Immunization with *Neisseria meningitidis* outer membrane vesicles prevents bacteremia in neonatal mice

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Received 14 May 2005; received in revised form 24 September 2005; accepted 27 September 2005
Available online 12 October 2005

Abstract

Although vaccines based on outer membrane vesicles (OMV) of *Neisseria meningitidis* have been developed and administered to children, little is known about the magnitude and quality of the immune response in animal models of early life immunization. We investigated the immunogenicity of meningococcal OMV, and the influence of route and immunization schedule, in neonatal mice. The administration of two intraperitoneal doses of OMV, given at 7 and 14 days after birth, induced a significant antibody response and was highly effective in conferring protection against bacteremia in 21-day-old mice challenged with meningococci. Intranasal immunization was less effective and did not generate a protective immune response. The antibodies elicited by intraperitoneal immunization were cross-reactive with several meningococcal strains and a memory response was demonstrated when mice immunized as neonates were given a booster immunization at 6 weeks of age.

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Keywords: *Neisseria meningitidis*; Outer membrane vesicle; Neonatal immunization

1. Introduction

The immune system of neonatal period is characterized by high susceptibility to infectious diseases and poor immune responses. However, early deaths are caused by a limited number of viral and bacterial pathogens [1]. Therefore, the ultimate goal of neonatal vaccinology is to develop safe and effective vaccines that could be administered soon after birth. This is particularly challenging, given the quantitative and qualitative differences that distinguish early life antibody responses from those generated later in life [2].

Since identification of the determinants of early life vaccine immunogenicity in human infants is difficult, numerous studies have used newborn or infant animals from various species, mostly mice or rats [3]. Murine models of early life immunization may reproduce the main known characteristics of neonatal and infant vaccine antibody responses, providing that immunization is initiated ≥ 7 days of age rather than in the immediate neonatal period [2]. Although meningococcal disease is rare in industrialized nations, *Neisseria meningitidis* holds a prominent position amongst agents causing pediatric infections because of the dramatic clinical presentation of the disease, high mortality, epidemic potential and the recent disappearance of many other important infectious diseases in developed countries through improvements in public health and vaccination [4]. Between 1 and 12 cases of invasive meningococcal disease occur per 100,000 population in industrialized nations each year with a peak incidence in children from 6 months to 2 years of age [5]. Based on the immunological characteristics of the surface capsular polysaccharide, the causal agent of the meningococcal disease is classified into 13 serogroups, with prevalence of the serogroups A, B and C [6]. Meningococci belonging to one of these serogroups are responsible for more
that 90% of the epidemics of meningococcal meningitis in the world [7].

Meningococcal disease produced by organisms classified within the serogroups A and C can be prevented by plain polysaccharide vaccines and by conjugated polysaccharide vaccines [8]. However, this immunization principle cannot be applied for prevention of serogroup B meningococcal disease, because of the low immunogenicity of this polysaccharide in humans [9]. A promising approach to the development of an effective group B meningococcal vaccine was the use of lipopolysaccharide-depleted outer membrane protein vaccines [8].

It is established that meningococcal outer membrane vesicles (OMV), also called proteoliposomes, are potent immunogens [10–14], which induce strong and long-lasting antibody responses, as determined in animal models of immunogenicity [15,16]. In addition, several vaccines based on these antigens have been tested in clinical trials, showing their immunogenic capacity in humans and proving to be efficacious against serogroup B meningococcal disease in Norway and Cuba [17–19]. At present OMV vaccines have been used more widely than any other serogroup B vaccine [20]. Currently, a public health intervention is going on in order to control a serogroup B epidemic in New Zealand, once the scale-up and standardization of vaccine production required for controlling the New Zealand epidemic was achieved [21]. The availability of licensed OMV vaccines highlights the concept of “tailor-made”, protein-based, outer membrane vesicle vaccines against meningococcal disease. “Tailor-made” vaccines, focusing on the sub-capsular antigens may also be considered for use in sub-Saharan Africa for the prevention of the recurrent outbreaks by serogroups A and W135 meningococci [22].

