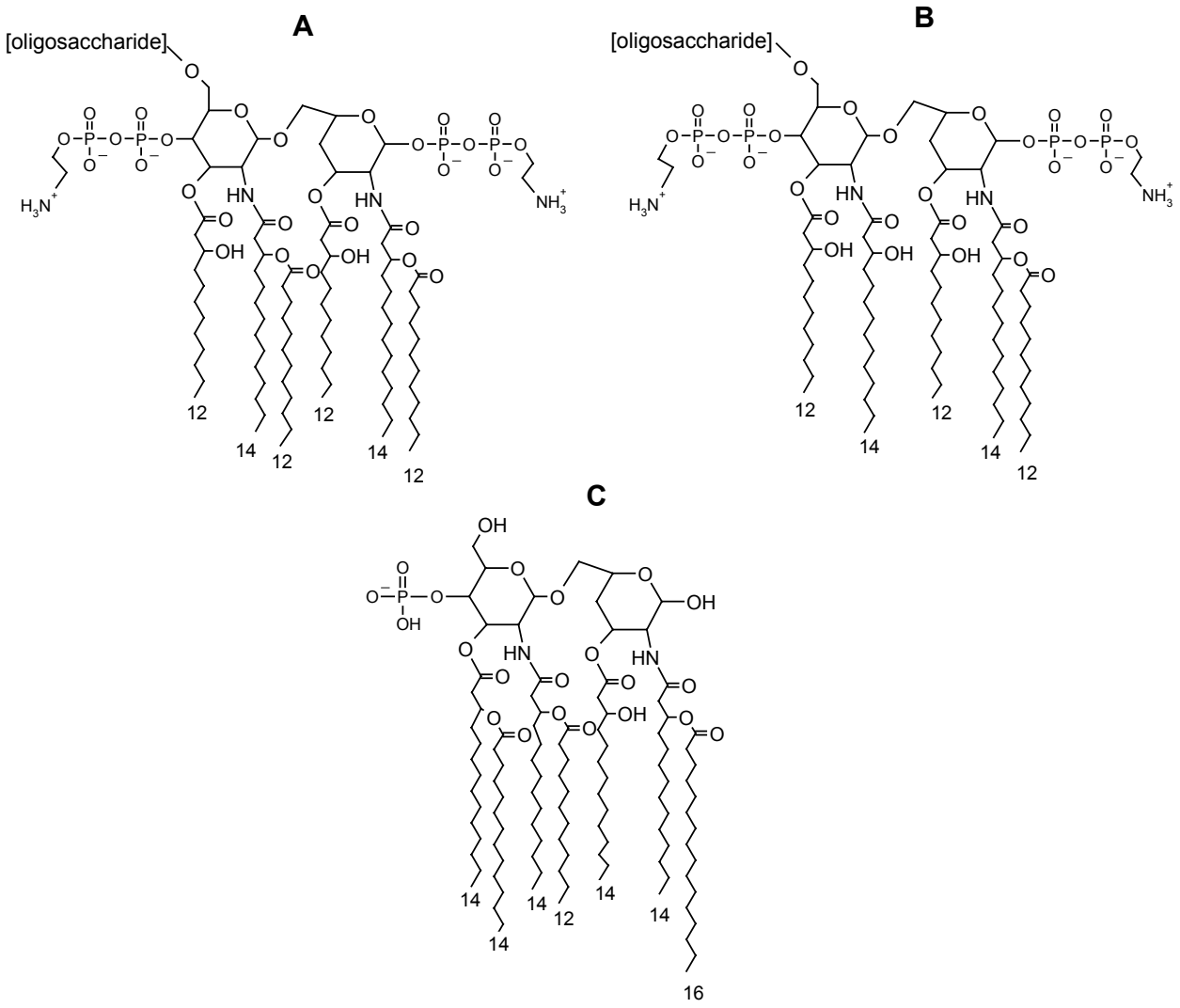

Figure 1. Lipid A structure of LPS and derivatives used in this study. **A**: wild type LPS of *Neisseria meningitidis*, **B**: *lpxL*-mutant of *Neisseria meningitidis*, **C**: monophosphoryl lipid A from *Salmonella minnesota.*

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 ($AlPO_4$) 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*.

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 $AlPO_4$ 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 –20ºC 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

[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 (lacking 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 HI5 (PorA$^-$). Sera from individual mice were heat inactivated for 30 min at 56ºC 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 2×10^5 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 37ºC. On day 3, 50 µl aliquots of the supernatants were taken and stored at -70ºC 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[a].

Adjuvant	*protein:lipid ratio (µg/µmol)*	*protein:adjuvant ratio (w/w)*	*Particle size (nm)[b]*	*PD[c]*	*Antigenicity[d]*
-	32	-	205 ± 7	0.4	0.94 ± 0.12
MPL	24	2.0	242 ± 4	0.4	1.04 ± 0.11
galE LPS	32	1.3	248 ± 7	0.3	0.87± 0.04
LpxL1 LPS	29	1.4	219 ± 3	0.4	0.82 ± 0.05

[a] Data in this table are representative for PorA-liposomes used in the experiments described.
[b] Particle size average ± SD of three measurements.
[c] Polydispersity: indication of the size distribution of the liposomes; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.
[d] Ratio 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 $\log_{10}$ 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 $AlPO_4$ 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.

Group	*Adjuvant*	*Anti-PorA titer*[a]					*IgG2a/IgG1 ratio*[b]	*Non-specific IgG*[c]
		IgG	*IgG1*	*IgG2a*	*IgG2b*	*IgG3*		
OMVs	*galE* LPS[d]	3.20	3.32	3.46	3.44	2.58	1.05 ± 0.16	1.92
liposomes	-	3.02	3.01	2.98	3.09	2.40	0.99 ± 0.04	1.36
liposomes	$AlPO_4$	3.21	3.13	3.34	3.43	2.49	1.07 ±0.10	0.27
liposomes	MPL	3.15	2.95	3.26	3.45	2.22	1.11 ±0.10	0.00
liposomes	*galE*	3.94*	2.96	4.18*	3.25	2.69	1.42 ±0.18	0.41
liposomes	*LpxL1*	3.74*	3.59*	3.76*	3.49	2.85	1.05 ± 0.10	1.57

[a] The titer of each anti-PorA 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)=0.29; $LSD_{0.05}$ (IgG1)=0.22; $LSD_{0.05}$ (IgG2a)=0.25; $LSD_{0.05}$ (IgG2b)=0.27; $LSD_{0.05}$ (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 $\log_{10}$ titer. The means were compared by the LSD test with a confidence level of 95%: $LSD_{0.05}$ (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).

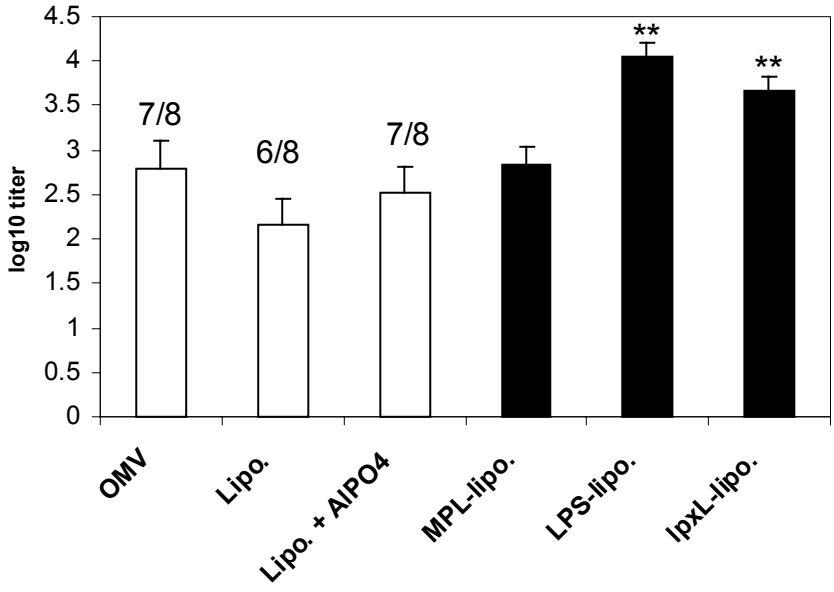

Figure 2. Serum bactericidal activity of mice immunized with OMV and (adjuvated) PorA-liposomes.
Bars indicate the average bactericidal activity of sera of responder ± SEM. White bars: groups with non-responders; the number of responders is indicated above the bars.
Black bars: groups with 100% responders.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

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 $AlPO_4$ 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 HI.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.

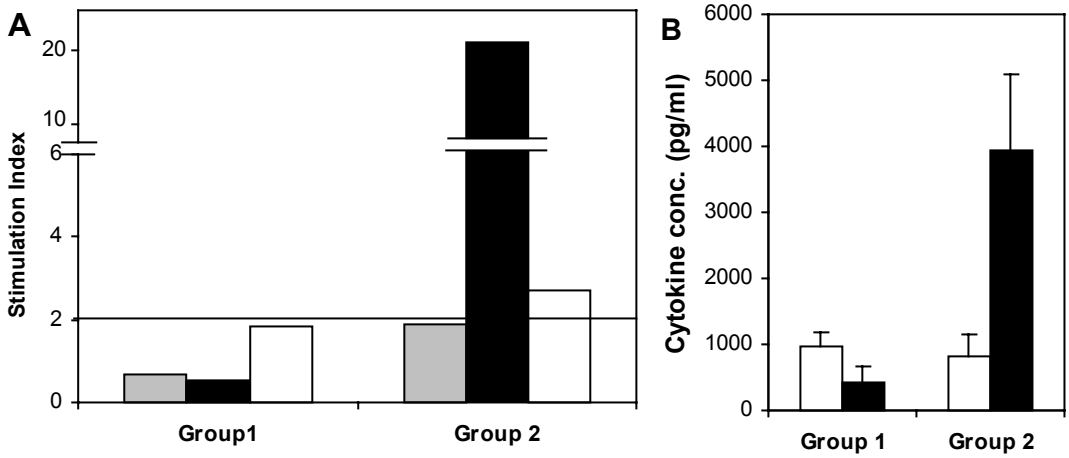

Figure 3. Proliferation and cytokine production of cells isolated from lymph nodes of mice immunized with non-adjuvated PorA-liposomes (Group 1) or PorA-liposomes adjuvated with *lpxL* (Group 2) and re-stimulated with the corresponding formulations. **Panel A:** Proliferation of cells. The bars indicate 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 *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 *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 *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 *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 *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 $AlPO_4$ 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

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 l*pxL1* 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 $AlPO_4$ 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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CHAPTER 7

Immunogenicity of vesicular PorA formulations encapsulated in biodegradable microspheres

Carmen Arigita[a,b], Joost van den Berg[a], Karin Wensink[a], Mies van Steenbergen[a], Wim E. Hennink[a], Daan J.A. Crommelin[a], Gideon F.A. Kersten[b], Wim Jiskoot[a]

[a]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands

[b]Laboratory for Product and Process Development, Netherlands Vaccine Institute, Bilthoven, the Netherlands

(*submitted)*

Abstract

The purpose of our study was to investigate the possibility to microencapsulate PorA-liposomes and outer membrane vesicles (OMV) in biodegradable dextran- and mannan-based microspheres and the effect of the microencapsulation on the immunogenicity of PorA.

Calcein-containing model liposomes, PorA-liposomes and OMV were encapsulated in dextran- or mannan-based microspheres using an aqueous two-phase system consisting of polyethylene glycol and methacrylated-dextran or -mannan solutions. Both types of microspheres were designed to have similar size and similar release profiles. The following *in vitro* characteristics were determined: size and size distribution, encapsulation efficiency, release profile in aqueous medium at 37ºC, integrity and antigen recovery of PorA after release. Finally, the immunogenicity of the formulations containing PorA was determined in mice after subcutaneous immunization.

Liposomes were encapsulated in dextran- and mannan-based microspheres with a high efficiency (70-90%). During day 1 to day 5 of incubation no release of the model calcein liposomes was detected. All formulations exhibited zero-order release kinetics of calcein liposomes between day 5 and day 10 of incubation for the chosen conditions. The total release was 80% and 100% from mannan and dextran microspheres, respectively. Although the trimeric PorA conformation was preserved in the released liposomes and OMV, the antigen recovery was not 100%. The immunogenicity of PorA-liposomes and OMV encapsulated both in dextran and mannan microspheres was preserved.

In conclusion, PorA-liposomes and OMV could be encapsulated in dextran- and mannan-based microspheres with high efficiency. The immunogenicity of encapsulated antigen was preserved.