During the development of OMV vaccines, the greatest amount of information about the preclinical performance of the immunogens is usually collected in adult mice [15,16]. Little is known about the magnitude and quality of the immune response in animal models that resemble the immune state of human newborns. However, the greatest meningococcal disease burden is in infant and young children [20]. An estimated 2600 cases occur each year in the United States, almost half among children 2 years old and younger [23].

Murine neonates have been previously employed to study the protective capacity conferred by meningococcal antigens. An infant rat infection model was developed in the 1980s [24], and antibodies against capsular and subcapsular meningococcal antigens were tested in the model. For example, the protective efficacy of monoclonal antibodies to the PorA protein [25] and to the rough lipopolysaccharide of N. meningitidis [26] was demonstrated in this murine infection model. Later, the infant rat model was adapted to evaluate human sera for protective immunity to group B meningococci [27]. However, in the infant rat model the antibodies are passively administered, by i.p. injection, 1 h before the injection of live meningococci by the same route. Hence, the model cannot be used to explore the immune response elicited after the active immunization with a vaccine candidate.

A previous study, conducted by Fukasawa et al. [28], tested formulations of polysaccharide C-OMV conjugate vaccines in the neonatal mouse model. They found that adjuvant can improve protection induced by OMV vaccine against N. meningitidis serogroups B/C in neonatal mice. However, in such a study animals were bled when they were 5 weeks old. In addition, these authors corroborated the functional activity of the antibodies by in vitro serum bactericidal assay. In the present work, we examine if active immunization of neonatal mice with meningococcal OMV protect them from bacteremia, after the challenge with live meningococci, when mice are as young as 3 weeks old.

To contribute to the understanding of the humoral immune response mounted after the active immunization with an OMV vaccine during early life, we took the advantage of the correlation between human and murine early life vaccine antibody responses [29]. The purpose of this study was to investigate the immunogenicity of meningococcal OMV in neonatal mice, following intraperitoneal (i.p.) or intranasal (i.n.) administration. Early life immunization was performed in 1-week-old mice, which correspond to the state of immune maturation of human newborns [2]. The immunization with two doses of OMV, given at 7 and 14 days after birth by i.p. route, induced a significant antibody response and was effective in reducing the levels of bacteremia in 21-day-old mice challenged with N. meningitidis.

2. Materials and methods

2.1. Antigen

OMV were obtained from the epidemic meningococcal strain CU385 (B:4,7:P1.19,15) [30]. The strain was grown on Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, United Kingdom) at 37 °C for 18 h in an atmosphere of 5% (v/v) CO2. The OMV were prepared by extraction of bacteria with 0.5% (w/v) sodium deoxycholate in 0.1 M Tris–HCl buffer (pH 8.6) containing 10 mM EDTA and were purified by differential centrifugation [31].

2.2. Mice

Pregnant female BALB/c (H-2d) mice were obtained from Centro para la Producción de Animales de Laboratorio, Havana, Cuba and housed in the Animal Care Unit during the experiments. Newborn mice were caged with their mothers during lactation, which lasted for 3 weeks.

2.3. Immunizations

To assess the early life immunogenicity of OMV, neonatal (1 week old) BALB/c mice were immunized with this
antigen either by i.p. or by the i.n. route. For i.p. immunization, 10 μg of OMV were administered, which corresponded to one fifth of the human dose for this type of antigen [32]. The antigen was absorbed onto 400 μg of aluminium hydroxide (Alhydrogel, Superfos Biosector, Frederikssund, Denmark) in a total volume of 50 μl/dose. For i.n. immunization, 50 μg of OMV diluted in 10 μl of phosphate buffered saline (PBS) were slowly delivered into the nares (5 μl/nare). This dose of antigen corresponds to one fifth of the dose previously employed in the intranasal immunization of human volunteers [33]. The immunization and blood sampling schedules for the different experiments are described in Section 3.