Introduction

Polymeric microspheres have been widely used as controlled release systems for antigens [1,2,3]. The most extensively studied polymeric microspheres are made of biodegradable and biocompatible polymers consisting of polylactic acid (PLA), polylactic-co-glycolic acid (PLGA) or mixtures of these [2,4,5]. The immunogenicity of microsphere-encapsulated vaccines can be varied to some extent by changing the physicochemical properties of the microspheres, e.g. size, surface properties and the release kinetics of the antigen from the microspheres [4]. Previous work has shown that mannosylated liposomes are efficiently taken up by dendritic cells (DC, see chapter 4 and 5). However the immunogenicity was not improved. We wondered how mannan microspheres, with their different characteristics, would perform. Dextran microspheres, a drug delivery system previously developed in our group, were studied in parallel. As compared to PLA/PLGA microspheres, mannan and dextran offer the following advantages: they are hydrophilic, they do not require organic solvents for their preparation and during the degradation phase no decrease of pH is occurring. This is particularly important when encapsulating proteins, as organic solvents and low pH may induce denaturation [6]. The microsphere preparation is based on the phase separation of a mannan-HEMA (hydroxyethyl methacrylate) or dextran-HEMA solution and a polyethylene glycol (PEG) solution. Upon polymerization, the HEMA attached to the mannan or dextran chains (Fig. 1) forms an oligomethacrylate chain, thereby crosslinking the dextran or mannan chains, resulting in microspheres with a hydrogel character. Dextran hydrogels and microspheres are degraded in time by hydrolysis of the carbonate esters. Factors to manipulate the release from dextran hydrogels and microspheres include water content and cross-link density [7,8,9]. These microspheres have been tested *in vitro* [10,11] and were well-tolerated *in vivo* [12]. It has also been demonstrated that liposomes are efficiently encapsulated in dextran microspheres and are released in their intact form *in vitro* after degradation of the microspheres [13].

We used mannan from *Saccharomyces cerevisiae* yeast to prepare microspheres. This polysaccharide consists of an α-1,6-linked mannose backbone with a high percentage of α-1,2- and α-1,3-side chains of different composition (Fig. 1) [14]. The dextran used for preparation of the microspheres was derived from *Leuconostoc spp*. Dextran mainly consists of an α-1,6-linked dextrose backbone with

only a low percentage (ca. 5%) of α-1,2, α-1,3 and α-1,4 linked dextrose side chains [15] (Fig. 1).

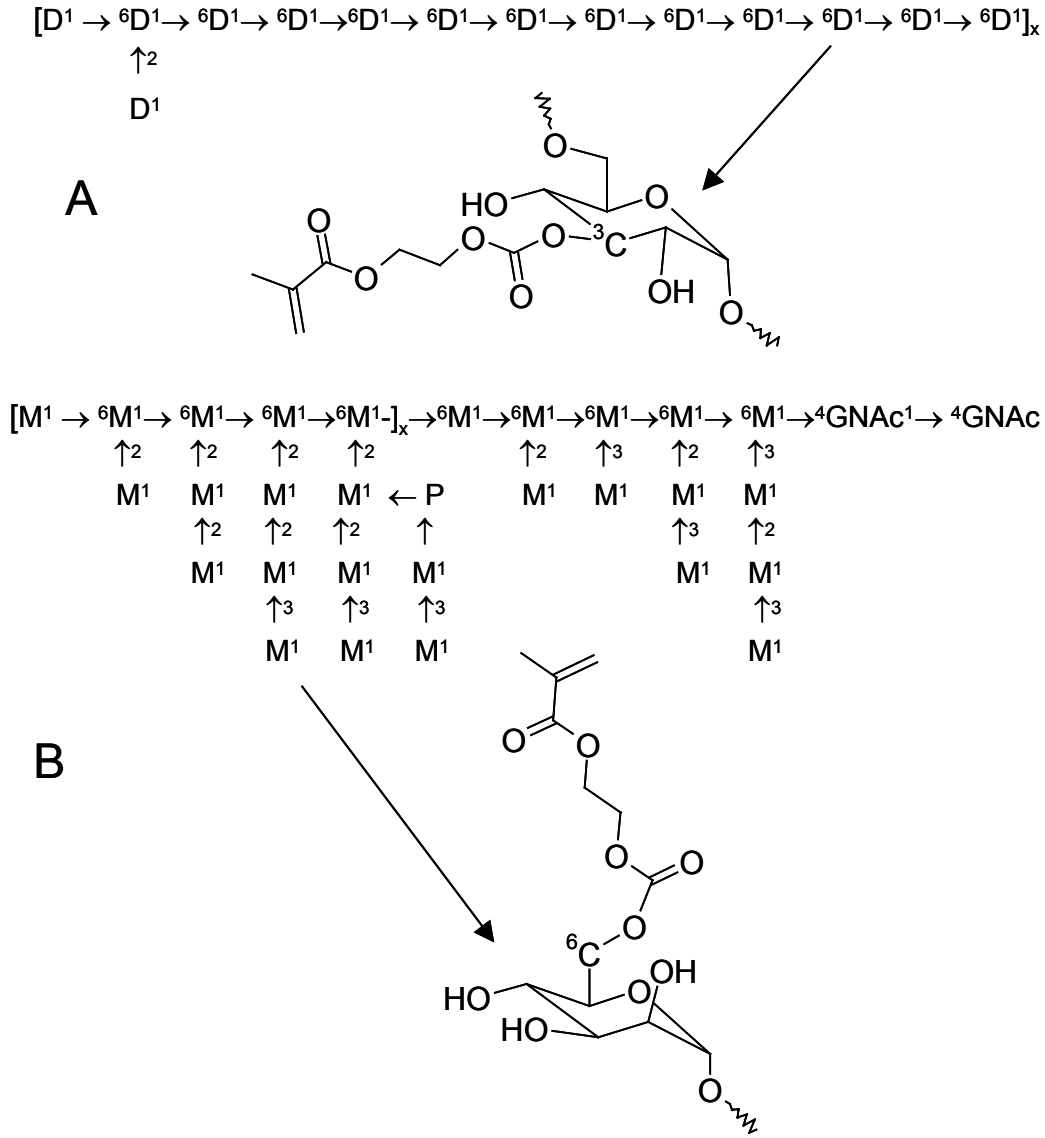

Figure 1. Structure of modified dextran (A) and mannan (B). The overall structures of the polymers are schematically indicated above the chemical structure of the HEMA-derivatized monomers. In dextran, HEMA is coupled to the ^{3}C and ^{2}C of a dextrose residue in the α-1,6-linked backbone of dextran [38]. In mannan, most HEMA groups are likely coupled to the ^{6}C of the mannose residues of the grafts. D: dextrose; M: mannose; P: phosphate; GNAc: *N*-acetylglucosamine [14].

We have previously described the use of liposomes as antigen delivery system for the *Neisseria meningitidis* type B antigen PorA [16]. Liposomes offer a well-defined and versatile alternative for vaccines based on outer membrane vesicles (OMV) [17]. Here, we study the effect of encapsulation of both PorA-liposomes and OMV in dextran and mannan microspheres on the immunogenicity of PorA. Both dextran and mannan microspheres were designed to provide similar, relatively fast, release profiles (i.e. full release in less than 10 days) of the encapsulated antigen

(PorA-liposomes and OMV). No attempts were made to obtain microspheres with a more prolonged release or pulsed release for use in 'single-shot' vaccination schedules. It was intended to prepare microspheres with a mean size below 10 µm. Microspheres smaller than 10 µm can be taken up efficiently by antigen-presenting cells (APC) into phagosomes [2]. On the other hand, microspheres larger than 10 µm can provide an extracellular depot for secondary immune responses by way of B-cell stimulation [1,4,18]. In addition to this, mannan microspheres were designed to study the possibility to specifically target APC, because they express the mannose receptor (MR) on their surface [19]. The MR is able to take up saccharide-containing components by receptor-mediated endocytosis. After this, the MR is recycled to the surface of APC to repeat this process. Thus, the MR is capable to increase the uptake of mannosylated antigens or vehicles enormously as compared to the non-mannosylated antigen [20,21].

The ultimate aim of our study is to investigate whether dextran- or mannan-based microspheres can improve the immunogenicity of encapsulated (physically entrapped, not covalently bound) antigen as compared to the non-encapsulated antigen (PorA-liposomes and OMV). As pointed above, the size and biophysical characteristics of the microspheres, their release properties, the presence of targeting ligands at their surface and the addition of co-entrapped adjuvants can likely modulate the immune response of encapsulated antigens. It is however very important that the antigen is released from the microspheres in its intact form. Thus, the aim of the present study was to evaluate whether PorA-liposomes and OMV could be entrapped with a high yield in dextran- and mannan-based microspheres and that the antigen could be released in its intact form capable to induce an immune response.

Materials and methods

Materials

Dextran, mannan, hydroxyethyl methacrylate (HEMA), N,N,N',N'-tetramethylethylenediamine (TEMED) and calcein were purchased from Fluka (Buchs, Switzerland). 1,1' carbonyldiimidazole (CDI) was obtained from Acros (Geel, Belgium). DNase (Benzonase), polyethylene glycol (PEG, Mw 20000), potassium

peroxodisulfate (KPS), chloroform and methanol were obtained from Merck (Darmstadt, Germany). Dimyristoyl phosphatidylcholine (PC) was purchased from Rhône-Poulenc Rorer (Köln, Germany). Dimyristoyl phosphatidylglycerol (PG) and egg phosphatidylcholine (EPC) were a gift from Lipoïd GmbH (Ludwigshafen, Germany). Cholesterol (Chol) and n-octyl β-d-glucopyranoside (OG) were obtained from Sigma (Zwijndrecht, The Netherlands). Triton X-100 was from BDH Laboratory Supplies (Poole, UK).

Preparation and characterization of OMV and liposomes

OMV were isolated from *Neisseria meningitidis* strain F91 (P1.7-2,4, $PorB^-$, $RmpM^-$, low expression of Opa/Opc) as previously described [16]. The OMV were used for purification of PorA as described [16]. Prior to incorporation of purified PorA into liposomes, the protein was precipitated with 80% (v/v) ethanol at –20ºC and solubilized in 150 mM OG in 50 mM Tris-HCl, 150 mM NaCl pH 7.4 (TBS). PorA-liposomes were made of PC, PG and Chol in a 8:2:2 molar ratio by detergent dilution as previously described [22]. The initial protein/lipid ratio used was 25 µg/µmol.

Calcein liposomes were prepared from EPC and Chol in a 2:1 molar ratio by lipid film hydration [23]. In short, appropriate amounts of each lipid were dissolved in absolute ethanol and a film was prepared by solvent evaporation in a rotavapor under reduced pressure. The film was hydrated by vigorous mixing with aid of glass pearls in 75 mM calcein in 50 mM Hepes, 150 mM NaCl, pH 7.4 (HBS). Subsequently, the liposomes were extruded through 0.2-, 0.1- and 0.05-µm polycarbonate filters. After extrusion, non-encapsulated calcein was removed by multiple ultracentrifugation steps (160,000 × g, 1 hour) (i.e. until no calcein could be detected in the supernatant), and the amount of encapsulated calcein was determined [24].

The mean particle size and polydispersity index of PorA-liposomes, calcein liposomes and OMV were measured by dynamic light scattering (DLS). The protein content of PorA-liposomes and OMV was determined according to Peterson [25] with BSA (Pierce, Rockford, IL) as standard. The phospholipid content of PorA-liposomes and calcein liposomes was determined according to Rouser [26] with sodium phosphate (Merck, Darmstadt, Germany) as standard. Trimer formation of PorA into

liposomes and OMV and integrity were monitored by SDS-PAGE in 'native' gels as previously described [27]. Antigenicity (ratio between the protein concentration determined by ELISA and the protein concentration determined according to Peterson [25]) of the P1.4 epitope of PorA in liposomes and OMV was tested by an inhibition ELISA, with the monoclonal antibody MN20B9.34, as described in chapter 3 of this thesis. In this system, analysis of antigen bound to large particles is possible, as the antigen-antibody interaction takes place in solution.

Preparation and characterization of dextran and mannan hydrogels and microspheres

The molecular weights of dextran and mannan were determined by gel permeation chromatography with a column calibrated with dextrans of known molecular weights. Dextran had a weight average molecular weight (M_w) of 32000 g/mol and a number average molecular weight (M_n) of 15000 g/mol. Mannan had a M_w of 57000 g/mol and a M_n of 34000 g/mol. HEMA-derivatized dextran (dex-HEMA) and mannan (man-HEMA) were prepared as described by van Dijk-Wolthuis et al [28]. The degree of substitution (DS: the number of HEMA groups per 100 dextran/mannan monomer units) as determined by ^{1}H-NMR [28] was 2.6 for dex-HEMA and 4.8 for man-HEMA.

Macroscopic hydrogels from dex-HEMA and man-HEMA with an initial water content of 70% (w/w) were made as previously described [7]. These macroscopic hydrogels were made to study the swelling properties of both derivatized mannan and dextran. The weight of the hydrogels was determined and used to calculate the swelling ratio (defined as the ratio of the weight of the hydrogels at time t (W_t) and the initial weight of the hydrogel (W_0)). The swelling properties give an indication of the degradation rate of the hydrogels.

Microspheres with an initial water content of 70% (w/w) were prepared according to Stenekes et al. [13]. The water content of dex-HEMA microspheres was calculated from the equilibrium dextran concentration with aid of a phase diagram [10], whereas the water content of man-HEMA microspheres was an estimation based on the phase diagram of dextran. In brief, solutions of 40% (w/v) PEG 20,000 in HBS and 20% (w/v) dex-HEMA or man-HEMA in HBS were flushed for 10 min with nitrogen and subsequently transferred to a scintillation vial in a 10/1 (w/w) ratio (total

weight 5 g). The two-phase system was vigorously mixed for 1 min to create a water-in-water emulsion, with the dextran-enriched phase as the discontinous phase. Next, the emulsion was allowed to stabilize for 15 minutes at room temperature, followed by addition of TEMED (100 µl, 20% v/v, adjusted to pH 7.0 with 4 M HCl) and KPS (180 µl, 50 mg/ml). This system was incubated for 30 minutes at 37°C to polymerize the methacrylate groups coupled to the mannan or dextran chains. Subsequently, the PEG phase was removed by three washing and centrifugation steps (first time 10 min at $2.2\times10^3 \times$ g, second and third time 5 min at $1.1\times10^3 \times$ g). The microspheres were resuspended in HBS and used immediately after preparation. The particle size and size distribution were measured with a laser light blocking technique (Accusizer™, model 770, Particle Sizing Systems, Santa Barbara, CA, USA).

PorA-liposomes, calcein liposomes or OMV were encapsulated in the microspheres by dissolving dex-HEMA or man-HEMA in the dispersion (final polymer concentration 20% (w/v)), followed by addition of this mixture to the PEG solution. The initial polymer/PorA ratio used was 3300 (w/w). For calcein liposomes, the amount of encapsulated lipid was equivalent to that of encapsulated PorA-liposomes. The subsequent steps of the microsphere preparation were unchanged.

Encapsulation efficiency

The encapsulation efficiency of liposomes is defined as the amount of liposomes in the microspheres divided by the amount of liposomes added to the two-phase system $\times$ 100%. Because of interference of the microsphere formulations with the phosphate and/or protein assays the encapsulation efficiency of PorA-liposomes and OMV was estimated by using calcein liposomes as a model as described previously [13]. Briefly, freshly prepared microspheres were pelleted and resuspended in HBS containing 1% (w/v) Triton X-100. The addition of this detergent resulted in disruption of the liposomes encapsulated in microspheres and release of calcein into the surrounding buffer. The encapsulation was quantified by measuring the fluorescence of released calcein with a calibration curve made of (disrupted) calcein liposomes. The fraction of PorA-liposomes encapsulated in dextran microspheres was also determined by measuring the phospholipid content in the dextran microsphere pellet with the phosphate determination according to Rouser [26].

Release of OMV, liposomes and integrity of PorA

After preparation, the microspheres were resuspended in 2.5 ml HBS and incubated at 37ºC in a shaking water bath. At different time points, the microsphere suspension was centrifuged (5 min, $1.1\times10^3 \times$ g), after which the supernatant was removed. Next, the microsphere pellet was resuspended in 2.5 ml HBS and further incubated. The amount of calcein liposomes in the supernatants was determined as described above [13]. The integrity of calcein liposomes was determined by measuring the fluorescence in the supernatant of dextran and mannan microspheres in the absence of Triton X-100.

To determine the release profile of OMV from dextran and mannan microspheres and the integrity of PorA in the released OMV, the amount of PorA in both the supernatant and the resuspended microspheres was determined by an antigenicity assay, i.e. an inhibition ELISA as previously described [16]. For this purpose, microspheres were divided in aliquots and at each measuring point the antigen recovery of a different aliquot was determined and compared to the antigen content of non-encapsulated OMV. In parallel, the integrity of PorA in liposomes and OMV after complete release from microspheres was studied. Microspheres were incubated for 14 days at 37ºC. Then, samples were homogenized and analyzed by SDS-PAGE and ELISA, with non-encapsulated OMV and PorA-liposomes (mixed or not with empty microspheres degraded under the same conditions) as controls.

Immunization of mice and serum analysis

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 approved by the Animal Ethics Committee of the Netherlands Vaccine Institute.

Mice (6-8 weeks old, 8 animals/group) were immunized subcutaneously on day 0, 14 and 28 with PorA-liposomes or OMV, encapsulated or not in dextran or mannan microspheres (1.5 µg protein per mouse, 0.25 ml). Blood samples were taken on day 14 and 28, and mice were bled on day 42. Sera were collected and stored at -20ºC until analysis.

Antibody titers were determined for each individual serum against whole cells from the strains H44/76 (containing PorA) and HI.5 (lacking PorA) by ELISA as

described elsewhere [29]. A four-parameter curve fit was made for the optical density values (λ=450 nm) obtained with serial dilutions of the sera, and the antibody titers were calculated as the reciprocal dilutions corresponding to 50% of the maximum absorbance.

The serum bactericidal activity was assayed against strains H44/76 and HI.5 as described [30], using a final concentration of 80% (v/v) rabbit complement. Sera were heat-inactivated for 30 min at 56ºC prior to use. 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 negative controls. The serum bactericidal titer was expressed as the reciprocal serum dilution showing more than 90% killing of the number of bacteria used.

Results of antibody and bactericidal titers are expressed as the mean $\log_{10}$ titers of eight individual sera. Analysis of variance was used for statistical evaluation. The significance of the differences between the mean IgG values was determined by the least significant difference (LSD) test with 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 $\pm$ SD.

Results

Characterization of macroscopic hydrogels

The DS of dex-HEMA and man-HEMA used in our experiments was chosen in order to obtain similar degradation characteristics for dextran and mannan microspheres. Swelling characteristics are indicative of the hydrogel degradation rate. To simplify the choice of the DS, macroscopic hydrogels were made, as the preparation and swelling measurements of these macroscopic systems are easier than those of microspheres. The hydrolysis of the carbonate ester in the crosslinks resulted in a progressive swelling in time, followed by a dissolution phase for both dex-HEMA and man-HEMA hydrogels. The choice of a low DS for dex-HEMA, which would result in microspheres with a fast degradation rate, was based on previous studies [13]. For man-HEMA, a DS similar to that of dex-HEMA resulted in slower degradation rates (not shown). Comparable swelling kinetics and swelling ratios were

observed for hydrogels made of dex-HEMA and man-HEMA with a DS of 2.6 and 4.8, respectively (Fig. 2).

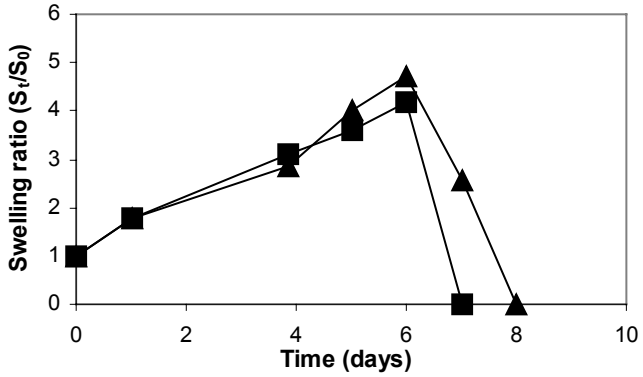

Figure 2. Swelling behavior of dextran-HEMA (squares) and mannan-HEMA (triangles) hydrogels in aqueous solution (pH 7.4, 37ºC). The initial water content of the hydrogels was 70% and the DS 2.6 and 4.8, respectively.

Characterization of the formulations

The microspheres obtained with both dex-HEMA and man-HEMA had a volume number mean diameter D [3,0] that typically ranged between 6 and 9 µm. Microspheres with a size above 10 µm were also present, as can be concluded from the size distribution of mannan and dextran microspheres (Fig. 3). The characteristics of PorA-liposomes, OMV and calcein liposomes to be encapsulated in mannan and dextran microspheres are shown in Table 1. Both liposome types had a mean particle size between 120 and 220 nm, whereas OMV were larger, with a size above 300 nm. The lowest polydispersity was obtained for (extruded) calcein liposomes (i.e. 0.17). PorA-liposomes and OMV had a relatively high polydispersity of 0.35 and 0.42, respectively. The antigenicity of PorA in liposomes and in OMV was similar.

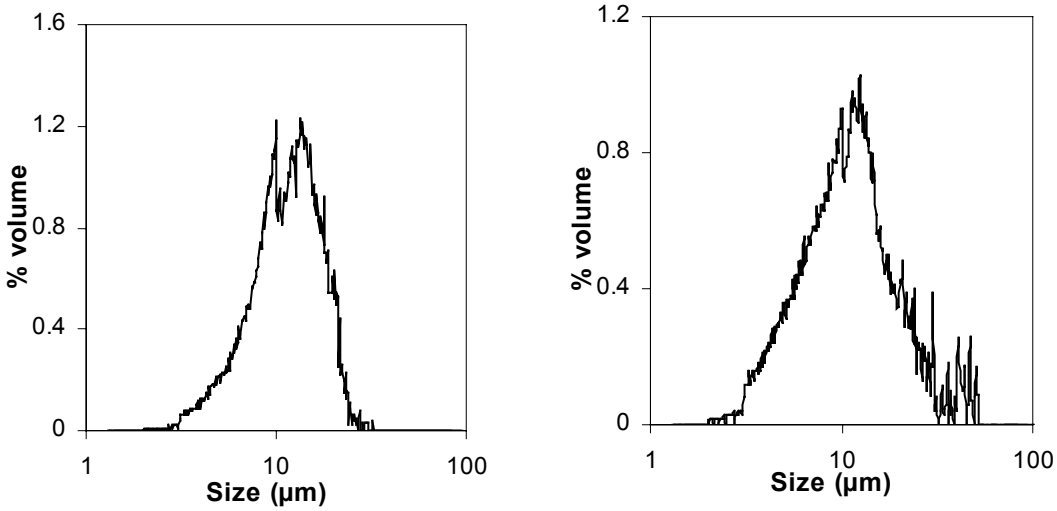

Figure 3. Volume weight diameter distribution D[3,0] of dextran (A) and mannan (B) microspheres.

Table 1. Characteristics of liposomes and OMV encapsulated in microspheres.

Formulation	*Composition*	*Size (nm)*	*PD*[a]	*Antigenicity*[b]
PorA-liposomes	PC:PG:Chol	218	0.35	0.94
Calcein liposomes	PC:Chol	126	0.17	-
OMV	Outer membrane lipids[c]	362	0.42	1

[a] Polydispersity: indication of the size distribution of the liposomes; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse suspension.
[b] Ratio between the protein concentration determined by ELISA and the protein concentration determined according to Peterson [25]. As a reference, the antigenicity of PorA in the outer membrane (OMV) was arbitrarily set at 1.
[c] [37].

The encapsulation efficiency of liposomes in mannan and dextran microspheres was determined by using calcein liposomes as a model (Table 2). As found previously [13], the encapsulation efficiency of liposomes in dextran microspheres was close to 100%. A similar encapsulation efficiency was found for PorA-liposomes in dextran microspheres, based on the phosphate determination. In contrast to the results obtained with dextran microspheres, the encapsulation efficiency of calcein liposomes in mannan microspheres was lower than 70%.

Phosphate determination could not be used for mannan microspheres because the phosphate groups present in mannan (Fig. 1) interfered with the assay. Assessment of the encapsulation efficiency of OMV in both types of microspheres was not possible because of interference of the polymers in the protein determination and the insufficient sensitivity of the phosphate assay to measure the phosphate content of OMV. Therefore, the encapsulation efficiency of OMV in dextran and mannan microspheres and PorA-liposomes in mannan microspheres was assumed to be similar to that of calcein liposomes.

Table 2. Encapulation efficiency of liposomes in mannan and dextran microspheres with an initial water content of 70%.

Polymer	*DS*	*Formulation*	*Encapsulation Efficiency*[a] *(%)*
Mannan-HEMA[b]	4.8	Calcein liposomes	64 ± 4
Dextran-HEMA[b]	2.6	Calcein liposomes	96 ± 5
Dextran-HEMA[c]	2.6	PorA-liposomes	92 ± 7

[a] Average $\pm$ SD of three independently prepared microsphere batches, based on fluorescence (calcein liposomes) or phosphate content (PorA-liposomes).
[b] Encapsulation efficiency calculated based on calcein determination.
[c] Encapsulation efficiency calculated based on the phospholipid content.

In vitro release of liposomes and OMV from microspheres

The release of liposomes from dextran and mannan microspheres was investigated by using calcein liposomes (Fig. 4). The liposome release profiles for

dextran and mannan microspheres were comparable, and similar to previous results obtained with dextran microspheres [13]. During the first 5 days, only 10% of the encapsulated calcein liposomes was released. Apparent zero-order release kinetics were observed during the next 5 days, after which the release curves leveled off at 100% release (for mannan) and 80% release (for dextran). Only marginal fluorescence was observed in the supernatants of calcein liposomes released from dextran microspheres before treatment with Triton X-100, indicating that the liposomes were intact upon release. In contrast, the liposomes released from mannan microspheres showed some fluorescence (20-30%), indicating destabilization of the liposome bilayer and leakage of the hydrophilic fluorescent marker. The low level of destabilization of the calcein liposomes occurred parallel to the degradation of mannan microspheres and release of the calcein liposomes.

The release of OMV from microspheres was studied by measuring the antigen recovery of PorA both in the supernatant of the microspheres and in homogenized samples (Fig. 5). Before incubation at 37ºC (t=0), no antigen could be detected, indicating that OMV were localized inside the microspheres and therefore PorA was not accessible to monoclonal antibodies. The antigen recovery was significantly higher in homogenized samples than in supernatants. This indicates that part of the released OMV co-precipitated with intact microspheres and/or degradation products and, consequently, only a fraction of the OMV was measured in supernatants. In homogenized samples, the antigen recovery increased progressively in time and reached a maximum level at day 12. After this time, the antigen recovery in homogenized dextran microspheres seemed to decrease slightly, but this was not significant. Interestingly, homogenized mannan microspheres showed a drastic decrease of the antigen recovery after day 12. In contrast to what was observed for homogenized samples, no antigen was measured in supernatants of microspheres loaded with OMV during the first 5 days of incubation at 37ºC. After this time, the amount of antigen detected in the supernatants increased, reaching a maximum at day 12. In line with the decrease in antigen recovery observed for homogenized samples, the antigen recovery also decreased in the supernatants after day 12 (Fig. 5).

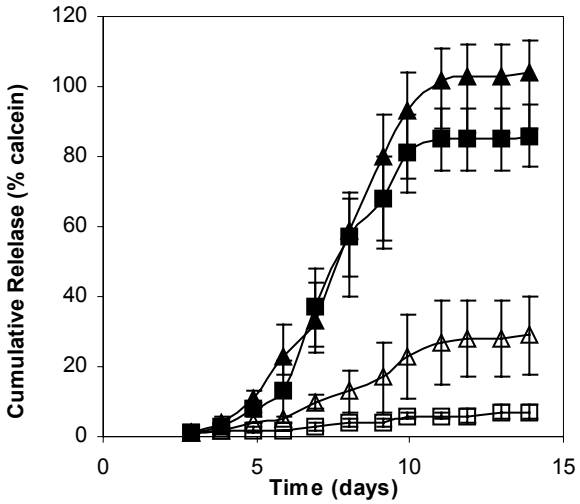

Figure 4. Cumulative release and leakage of calcein liposomes from dextran (squares) or mannan microspheres (triangles). Closed symbols: supernatants treated with Triton X-100 (cumulative release); open symbols: control supernatants, untreated (cumulative calcein leakage). Values represent the mean ± SD of three independently prepared batches.

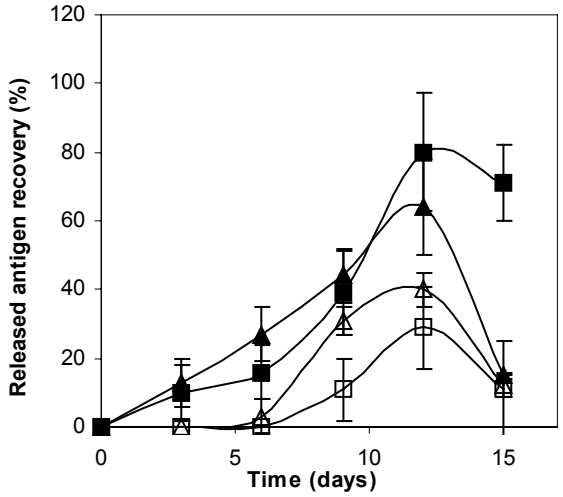

Figure 5. Antigen recovery of OMV released from dextran (squares) or mannan microspheres (triangles). Microspheres were divided in alliquots and at each measuring point the antigen recovery of a different alliquot was measured (both supernantant and homogenized samples) and compared to the antigen content of non-encapsulated OMV. Closed symbols: homogenized samples. Open symbols: supernatants. Values are mean ± SD of three independently prepared batches.

In a separate experiment, the integrity of PorA liposomes and OMV after complete release from the microspheres was studied by ELISA and SDS-PAGE. The antigen recovery for PorA-liposomes and OMV in homogenates from microspheres incubated at 37ºC for 14 days is shown in Fig. 6. In these samples, only 20 to 50% of the initial antigen content (i.e. antigen content of the non-encapsulated PorA-liposomes or OMV) was recovered. A decreased recovery of antigen (50% for PorA-liposomes and 80% for OMV) was also observed when OMV or PorA-liposomes were incubated at 37ºC during 14 days mixed with empty mannan and dextran microspheres. However, the antigen recovery measured for PorA-liposomes or OMV was maintained after incubation for 14 days in the absence of microspheres (not shown).

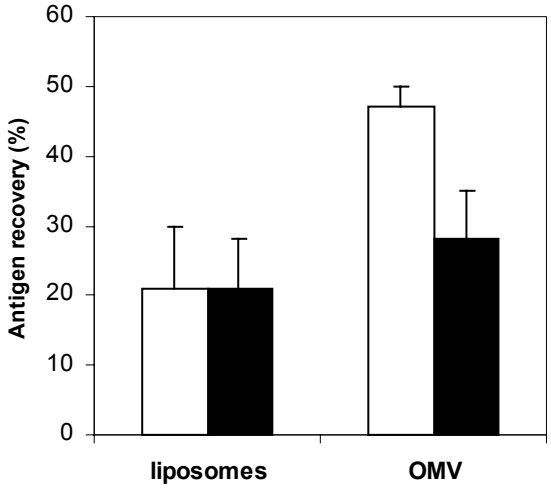

Figure 6. Antigen recovery of PorA in liposomes and OMV released from dextran (white bars) or mannan (black bars) microspheres after 14 days incubation at 37ºC. The antigenicity is expressed as percentage of the antigenicity of non-encapsulated samples. Samples were homogenized prior to the measurement. Values are mean ± SD of the measurements performed on three independently prepared batches.

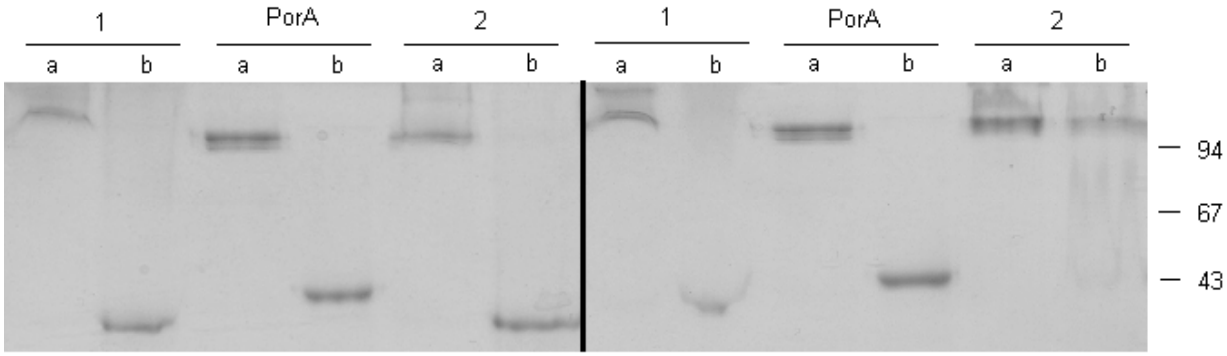

Figure 7. SDS-PAGE of PorA released from dextran microspheres (left) or mannan microspheres (right). Lanes 1: released PorA-liposomes; lanes PorA: non-treated purified PorA; lanes 2: released OMV. Samples were taken from homogenized microspheres after 14 days incubation in aqueous solution (pH 7.4, 37ºC). Each sample was solubilized in sample buffer containing 0.05% SDS and incubated for 10 min either at room temperature (a-lanes) or at 100ºC (b-lanes). Numbers on the right indicate the position of molecular weight markers.

The integrity of the PorA trimers in homogenates of microspheres loaded with PorA-liposomes and OMV was measured by 'native' SDS-PAGE [27] after 14 days incubation at 37ºC (Fig. 7, a-lanes). In all samples the protein was still present as trimers. When incubated at 100ºC, monomers were present (Fig. 7, b-lanes). Surprisingly, the monomer bands of release samples showed a slightly higher electrophoretic mobility than those of pure PorA.

Immunogenicity of encapsulated PorA-liposomes and OMV

The immunogenicity of PorA-liposomes and OMV encapsulated in dextran and mannan microspheres was studied in Balb/c mice. Two weeks after injection, bumps

containing residual amounts of microspheres were observed at the injection site, as determined by light microscopy (not shown). These bumps may be caused by a foreign body reaction, which has been previously observed with similar systems [12,31].

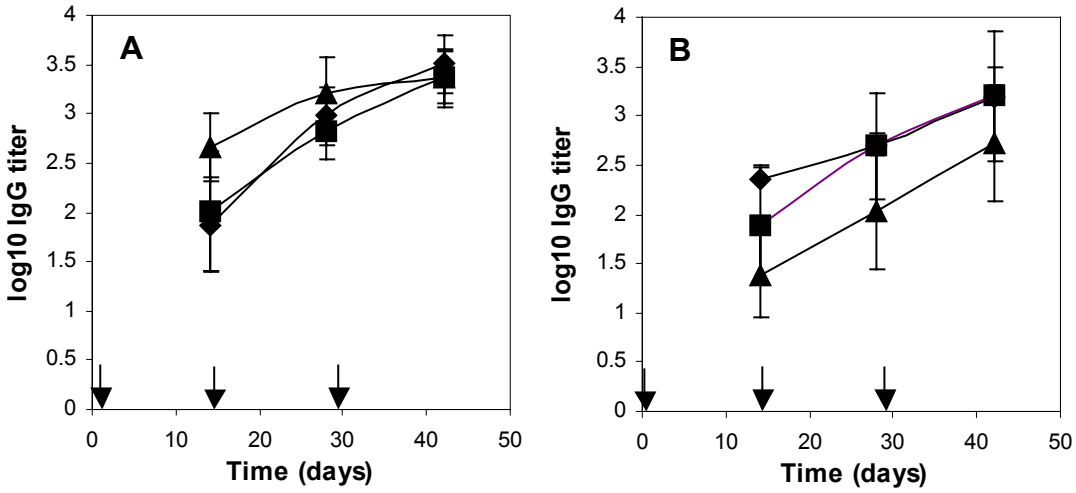

Figure 8. IgG titers found in mice immunized with OMV (panel A) or PorA-liposomes (panel B). Squares: non-encapsulated; triangles: encapsulated in dextran microspheres; diamonds: encapsulated in mannan microspheres. Results are shown as averaged $\log_{10}$ titers of 8 mice ± SD. Arrows indicate the moments of immunization.

The IgG titers of individual mice induced by PorA-liposomes and OMV, both free and encapsulated in dextran or mannan microspheres, were monitored in time (Fig. 8). In general, no substantial differences were observed between free antigen (i.e. PorA-liposomes or OMV) and antigen encapsulated in dextran microspheres. This was also true for OMV encapsulated in mannan microspheres. However, PorA-liposomes encapsulated in mannan microspheres elicited significantly lower IgG titers than non-encapsulated PorA-liposomes (Fig. 8B).

The IgG subclass distribution was studied in sera of mice after 3 immunizations (Table 3). Again, no significant differences could be found among the different formulations on the IgG subclass distribution, with substantial IgG2a and IgG2b titers. Both IgG2a and IgG2b subclasses are important, as they are able to activate complement and induce a bactericidal response [30].

In order to determine the PorA-specificity of the immune response, IgG titers against other structures than PorA were measured using a *Neisseria meningitidis* strain that does not express PorA (HI.5 strain). In all groups, only serum of one or two mice showed low reactivity against HI.5 (not shown). This indicates that the response was largely PorA-specific.

The bactericidal titers induced in serum were similar for all formulations and were mainly directed against PorA (not shown). In the groups of mice immunized with PorA-liposomes encapsulated in mannan microspheres or OMV encapsulated in dextran microspheres, only 5 and 4 mice out of 8 mice, respectively, showed a bactericidal response. In the other groups all mice had bactericidal serum activity.

Discussion

In this study we investigated the possibility to encapsulate PorA-liposomes and OMV into dextran- and mannan-based microspheres and the effect of this microencapsulation on the PorA-specific immune response.

The swelling behavior of macroscopic dex-HEMA and man-HEMA hydrogels gave a reasonably good indication of the degradation rate of the hydrogels. Although the DS of dex-HEMA was lower than that of man-HEMA, both systems showed comparable swelling kinetics and swelling ratios. This may be due to the fact that mannan is more branched than dextran (see Fig. 1), which may result in large amounts of intramolecular crosslinks fro mannan-HEMA and therefore similarly dense network for mannan and dextran microspheres. Macroscopic hydrogels were completely degraded in 7-8 days (Fig. 2). In line herewith, the release studies of the model calcein liposomes showed complete release after 10-11 days (Fig. 4).

Calcein liposomes provided a useful model to investigate the encapsulation efficiency and the release of liposomes from microspheres. The encapsulation efficiency for both PorA-liposomes and calcein liposomes in dextran microspheres was high (~90%), despite differences in particle sizes between these liposomes and the larger polydispersity of PorA-liposomes. This is in agreement with previous studies, where smaller liposomes were encapsulated in dextran microspheres [13]. Unfortunately, the encapsulation efficiency of OMV in either dextran and mannan microspheres or PorA-liposomes in mannan microspheres could not be measured directly. The encapsulation efficiency of calcein liposomes in mannan microspheres was lower (64%) than for the dextran microspheres (96%). This might be due to the less favorable partition of the liposomes over the Man-HEMA/PEG two-phase system.

Calcein liposomes were less stable upon release from mannan than after release from dextran microspheres. As no instability of calcein liposomes was observed in the surrounding buffer during the first five days, we can conclude that the instability of the liposomes was not caused by the microsphere preparation. Degradation products of man-HEMA may be responsible for the observed destabilization of the liposomes and leakage of calcein. Furthermore, PorA (from OMV or liposomes) showed decreased antigen recovery upon release from both dextran and mannan microspheres (Fig. 6). This could be due to protein denaturation, or to binding of degradation products to the P1.4 epitope. Both possibilities would impede the interaction of PorA with the monoclonal Ab. The monomer bands of PorA in homogenates from microspheres incubated for 14 days at 37ºC and visualized by SDS-PAGE gels were located lower than those of non-encapsulated PorA (Fig. 7), suggesting hydrolysis of the PorA polypeptide chain. However, this could also be due to the presence of the polymers and degradation products thereof, which might protect PorA against SDS-induced unfolding in linear monomers. This would result in a more globular form of PorA that would run faster in the SDS-PAGE gel. Decreased antigen recovery has been reported previously for tetanus toxoids released from PLGA microspheres [32]. In this case the decreased amount of released antigen was explained by surface adsorption of the toxoid to hydrophobic surfaces such as PLGA microspheres. In our system, however, the adsorption mechanism is probably different, as the polymers used are not hydrophobic.

The release profiles measured in the supernatants of microspheres with calcein liposomes (fluorescence) or OMV (antigen recovery) were similar (Fig. 4 and 5). In both cases, no release was detected the first 5 days of incubation and close to zero-order release was observed from day 5 to day 10. However, in homogenized samples the antigen was already detected after 2-3 days. Swelling of the microspheres and partial degradation of the crosslinks causes increased mesh size. This increased mesh size could allow the monoclonal antibodies to partially enter the microspheres and therefore interact with the antigen located near the microsphere surface.

The particle size distribution of dextran and mannan microspheres showed that microspheres smaller and larger than 10 µm were present. These microspheres

will probably follow different processing pathways *in vivo*. The small microspheres can be taken up easily by APC, whereas the large microspheres cannot be phagocytosed but will probably provide a reservoir of antigen [2]. In Fig. 3, the particle size distribution is expressed as volume weight diameter. Provided that the encapsulation of the antigen (i.e. OMV and PorA-liposomes) was homogeneous in both microsphere populations, about 40-45% of the PorA dose would be present in the small microspheres (<10 μm) and 55-60% in the larger microspheres, which cannot be endocytosed by APC. The separation of the PorA dose in a fraction that can be phagocytosed and a fraction that cannot be phagocytosed may have consequences for the immunogenicity. Encapsulated PorA-liposomes or OMV maintained their immunogenicity as compared to non-encapsulated PorA formulations (Table 3). The presence of two types of microspheres, i.e. large and small microspheres may affect the immunogenicity of encapsulated antigens. The smaller microspheres are probably taken up by APC, where they may end up in endosomal compartments. As the degradation of ester bonds is catalyzed by hydroxyl ions [33], the acidic environment of the endosomes probably results in slowing down of the microsphere degradation, thereby inhibiting intracellular antigen release. Future studies are needed to determine the intracellular fate of small microspheres. Further, it would be interesting to compare the immunogenicity of small and large microspheres loaded with antigen.

The use of modified mannan instead of dextran to prepare microspheres did not improve the immunogenicity of the encapsulated PorA. Mannan has been shown to have an adjuvant effect [34], presumably via recognition by the mannose receptor of APC. In the study by Apostopoulos et al., mannan conjugated to the antigen was used. In a recent study, the improvement of the uptake of mannosylated liposomes by APC expressing the mannose receptor has been related with the spacer length between the liposome and the mannose head group [35]. In our microspheres, the DS is quite low, which would result in large amounts of mannose residues on the surface of the microspheres. However, the orientation of the mannose residues at the microsphere surface is unknown and may not be optimal for interaction with the mannose receptor. Another reason for the lack of extra effect of mannan on the immune response as targeting device for APC as compared to dextran could be the presence of other receptors on the surface of APC with affinity for dextran. Indeed, it has been shown recently in mice that APC express a receptor homologue to

DC-SIGN with high affinity for dextran [36]. Whether or not this receptor also has affinity for mannan is unknown. Further studies are needed to determine the uptake of mannan and dextran microspheres by APC.

In conclusion, in this study we have demonstrated that PorA-liposomes and OMV can be encapsulated with a high efficiency (70-90%) in dextran and mannan microspheres. *In vitro* studies showed that encapsulated calcein liposomes were released almost quantitatively. Although the antigen was not completely recovered, its trimeric structure was preserved. Importantly, *in vivo* studies revealed that the immunogenicity of the encapsulated antigen was maintained. This opens possibilities to use these systems for improved vaccines. Points for further investigation are the size of the microspheres, improvement of the antigen recovery, study of targeting to APC and co-encapsulation of adjuvants in the microspheres. Moreover, it can be foreseen that by combination of non-encapsulated antigens and microspheres varying in surface and release characteristics, single shot vaccines can be obtained.

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CHAPTER 8

Summary and perspectives

Summary

There is a great need for vaccines against *Neisseria meningitidis* serogroup B. This is especially important in Western European countries, were approximately two thirds of the cases of meningococcal disease can be attributed to serogroup B strains [1,2]. Against this serogroup, traditional vaccines based on capsular polysaccharides are not effective. The immunogenicity of the capsular polysaccharide of group B meningococci is low, due to structural similarity with epitopes expressed by host neural tissue [3]. During the search for vaccine candidates against serogroup B meningococci, most attention has been focused on PorA. Por A is considered as an immunondominant antigen [4]. The vaccine potential of PorA embedded in outer membrane vesicles (OMV) developed by different groups has been studied in various clinical trials [5,6,7]. The vaccine presently being developed by the Netherlands Vaccine Institute (NVI) is based on OMV purified from genetically modified meningococcal strains each expressing three different PorA serosubtypes (trivalent OMV) [8]. The vaccine contains a mixture of two different trivalent OMV, which together represent the majority of PorA serosubtypes circulating in the Netherlands and other European countries [9]. The serosubtype P1.7-2,4 is present in up to 40% of the serogroup B strains causing bacterial meningitis [2]. Thus, a PorA-based vaccine should cover at least this subtype. On the other hand, PorA P1.7-2,4 is one of the least immunogenic serosubtypes in OMV [5,10]. For these reasons, PorA P1.7-2,4 was the serosubtype of choice for our studies. The objective of the work described in this thesis was to improve the immunogenicity of PorA by optimization of the presentation form. For this purpose, three lines of investigation were followed:

- A detailed study of physicochemical and immunochemical characteristics of PorA and its presentation form. The tools developed to characterize PorA and their presentation forms were used to monitor the stability of PorA formulated in different types of OMV.
- Preparation of well-defined liposomal PorA formulations and exploitation of various targeting strategies for dendritic cells (DC).
- Utilization of adjuvants co-incorporated in PorA-liposomes.

In **chapter 1**, an introduction is given to meningococcal disease, the surface structures of meningococci and vaccine candidates against serogroup B meningococci. Also, mechanisms involved in the immune response after vaccination

are described. Furthermore, an overview is given on antigen delivery systems and adjuvants that can be used to obtain improved immunogenic PorA formulations. Finally, the aim and outline of this thesis are provided in this chapter.

The composition of OMV may have an effect on the stability of the formulations. In **chapter 2**, three types of OMV were compared for their stability. The OMV investigated contained either one (monovalent) or three different PorA serosubtypes (trivalent), the latter with and without class 4 outer membrane protein (RmpM). OMV were sensitive to elevated temperatures (37ºC and 56ºC), resulting in destruction of the OMV structure and denaturation of PorA, followed by chemical degradation, as well as a decrease or complete loss of immunogenicity. When stored under conventional storage conditions (4ºC), all three OMV were stable for at least one year in both the liquid and the freeze-dried state.

A purification method for PorA P1.7-2,4 was developed in **chapter 3**. Purified, lipopolysaccharide (LPS) depleted PorA was characterized in detail: its conformation was monitored by electrophoretic and spectroscopic techniques, its antigenicity was measured using a bactericidal anti-P1.4 monoclonal antibody and its immunogenicity was determine in Balb/c mice. No differences were detected in the conformation of purified PorA as compared to PorA embedded in OMV or outer membrane complexes (OMC). Although the ability of purified PorA to interact with a specific anti-P1.4 bactericidal monoclonal antibody was increased upon purification, purified PorA failed to induce a bactericidal antibody response in mice, as expected. The failure to induce a serum bactericidal response was also observed for heat denatured PorA in OMV. The immunogenicity of purified PorA was recovered upon incorporation into plain liposomes, i.e. in the absence of other adjuvants. These results led us to conclude that both the conformation and the presentation form of PorA dictate the quality of the immune response.

Next, the possibility of improving the immunogenicity of liposomal PorA through targeting to DC was explored. Dendritic cells are the most efficient antigen presenting cells (APC), being able to initiate and modulate immune responses [11]. In **chapter 4**, the interaction with and uptake of PorA-liposomes by murine DC was studied. Several types of PorA-liposomes were used, differing in membrane composition. The liposomal formulations were designed to optimize their uptake by DC through different mechanisms: a) by targeting to the mannose receptor by use of mannosylated liposomes; b) by targeting to the phosphatidylserine (PS) receptor

using liposomes with PS in the bilayer or c) by a non-specific electrostatic interaction with DC, using positively charged liposomes. All these strategies resulted in improved liposome uptake by DC *in vitro* when compared to plain liposomes. The improved uptake was accompanied by increased expression of maturation markers and induction of IL-12 production. Liposomes targeted to the mannose receptor of DC were also found to target APC more efficiently *in vivo*: they showed enhanced localization in APC of draining lymph nodes after subcutaneous immunization, as compared to non-targeted liposomes. After immunization of mice, the percentage of responding mice per group was increased in groups immunized with targeted liposomes. However, in the responding mice, targeted liposomes showed equal functional immunogenicity (i.e. induction of bactericidal antibodies) as non-targeted liposomes and OMV. In conclusion, the marked positive effect of targeting observed *in vitro* was not translated to the *in vivo* response.

In another set of experiments (**chapter 5**), the interaction with and uptake by DC of targeted and non-targeted liposomes (i.e. similar formulations as described in **chapter 4**) was studied *in vitro* both in human DC and murine DC. In these systems, liposomes targeted to the mannose receptor or interacting with DC through non-specific electrostatic forces showed enhanced association with and uptake by DC. The uptake of mannosylated liposomes could be blocked with mannan in the cultures with human DC, but not with murine DC. This difference was ascribed to differences in culture conditions and origin of the cells (monocyte-derived in the human system and bone-marrow derived in the murine system). The overall conclusion of these experiments was that liposomes could be efficiently targeted to both human and murine DC.

In **chapter 6**, the use of adjuvants to improve the immunogenicity of PorA was explored. For this purpose, LPS-derived adjuvants: monophosphoryl lipid A (MPL) and the detoxified *lpxL1* mutant of LPS were used. LPS-derived adjuvants were chosen because they are easily incorporated into the liposomes and their structure is similar to that of LPS, the natural adjuvant present in the outer membrane of meningococci. As a positive controls, liposomes were adjuvated with *galE* LPS, a toxic mutant of LPS with high adjuvant activity, and with $AlPO_4$. An improved immune response (i.e. significantly increased induction of bactericidal antibodies and 100% responders in the groups) was observed with *galE* LPS and *lpxL1* LPS. However, the type of response was different, as only *galE* LPS resulted in an improved Th1 type

immune response, with increased production of IgG2a antibodies relative to IgG1 antibodies. This is desirable, as IgG2a and IgG2b subclasses are capable to activate the complement system and induce a bactericidal immune response [12]. In mice immunized with *lpxL1* LPS-adjuvated liposomes an overall increase in the levels of IgG of all subtypes was observed. The increased induction of bactericidal serum activity observed in mice immunized with *lpxL1* LPS-adjuvated liposomes was accompanied with an improved (PorA-specific) proliferation of lymph node cells after *in vitro* restimulation with PorA, as compared to non-adjuvated PorA-liposomes. In addition, proliferating cells produced high levels of IL-2 (a Th1 type cytokine), whereas the production of IL-10 (a Th2 type cytokine) was not increased.

Biodegradable microspheres were investigated in **chapter 7** as potential antigen delivery systems for both PorA-liposomes and OMV. Small microspheres (<10 μm) can be phagocytosed by APC and larger microspheres (> 10 μm) may act as a depot at the injection site while attracting APC. Dextran- and mannan-based microspheres with comparable size distribution were prepared containing PorA-liposomes or OMV. Both systems were designed to obtain a fast release of the antigen after administration. The ultimate purpose of these formulations is to improve the delivery of PorA to APC. Mannan, a polymer of mannose, has adjuvant properties [13]. Mannose residues located on the surface of the mannan microspheres may result in an improved microsphere uptake by APC and, consequently, in an improved immunogenicity of PorA-liposomes or OMV encapsulated in these microspheres. PorA liposomes or OMV were released *in vitro* from both dextran and mannan microspheres in a sustained manner between day 5 and day 10-11 when incubated at 37ºC. The trimeric structure of PorA was maintained after release from both microsphere types. Despite incomplete recovery of released antigen *in vitro*, the immunogenicity of microencapsulated PorA-liposomes and OMV was maintained.

Perspectives

From the studies presented in this thesis it is clear that liposomes are a very suitable presentation form for PorA. Not only are the physicochemical characteristics and the immunogenicity of the protein maintained, but liposomes also allow the incorporation of adjuvants and the coupling of targeting ligands to the surface of the

liposomes to enhance the PorA-specific immune response. Moreover, the bilayer building liposome components (phospholipids) have low intrinsic immunogenicity.

In comparison with liposomes, the composition of OMV is not well defined. The composition of OMV varies among different strains and is also influenced by the growth conditions and the isolation method. Besides, the presence of a number of outer membrane proteins predicted by reversed vaccinology has been demonstrated [14]. These outer membrane proteins are not detected by conventional techniques (because they are normally present in minute amounts in the outer membrane) and add to the lack of knowledge about the exact composition of OMV. The presence of outer membrane proteins other than PorA may have a positive effect on the immune response against OMV, by inducing cross-reactive antibodies that would increase the coverage of the vaccine. Here again, the use of liposomes would be advantageous to separately study the contribution of each outer membrane protein to the immunogenicity of the vaccine.

In **chapter 2** of this thesis we demonstrated that both trivalent and monovalent OMV were stable for 1 year when stored at 4ºC. However, for a commercial product a shelf life of at least 2 years is desired. If stability of OMV in the liquid state for prolonged periods would turn out to be problematic (as might be derived from the accelerated stability studies at 37ºC and 56ºC), freeze-drying may be an alternative. In that case, however, the freeze-drying process of OMV has to be further investigated in order to prevent aggregation of the vesicles. This can be done by monitoring the effects of different process conditions, i.e. freezing rate, primary and secondary drying phase, or reconstitution [15] on their physicochemical stability and immunogenicity. The critical step(s) in these processes should be identified and optimized. Furthermore, the aggregation of OMV is possibly preventable by use of other lyoprotectants than sucrose (e.g. maltose, trehalose). The stability of the liposomal formulations used in other chapters of this thesis remains to be investigated. Liposomes can be prepared with high physical stability [16] and freeze-drying of liposomes is feasible [17,18]. Because of the similarities between PorA-liposomes and OMV, the tools used to investigate the stability of OMV can also be applied for monitoring the stability of liposomal PorA formulations.

In the experiments with liposomal PorA described in this thesis, PorA was purified from monovalent OMV. Another source of PorA that was not studied in this thesis are inclusion bodies derived from *Escherichia coli.* This recombinant PorA may

be advantageous to use with respect to purity and yield, as compared to PorA purified from OMV. Indeed, in the PorA purified from monovalent OMV, traces of neisserial LPS were still present (**chapter 3**). A study should be done comparing the structures of PorA derived from these two sources. The use of recombinant PorA allows the investigation of other important PorA serosubtypes as well. For instance, making an artificial trivalent OMV (i.e., liposomes containing three different recombinant PorA serosubtypes). In this way, the decreased immunogenicity that was observed for PorA P1.7-2,4 in trivalent OMV [5] can be studied in detail.

Successful DC targeting *in vitro* is no guarantee for enhanced functional immunogenicity *in vivo*, as was shown in **chapter 4**. The lack of correlation between effects of targeting *in vitro* and bactericidal response *in vivo* may be due to the complexity of the interactions between liposomes and various cell types and tissues *in vivo.* For instance, the mannose receptor to which mannosylated liposomes were targeted is not only present on DC or APC, but in a variety of other cells and tissues as well. Also, there is not only one receptor with affinity for mannose, but a whole family of receptors [19]. In future studies, the use of more specific targeting ligands (e.g. use of monoclonal antibodies directed to receptors expressed exclusively by DC) may result in improved functional immunogenicity. It is difficult, however, to find a specific DC target. DEC-205, a receptor for endocytosis has been described as a possible specific target on DC [20]. However, it has been demonstrated that DEC-205 is not only expressed by DC, but also by thymic, pulmonary and intestinal epithelia as well as B-cells [19]. A unique DC receptor is yet to be found. Antibodies against Fc receptors can also be used as targeting devices. Fc receptors are expressed on the surface of many APC (e.g. B-cells, macrophages) [21]. A question that remains to be answered is whether specific DC targeting is more effective in raising a functional immune response than targeting to APC in general.

In addition to difficulties related with the extrapolation of *in vitro* data to *in vivo* effects, the predictability of animal experiments for the human situation remains a critical issue. This is illustrated by the fact that murine DC and human DC seem to differ regarding recognition and uptake of (targeted) PorA-liposomes *(***chapter 5***)*. However, to further unravel and properly compare the interactions of antigen presentation forms with murine and human DC, efforts should be made to obtain well-characterized murine and human DC with similar characteristics (e.g. receptor and maturation markers expression). Furthermore, *in vitro* culture conditions should

be relevant to the *in vivo* situation of the cells. Also, the use of more specific targeting ligands for both human and murine DC can help to understand the pathways of antigen processing (e.g. uptake mechanism involved) that result in an improved immune response. With this information, more appropriate ligands could be designed and tested, coupled to antigen delivery systems (e.g. liposomes).

The results presented in **chapter 6** suggest that an enhanced PorA-specific Th1 type immune response correlates with an improved functional immunogenicity against meningococci. However, absolute and relative Th1 and Th2 type response levels leading to optimum protection against meningococcal disease are yet to be established. Further PorA-specific T-cell proliferation studies are needed to determine the production pattern of cytokines (e.g. IL-2, IL-10, INF-γ, TNF-α, IL-12) by activated T-cells and the kinetics of the production of these cytokines. This will help to confirm the type of response (Th1/Th2) induced by adjuvated PorA-liposomes and to gain insight in the desired characteristics of PorA-formulations. In follow-up studies, the effect of the formulation (e.g. use of other adjuvants, effect of targeting to DC) on the T-cell activation induced by liposomal PorA should be tested, in order to characterize in detail the type of T-cell responses associated with improved PorA-specific bactericidal responses.

The studies presented in **chapter 7** show that dextran- and mannan-based microspheres are promising delivery systems for PorA-liposomes or OMV, provided that optimization of the microsphere characteristics is pursued. The microspheres used in our studies had a rather broad size distribution, with small microspheres (size < 10 μm) and large microspheres (size > 10 μm). The presence of large and small microspheres results in mixed antigen processing routes. The small microspheres were probably taken up by APC, and would then end up in endosomal compartments. It cannot be predicted what would happen at the low pH of late endosomal compartments, as the degradation of microspheres is retarded by low pH, and the antigens may not become available in time for presentation by MHC molecules. Information regarding the release rate could be collected by monitoring the stability of antigen loaded in microspheres incubated at low pH. On the basis of this information, microspheres might be designed with faster release kinetics at low pH. In contrast to small microspheres, the larger microsphere particles will probably not be taken up by APC, but will rather form a depot at the injection site. The release of the antigen will occur upon degradation of the microspheres. Follow-up

experiments are needed to study the effect of the microsphere size on the immunogenicity of the encapsulated PorA formulations, i.e. separating large microspheres from small microspheres and studying the effect of microsphere size on the immunogenicity of the encapsulated antigen. Finally, the maintained immunogenicity of PorA-liposomes and OMV upon encapsulation brightens the prospects to use these formulations for single-shot PorA vaccination schedules. In our system, the antigen was released after 5 days of incubation at 37ºC. By modifying characteristics of the polymer (e.g. degree of substitution, type of crosslinks) and/or the microspheres (e.g. initial water content), longer delay periods for the release of encapsulated antigen can be obtained [22]. By combining in one injection immediately available PorA-liposomes or OMV with presentation forms that release the antigen pulse-wise after a delay time of weeks or even months, single-shot systems could be obtained. In these systems, one injection would mimic the immunization and booster administration of the vaccine.

In this thesis, liposomes were studied as a presentation form for PorA, as an alternative for OMV. Other presentation forms, such as ISCOMs or virosomes remain to be studied. ISCOMs are very stable spherical micellar assemblies composed of the saponin mixture QuilA, cholesterol and phospholipids, which present membrane proteins as multimers. The use of ISCOMs may offer advantages as compared to plain liposomes because of the adjuvant effect derived from the presence of QuilA [23]. Another presentation form that may be suitable for PorA are virosomes. Virosomes have a similar structure to liposomes, but contain influenza hemagglutinin (HA) in addition to phospholipids. The presence of HA confers virosomes a fusogenic activity, and acts as a targeting device; binding to sialic acid residues present on APC [23]. These characteristics allow virosomes to stimulate both humoral and cellular immune responses.

The PorA dose used in our immunization studies was relatively low (1.5 µg) as compared to the dose used in investigations from other groups (10-20 µg) [24,25]. A dose-response study was only performed with plain (i.e. non-targeted, non-adjuvated PorA) liposomes (**chapter 3**). In this case, the increase of the PorA dose did not result in increased immunogenicity. Based on this study, the lowest PorA dose tested (1.5 µg) was used for further experiments described in this thesis. Still, an even lower dose of PorA might have served to unmistakably observe enhanced immunogenicity of targeted and/or adjuvated liposomes as compared to plain ones. Additional studies

should be done with the (improved) PorA-liposomes to determine whether the PorA dose needed to induce a bactericidal response could be decreased or not.

The administration route used in the immunogenicity studies described in this thesis was the subcutaneous route. The administration route chosen should be in accordance with the presentation form of the vaccine and the effect pursued. Thus, when studying presentation forms with specific targeting moieties for dendritic cells, the intradermal administration route could be advantageous. Through this route, the layer of Langerhans cells (immature DC) located in the epidermis [26] may be easily reached. However, the administration of vaccines via the intradermal route is difficult and the injections may be quite painful. Moreover, the mouse skin is very thin compared to human skin and only low volumes can be injected (maximal 100 µl per injection site). Attempts were made to administer targeted PorA-liposomes via the intradermal route (results not shown). This did not lead to improved immunogenicity of the formulations with respect to the subcutaneously administered ones. Studies with confocal microscopy on the localization of fluorescently labeled liposomes at the injection site reveled that only a small fraction of the liposomes administered was located near the immune cells of the skin, and the majority of the dose was located subcutaneously. To overcome the drawbacks of intradermal injections, new delivery devices are being developed. Among these are Macroflux® microprojection array patches [27]. In this system, antigens are coated as a dry film on the microneedles in the patch, and it was proven that the depth of skin penetration was sufficient to allow antigens to reach skin immune cells (i.e. Langerhans cells). Detailed formulation studies have to be done in order to adapt this system to (liposomal) PorA formulations.

In conclusion, the studies presented in this thesis demonstrate the potential of liposomes as PorA delivery systems.

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Appendices

Achtergrond

De meningokok ofwel *Neisseria meningitidis* is een gramnegatieve bacterie die bacteriële meningitis (hersenvliesontsteking, nekkramp) en bloedvergiftiging (sepsis) kan veroorzaken. Ongeveer 10% van de bevolking draagt *N. meningitidis* in de keelholte. In de meeste gevallen treden geen ziekteverschijnselen op. De bacterie wordt overgedragen via direct contact met nasaal of oraal slijm of via inademing van kleine druppeltjes, bijvoorbeeld door niezen of hoesten. In een klein aantal mensen leidt dragerschap tot meningitis of bloedvergiftiging. De meeste gevallen komen voor bij kinderen jonger dan vijf jaar en adolescenten. Tien tot dertig procent van de patiënten overlijdt aan de ziekte. In veel gevallen houdt de patiënt ernstig blijvend letsel over van de infectie

In West-Europese landen wordt ongeveer twee derde van de infecties door meningokokken veroorzaakt door serogroep B. Daarom is er grote behoefte aan vaccins tegen *N. meningitidis* serogroep B. Traditionele vaccins tegen meningokokken, welke gebaseerd zijn op polysacchariden (suikerketens) uit het kapsel, zijn niet werkzaam tegen serogroep-B-meningokokken. Polysacchariden van serogroep B zijn namelijk nauwelijks immunogeen. Dit komt waarschijnlijk doordat hun structuur gelijkenis vertoont met die van suikerketens welke in de hersenen voorkomen. Tijdens het zoeken naar vaccinkandidaten werd de meeste aandacht gericht op PorA, een porievormend buitenmembraaneiwit. PorA is in staat bacteriedodende (bactericide) antistoffen op te kwekken en wordt beschouwd als een immunodominant antigeen. Bactericide antistoffen zijn nodig voor de bescherming tegen meningokokkenziekte. Het vaccin dat nu in ontwikkeling is bij het Nederlands Vaccin Instituut (NVI) is gebaseerd op buitenmembraan*vesicles* (in het Engels *outer membrane vesicles*, OMV). Deze worden gezuiverd uit genetisch gemodificeerde meningokokkenstammen waarin drie verschillende PorA's tot expressie komen (trivalente OMV). Het vaccin is een mengsel van twee verschillende trivalente OMV, waarin de PorA's vertegenwoordigd zijn die het meest voorkomen in Nederland en andere Europese landen. Van de door serogroep B stammen veroorzaakte meningitis gevallen wordt bijna 40% veroorzaakt door het subtype P1.7-2,4. Een op PorA gebaseerd vaccin zal dus dit subtype moeten bevatten. Aan de andere kant is PorA P1.7-2,4 een van de minst immunogeen subtypen in OMV. Om deze redenen is PorA P1.7-2,4 gekozen als model voor onze studies.

Het doel van het in dit proefschrift beschreven onderzoek was het verbeteren van de immunogeniciteit van PorA P1.7-2,4 door gebruik te maken van betere presentatievormen voor het eiwit. Om dit doel te bereiken werden drie onderzoeksrichtingen gevolgd:

- Een gedetailleerd onderzoek van de fysisch-chemische karakteristieken en de formulering van PorA. De methoden ontwikkeld om PorA en de presentatievorm van PorA te karakteriseren werden gebruikt om de stabiliteit van PorA in verschillende OMV-types te bestuderen.
- De bereiding van goed gedefinieerde liposomale PorA-formuleringen en het gebruik van moleculen aan het oppervlak van de liposomen die een verbeterde interactie met dendritische cellen (DC), een belangrijke celtype van het afweersysteem, tot stand brengen.
- Het gebuik van adjuvantia, stoffen die in staat zijn de immunogeniciteit van (liposomaal) PorA te verbeteren.

Opbouw van het proefschrift

Hoofdstuk 1 geeft een introductie over de meningokokkenziekte en beschrijft de oppervlakte-eiwitten van meningokokken. Tevens wordt aangegeven welke van deze eiwitten mogelijke vaccinkandidaten zijn tegen serogroep-B-meningokokken. Vervolgens worden de mechanismen die het afweersysteem gebruikt na vaccinatie beschreven, samen met de mogelijke formuleringen en adjuvantia die gebruikt kunnen worden om de immunogeniciteit van PorA te verbeteren. Tenslotte worden het doel en de opbouw van het proefschrift aangegeven.

De samenstelling van OMV zou een effect kunnen hebben op de stabiliteit van de formuleringen. In **hoofdstuk 2** wordt de stabiliteit van drie verschillende typen OMV vergeleken. De in dit hoofdstuk bestudeerde OMV bevatten één PorA (monovalente OMV) of drie PorA's (trivalente OMV). Twee typen trivalente OMV zijn bestudeerd: met en zonder klasse-4-buitenmembraaneiwit (RmpM). Wanneer de OMV in vloeibare of gevriesdroogde vorm bewaard werden bij 4ºC, de standaard bewaartemperatuur, bleven ze gedurende minstens een jaar stabiel, ongeacht hun samenstelling. Na versnelde stabiliteitstudies, d.w.z. opslag bij 37ºC en 56ºC, waren duidelijke effecten waarneembaar: de structuur van zowel de vesicles als PorA ging verloren. Hierbij ging de immunogeniciteit van PorA gedeeltelijk of geheel verloren.

Een zuiveringsmethode voor PorA P1.7-2,4 werd ontwikkeld in **hoofdstuk 3**. Gezuiverd PorA werd in detail gekarakteriseerd: de vouwing van PorA werd bestudeerd met elektroforetische en spectroscopische technieken, de antigeniciteit (het vermogen herkend te worden door antistoffen) werd gemeten met gebruik van bactericide anti-P1.4-antistoffen en de immunogeniciteit (het vermogen om een immuunrespons op te wekken) werd bepaald in Balb/c-muizen. De conformatie van gezuiverd PorA kwam overeen met die van PorA in OMV of in buitenmembraancomplexen (OMC). Hoewel gezuiverd PorA een betere interactie met specifieke anti-P1.4-antistoffen (antigeniciteit) gaf dan PorA in OMV en in muizen antistoffen opwekte, bleken die niet functioneel, d.w.z. bacteriedodende te zijn, evenmin als gedenatureerd PorA. De functionele immunogeniciteit van gezuiverd PorA werd volledig hersteld tot het niveau verkregen met OMV als het in liposomen ingebouwd werd. De conclusie van deze studie is dat zowel de conformatie als de presentatievorm van PorA de kwaliteit van de immuunrespons bepalen.

Vervolgens werd in **hoofdstuk 4** de mogelijkheid onderzocht om de immunogeniciteit van PorA bevattende liposomen verder te verbeteren. Hiertoe werden aan het oppervlak van de liposomen structuren aangebracht waardoor dat ze makkelijker binden en vervolgens worden opgenomen door DC, welke beschouwd worden als de meest efficiënte antigeenpresenterende cellen (APC). De interactie tussen zulke doelzoekende PorA-liposomen en DC afkomstig van de muis (murine DC) werd onderzocht. De liposomale formuleringen werden zo gekozen dat de opname van PorA-liposomen door DC via verschillende mechanismen zou kunnen worden verbeterd: a) gemannosyleerde liposomen: liposomen die aan hun oppervlak voorzien zijn van mannosegroepen waardoor ze door de mannosereceptor op de DC herkend en via deze receptor opgenomen kunnen worden; b) liposomen voorzien van fosfatidylserine waardoor ze via de fosfatidylserinereceptor op de DC opgenomen kunnen worden; of c) positief geladen liposomen die aspecifieke elektrostatische interacties met negatief geladen DC aangaan. Elk van deze doelzoekstrategieën had een verbeterde opname van liposomen door DC, een verhoogde expressie van maturatiemarkers en inductie van IL-12-productie tot gevolg. Gemannosyleerde liposomen werden na immunisatie in hogere mate teruggevonden in lymfkliercellen dan ‘kale’ (negatief geladen), niet doelzoekenden liposomen. Het percentage muizen met bacteriedodende antistoffen was hoger in de

groepen die geïmmuniseerd waren met doelzoekende PorA-liposomen dan in de groep muizen die niet doelzoekende PorA-liposomen toegediend hadden gekregen. De concentratie aan bacteriedodende antistoffen in de muizen die geïmmuniseerd waren met doelzoekende liposomen was echter niet hoger dan in de muizen die niet doelzoekende liposomen of OMV toegediend hadden gekregen. De conclusie is dat het positieve effect van doelzoekende PorA-liposomen dat gezien werd *in vitro* niet volledig werd vertaald naar de respons *in vivo*.

In andere experimenten (**hoofdstuk 5**) werd *in vitro* de interactie met en opname door DC van doelzoekende en niet doelzoekende liposomen (dezelfde formuleringen als in **hoofdstuk 4** werden gebruikt) onderzocht in humane en murine DC. In beide systemen lieten gemannosyleerde liposomen en positief geladen liposomen een verhoogde associatie met en opname door DC zien. De opname van gemannosyleerde liposomen door humane DC werd geblokkeerd door mannaan, in tegenstelling tot murine DC. Dit verschil zou veroorzaakt kunnen zijn door de verschillende kweekcondities en oorsprong van de cellen (uit monocyten in het humane systeem en uit het beenmerg in het muissysteem). Uit deze studies werd geconcludeerd dat doelzoekende liposomen efficiënt afgeleverd kunnen worden aan humane en murine DC.

In **hoofdstuk 6** werden adjuvantia gebruikt om de immunogeniciteit van liposomaal PorA te verbeteren. Voor dit doel werden van LPS afgeleide adjuvantia gebruikt: het veelgebruikte monofosforyl lipid A (MPL) en een meningokokken LPS met verminderde toxiciteit, gemaakt door de zg. *pxL1*-mutant. De keuze van deze adjuvantia was gebaseerd op het feit dat deze makkelijk ingebouwd kunnen worden in liposomen en de structuur vergelijkbaar is met die van LPS, het endotoxine van meningokokken dat ook als adjuvans kan fungeren. Als positieve controles werden liposomen geadjuveerd met *galE*-LPS, een toxische mutant van meningokokken-LPS met een hoge adjuvansactiviteit, en met colloïdaal aluminiumfosfaat. Verhoogde immunogeniciteit (ofwel verhoogde productie van bacteriedodende antistoffen) werd gezien bij liposomen geadjuveerd met *galE*-LPS en *lpxL1*-LPS. Het type immuunrespons was echter anders omdat alleen *galE*-LPS een betere Th1-type respons veroorzaakte met een verhoogde productie van IgG2a-antistoffen ten opzichte van IgG1-antistoffen. Dit is gewenst omdat IgG2a- en IgG2b-antistoffen het complementsysteem kunnen activeren om bacteriën te doden. In muizen die geïmmuniseerd waren met *lpxL1*-LPS-geadjuveerde PorA-liposomen waren de

serumconcentraties van alle IgG-isotypen verhoogd ten opzichte van niet-geadjuveerde liposomen. Geïsoleerde lymfklierknopen van muizen die geïmmuniseerd waren met *lpxL1*-geadjuveerde liposomen lieten, na restimulatie *in vitro* met PorA, een sterkere PorA-specifieke celproliferatie zien ten opzichte van niet-geadjuveerde liposomen. Tegelijkertijd produceerden deze cellen ook grote hoeveelheden IL-2 (een Th1-type cytokine), terwijl de productie van IL-10 (een Th2-type cytokine) niet was verhoogd.

Afbreekbare microsferen werden in **hoofdstuk 7** onderzocht op hun toepasbaarheid als afgiftesystemen voor PorA-liposomen en OMV. Kleine deeltjes (< 10 µm) kunnen opgenomen worden door antigeenpresenterende cellen (APC), terwijl grotere deeltjes (> 10 µm) op de injectieplaats als depot kunnen fungeren en daarbij wellicht APC aantrekken. De gebruikte microsferen waren gebaseerd op dextraan of mannaan en hadden een vergelijkbare grootte. In de microsferen waren porA of OMV ingebouwd. Beide systemen waren ontwikkeld om ingebouwde PorA-liposomen of OMV na toediening snel af te geven. Het uiteindelijke doel van deze formuleringen was een verbeterde aflevering van PorA aan APC. Mannaan, een polymeer van mannose, heeft adjuvanseigenschappen. Mannoseresiduen aan het oppervlak van de microsferen zouden herkend kunnen worden door APC veroorzaken, waardoor de immunogeniciteit van de ingebouwde PorA-liposomen of OMV verbeterd zou kunnen worden. Tijdens *in vitro* incubatie (37ºC) van beide typen microsferen (dextraan en mannaan) werden PorA-liposomen en OMV tussen dag 5 en dag 11 gelijkmatig afgegeven. De trimeerstructuur van PorA na afgifte uit beide microsferen was behouden. De immunogeniciteit van de ingebouwde PorA-liposomen en OMV bleef gehandhaafd ondanks het feit dat het antigeen niet volledig afgegeven werd.

Conclusie

Dit proefschrift toont aan dat liposomen heel geschikte presentatievormen zijn voor op PorA gebaseerde vaccins. De fysisch-chemische karakteristieken en de immunogeniciteit van het eiwit blijven behouden. Bovendien kan de immunogeniciteit van PorA verbeterd worden door adjuvantia en APC-herkennende moleculen in de liposomen in te bouwen.

Introducción

Neisseria meningitidis, también llamados meningococos, es una bacteria gram-negativa causante de meningitis y septicemia. Aproximadamente un 10% de la población es portadora de esta bacteria en la laringe, sin desarrollar síntomas de la enfermedad. Este tipo de bacterias se transmiten bien por contacto directo con las mucosas nasales y orales, o bien por inhalación de pequeñas partículas que contienen la bacteria, por ejemplo por estornudos o tos. En un pequeño número de casos, el portador de la bacteria desarrolla la enfermedad. La mayoría de los casos de meningitis o septicemia se dan en niños menores de cinco años y en adolescentes, de los cuales entre el 10 y el 30% de los casos son mortales.

Existe una gran necesidad de vacunas contra la meningitis causada por meningococos del serogrupo B. Esta necesidad es especialmente acuciante en países de Europa occidental, donde prácticamente dos tercios de las infecciones causadas por meningococos son atribuibles al serogrupo B. Vacunas tradicionales, basadas en polisacáridos (azúcares) de la cápsula bacteriana, no son efectivas contra meningococos del serogrupo B, ya que los polisacáridos del serogrupo B no producen una respuesta inmunológica adecuada. Probablemente esto se deba a la similitud entre las estructuras de estos polisacáridos y otros azúcares presentes en tejidos cerebrales. Durante la búsqueda de antígenos que pueden ser utilizados en vacunas contra meningococos del serogrupo B, ha suscitado el mayor interés PorA, una proteína que forma poros en la membrana exterior de la bacteria. La mayor parte de los anticuerpos que se dirigen hacia esta proteína son capaces de destruir bacterias por medio de mecanismos que implican la activación del sistema del complemento, lo cual es supuestamente necesario para la protección contra infecciones causadas por meningococos. La vacuna que está en desarrollo en el Instituto Holandés de Vacunas (Nederlands Vaccin Instituut, NVI) está basada en vesículas aisladas de la membrana exterior de la bacteria (en Inglés: outer membrane vesicles, OMV), purificadas de meningococos genéticamente modificados, los cuales expresan tres subtipos diferentes de PorA (OMV trivalentes). La vacuna consiste en una mezcla de dos tipos de OMV trivalentes, en la que las distintas moléculas de PorA representan a la mayoría de los subtipos que circulan en los Países Bajos y Europa occidental. PorA del subtipo P1.7-2,4 está presente en aproximadamente un 40% de los meningococos aislados en pacientes. Así, una

vacuna basada en PorA debe contener este subtipo. Por otra parte, PorA P.17-2,4 es uno de los subtipos menos inmunogénicos en OMV. PorA P1.7-2,4 ha sido elegido como modelo en esta investigación. El objetivo del trabajo descrito en esta tesis es la mejora de la respuesta inmunológica dirigida contra PorA P1.7-2,4, por medio del uso de formas de presentación más adecuadas. Para conseguir este objetivo, se han seguido tres líneas de investigación:

- La investigación detallada de las características físico-químicas de PorA y sus formas de presentación. Los métodos desarrollados para caracterizar PorA y sus formas de presentación han sido utilizados para el estudio de la estabilidad de PorA en varios tipos de OMV.
- La preparación de formulaciones liposomales de PorA bien definidas y el uso de moléculas en la superficie de dichas fórmulas específicamente dirigidas hacia células dendríticas (CD), que constituyen uno de los tipos de células más importantes del sistema inmunológico.
- El uso de compuestos capaces de mejorar la respuesta inmunológica de antígenos pobres, también llamados adyuvantes, incorporados en las fórmulas liposomales de PorA.

Organización de la tesis

El **capítulo 1** es una introducción sobre la meningitis, las estructuras de la superficie de meningococos y los posibles candidatos para vacunas contra la meningitis causada por meningococos del serogrupo B. Asimismo, se describen los mecanismos que generan la inmunidad creada por vacunas y las posibles formulaciones que pueden utilizarse para mejorar la respuesta inmune de PorA. Por último, se introducen los objetivos y organización de la tesis.

Dado que es probable que la composición de las OMV afecte a la estabilidad de las formulaciones, el **capitulo 2** de esta tesis está dedicado a estudiar la estabilidad de tres tipos de OMV diferentes. Las OMV estudiadas contienen un solo subtipo de PorA (OMV monovalentes) o tres subtipos de PorA (OMV trivalentes). Igualmente, se han estudiado dos tipos de OMV trivalentes, que contienen o no proteína de la membrana exterior de clase 4 (también llamada RmpM). Las OMV demostraron ser susceptibles a temperaturas elevadas (37ºC o 56ºC), lo cual dio lugar a la destrucción de la estructura vesicular de las OMV, la desnaturalización y

degradación de la proteína, y la pérdida parcial o total de la capacidad de inducir una reacción inmunológica. OMV de los tres tipos estudiados, mantenidas a 4ºC, son estables al menos durante un año en forma líquida ó liofilizadas.

En el **capítulo** 3, se aborda la descripción de un método para la purificación de PorA P1.7-2,4. Asimismo, se caracteriza de forma detallada PorA purificada y parcialmente libre de lipopolisacárido (LPS): la conformación de la proteína se estudió por medio de métodos electroforéticos y espectrofotométricos, la antigenicidad de la proteína se precisó con anticuerpos monoclonales bactericidas específicos, y la capacidad de inducir une respuesta inmune fue determinada en ratones Balb/c. No se detectaron diferencias en la conformación de PorA purificada o PorA en OMV o en complejos de la membrana exterior (en Inglés: outer membrane complexes, OMC). Aunque la proteína purificada demostró una mejor capacidad de interacción con anticuerpos monoclonales, de acuerdo con resultados previos de otros grupos, PorA purificada no fue capaz de inducir anticuerpos bactericidas en ratones. De la misma forma, PorA desnaturalizada por calor tampoco indujo anticuerpos bactericidas en ratones. Por otra parte, la respuesta inmune producida por PorA fue completamente recuperada al formular la proteína en liposomas, sin necesidad de añadir adyuvantes. La conclusión de este capítulo es que tanto la conformación de la proteína como su forma de presentación determinan el tipo de respuesta inmunológica obtenida con el antígeno.

El siguiente paso (**capítulo 4**) consistió en valorar la posibilidad de incrementar la respuesta inmunológica de PorA en liposomas por medio de vectorización a células dendríticas. Este tipo de células son presentadoras de antígenos muy eficientes y pueden considerarse únicas, ya que son capaces de iniciar y modular la respuesta inmune. En este capítulo se investigó la interacción e internalización de PorA liposomal en CD de ratón. Para ello, se produjeron diferentes tipos de liposomas, con diferentes composiciones de la membrana liposomal. Estos liposomas han sido diseñados como sistemas de vectorización de CD: a) vectorización dirigida hacia receptores de manosa en la membrana de CD (liposomas con residuos de manosa en la superficie); b) vectorización dirigida al receptor de fosfatidilserina (liposomas con fosfatidilserina en la membrana); o c) por medio de interacciones no específicas, de tipo electrostático entre liposomas con carga positiva y la membrana celular (cargada negativamente). Estas tres estrategias dieron como resultado una mejora de la interacción e internalización de

liposomas por parte de las CD. Al mismo tiempo, se incrementaron de los niveles de expresión de marcadores de maduración celular en CD y producción de interleukina 12 (IL-12) por parte de éstas. En un siguiente paso, el uso de liposomas vectorizados hacia el receptor de manosa en CD tuvo como consecuencia un incremento de la internalización de liposomas por células presentadoras de antígenos (CPA) en ratones: la localización de estos liposomas se incrementó en nódulos linfáticos eferentes tras administración subcutánea, comparada con la localización de liposomas no vectorizados. El porcentaje de animales que mostraron producción de anticuerpos bactericidas fue superior en ratones inmunizados con liposomas vectorizados que en ratones inmunizados con liposomas no vectorizados o OMV. A pesar de esto, los niveles de anticuerpos bactericidas en ratones con respuesta fueron similares en todos los grupos. En conclusión, el efecto marcadamente positivo observado en cultivos celulares no se traslada completamente en la respuesta inmunológica.

En otros experimentos, descritos en el **capítulo 5,** la interacción e internalización por CD de liposomas vectorizados o no (formulaciones similares a las usadas en el **capítulo 4**) se estudió en cultivos celulares de CD de origen humano o de ratón. En ambos sistemas, el uso de liposomas vectorizados hacia el receptor de manosa o vectorizados por medio de interacción electrostática, obtuvo como resultado un incremento de la interacción e internalización por CD. En cultivos de CD de origen humano, la interacción de liposomas vectorizados con manosa se puede bloquear usando manan, un polímero de manosa, lo cual no fue observado en cultivos de células de ratón. Esta diferencia puede estar causada por las diferentes condiciones de los cultivos, o por el diferente origen de las células (monocitos en el caso de las CD de origen humano, o de la medula ósea en ratones). La conclusión de este estudio es que liposomas conteniendo PorA pueden ser eficientemente vectorizados hacia CD.

El **capítulo 6**, se centró en investigar el uso de adyuvantes para mejorar la respuesta inmune contra PorA en liposomas. Así, se estudiaron adyuvantes derivados de LPS: monofosforil lípido A (MPL) y el menos tóxico *lpxL1* mutante de LPS. La elección de estos adyuvantes se basó en el hecho de que estas sustancias se pueden incorporar fácilmente en la membrana de liposomas y su estructura es similar a la de LPS, el adyuvante natural, aunque extremadamente tóxico, producido por meningococos. Se utilizaron como controles liposomas que contenían un

derivado de LPS, *galE* LPS, tóxico aunque con buena actividad adyuvante, o mezclados con $AlPO_4$. A partir de ahí se observo un incremento en la respuesta inmune de PorA (es decir, en el nivel de anticuerpos bactericidas en suero de ratones inmunizados) en liposomas que contenían *galE* LPS y *lpxL* LPS. De todas maneras, la reacción inmune obtenida con estas formulaciones fué diferente: *galE* LPS indujo una respuesta de tipo Th1, con incremento en la producción de anticuerpos del tipo IgG2a. Esto es beneficioso, ya que anticuerpos de los tipos IgG2a e IgG2b son capaces de activar el sistema del complemento necesario para una actividad bactericida. En ratones inmunizados con liposomas con el adyuvante *lpxL1* LPS, los niveles de todos los tipos de IgG se incrementaron, en comparación con animales inmunizados sin adyuvantes. El incremento en los niveles de anticuerpos bactericidas en ratones inmunizados con liposomas con el adyuvante *lpxL1* LPS fue acompañado de una mejora en la proliferación (específica hacia PorA) de células en los nódulos linfáticos tras la re-estimulación en cultivos con PorA, y en comparación con ratones inmunizados en ausencia de adyuvantes. Al mismo tiempo, las células en proliferación producen también niveles incrementados de IL-2 (una citoquina de tipo Th1), mientras que la producción de IL-10 (una citoquina de tipo Th2) no se incrementa.

El uso de microesferas biodegradable como sistemas de transporte de PorA en liposomas y OMV se estudió en el **capítulo 7**. Microesferas de pequeño tamaño (< 10 μm) pueden ser internalizadas por células presentadoras de antígenos (CPA) y microesferas de mayor tamaño (> 10 μm) pueden actuar como depósito en el lugar de administración, atrayendo así a CPA. Se prepararon microesferas basadas en dextran y manan con similar distribución del tamaño que contenían OMV o liposomas con PorA. Ambos sistemas fueron desarrollados para liberar en poco tiempo tras la administración del antígeno encapsulado. El objetivo final de estas formulaciones es mejorar la respuesta inmune contra PorA, por medio de una mejora de la internalización de PorA por CPA. Manan, un polímero de manosa, también tiene propiedades adyuvantes. Los residuos de manosa situados en la superficie de las microesferas probablemente mejoran la interacción e internalización por las CPA, lo que puede resultar en una mejora de la respuesta inmunológica contra PorA en liposomas o OMV encapsulados en las microesferas. Tanto liposomas como OMV son rápidamente liberados de las microesferas *in vitro* entre los dias 5 y 10-11 de incubación a 37ºC. La estructura de PorA (formando trímeros en la membrana) se

mantuvo tras la liberación de los dos tipos de microesferas. A pesar de que la liberación del antígeno no fué total, la respuesta inmune inducida por PorA en liposomas o OMV encapsulados en microesferas se mantuvo similar a la respuesta de estas formulaciones no encapsuladas.

Conclusión

Esta tesis doctoral demuestra que las formulaciones liposomales son muy favorables como formas de presentación para vacunas basadas en PorA. No solamente las características físico-químicas y la respuesta inmunológica producida por esa proteína se mantienen en su formulación en liposomas, sino que además este tipo de formulaciones también permite la incorporación de adyuvantes y moléculas en la superficie de los liposomas para la vectorización de éstos, lo cual resulta en una mejora de la respuesta inmune producida por PorA.

$AlPO_4$	aluminum phosphate
APC	antigen presenting cell
BCR	B-cell receptor
BMDC	bone marrow-derived DC
BSA	bovine serum albumin
CD	circular dichroism
Chol	cholesterol
CLSM	confocal laser scanning microscopy
CTL	cytotoxic T lymphocytes
DC	dendritic cell
Dex	dextran
DiD	3,3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate
DLS	dynamic light scattering
(DM)PC	dimyristoyl phosphatidylcholine
(DM)PE	dimyristoyl phosphatidylethanolamine
(DM)PG	dimyristoyl phosphatidylglycerol
(DM)TAP	dimyristoyl trimethylamoniumpropane
DOC	sodium deoxycholate
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EPC	egg phosphatidylcholine
FBS	fetal bovine serum
FACS	fluorescence activated cell sorting
FDA	food and drug administration
FITC	fuorescein isothiocyanate
GAM	goat anti-mouse
GM-CSF	granulocyte macrophage colony stimulating factor
Gnd-HCl	guanidinium hydrochloride
GPC	gel permeation chromatography
GC	gas chromatography
HEMA	hydroxyethyl methacrylate
HLA	human leukocyte antigen

HRP	horse-radish peroxidase
Ig	immunoglobulin
IL	interleukin
IM	inner membrane
KDO	keto-deoxyoctonate
LPS	lipopolysaccharide
LSD	least significant difference
MAb	monoclonal antibody
Man	mannose/mannosylated
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MoDC	monocyte-derived DC
MPL	monophosphoryl lipid A
MR	mannose receptor
NATrA	N-acetyl tryptophan amide
NATyA	N-acetyl tyrosine amide
NspA	Neisserial surface protein A
OG	octylglucopyranoside
OM	outer membrane
OMC	outer membrane complexes
OMP	outer membrane protein
OMV	outer membrane vesicles
Opa	Opacity protein
PD	polydispersity index
PorA	porin A
RmpM	reduction modifiable protein M
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Th	T helper
TCR	T-cell receptor
Trp	tryptophan
Tyr	tyrosine

Curriculum vitae

Carmen Arigita Maza was born on November 9th 1973 in Santander, northern Spain. In June 1992 she finished high school (Spanish system: COU) at the Institute 'la Albericia' in Santander. In September of the same year she started to study pharmacy at the University of Granada. During her study she participated in the European Erasmus Program, for which she spent 9 months in Nantes, France (1994-1995). During the last year of her study (1996-1997), she joined the Department of Pharmaceutics of the Faculty of Pharmacy from the University of Granada for a research project that resulted in a minor thesis entitled "Technological study on the formulation of ketoconazol in liposomes and lipid microspheres". In July 1997 she obtained her pharmacist' degree. In October 1997, Carmen moved to the Netherlands and joined the Department of Pharmaceutics of the Faculty of Pharmacy in Utrecht. She worked as research associate in a project entitled "Polymeric DNA delivery systems for gene therapy" under the supervision of Prof. Dr. W.E. Hennink and Dr. N.J. Zuidam. In February 1999, Carmen started a Ph.D. under the supervision of Dr. W. Jiskoot and Dr. G.F.A. Kersten, resulting in a thesis entitled "Towards and imporoved *Neisseria meningitidis* B vaccine: vesicular PorA formulations". This project was a collaboration between the Department of Pharmaceutics of the Faculty of Pharmaceutical Sciences, Utrecht University and the Laboratory for Product and Process Development from de Netherlands Vaccine Institute (NVI, formerly a division of the National Institute of Public Health and the Environment) in Bilthoven. From July 2003 Carmen is working as Technical Specialist at Centocor, in Leiden.

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