2.4. ELISA

Antibody levels in sera were determined by Enzyme Linked Immunosorbent Assay (ELISA). To detect anti-OMV antibodies, 96-well plates (High Binding, Costar, USA) were coated with 100 μl/well of OMV (10 μg/ml) in carbonate buffer (0.05 M Na₂CO₃, pH 9.6). Skimmed milk powder (3%, w/v, Oxoid, England) was used as a blocking reagent. Two-fold serum dilutions (starting dilution 1:100) were prepared in PBS with 0.05% (v/v) Tween 20 (Merck, Germany) and incubated for 1 h at 37°C in precoated and blocked plates. For detection of antibody binding, the plates were incubated with anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) diluted 1:12,000. The assay concluded as previously published [34]. All sera were titrated in duplicate and titers were determined as the reciprocal of the final dilution yielding twice the mean absorbance (at 492 nm) obtained for pre-immune serum. For the statistical processing of the data, a titer value of 50 was assigned to sera with an antibody titer lower than 100, which was the lower limit of the assay.

Antibody levels in lung homogenates and lung washes were determined by ELISA, as well. Mice were sacrificed using a humane method. To obtain lung homogenates, lungs were removed and homogenized in 1 ml PBS. Lung washes were obtained by tracheal cannulation and gentle washing with 0.3 ml of cold PBS. Homogenates and washes were then centrifuged and stored at −20°C. The ELISA procedure was similar to the one employed to determine IgG, with few modifications. Lung homogenates were initially diluted 1:100, and lung washes remained undiluted, before two-fold dilutions of them were prepared in PBS with 0.05% (v/v) Tween 20 and were incubated for 1 h at 37°C in precoated and blocked plates. For detection of antibody binding, an anti-mouse immunoglobulin A (IgA) conjugated with horseradish peroxidase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was employed, diluted 1:4000. Titers were determined as the reciprocal of the final sample dilution yielding twice the mean absorbance (at 492 nm) obtained for samples taken from non-immune mice.

2.5. Avidity ELISA

We determined the avidity index (AI) of antibodies using potassium thiocyanate (KSCN. Merck, Darmstadt, Germany), according to Vermont et al. [35]. Briefly, three-fold serial dilutions of serum samples (in PBS) were added in duplicates to antigen-coated plates and incubated for 1 h at 37°C. After three washings, 100 μl of 1.5 M KSCN solution in PBS was added to one sample replicate, and plain PBS was added to the other one. After incubation for 15 min at room temperature, all wells were washed three times, and for detection of antibody binding the plates were incubated with anti-mouse IgG conjugated with horseradish peroxidase (Sigma-Aldrich) at the recommended working dilution (1:12,000). The assay concluded as previously published [34]. The AI was the percentage of antibodies that remained bound at the antigen coat after treatment with KSCN and was calculated as follows: AI = (titer with KSCN)/titer without KSCN) × 100.

2.6. Bactericidal antibody assay

Bactericidal assay was performed as described previously [36] using N. meningitidis strain CU385 as target bacteria. Briefly, target bacteria were incubated with serial dilutions of serum samples (starting dilution 1:4), in the presence of rabbit serum as a source of exogenous complement. The serum bactericidal titer was defined as the highest serum dilution yielding ≥50% killing of bacteria after the 30 min incubation reaction. The lower limit of the detection in this assay was 1:4.

2.7. Bacterial challenge

To test active protection in 21-day-old mice, we adapted the infection model described for infant rats [24] to mice. The experimental conditions employed were selected based on our previous experience in the use of the infant rat model to evaluate protection against serogroup B [37] and C [38,39] meningococcal strains; and on preliminary experiments done to adjust the challenge dose, time of sampling, etc. The N. meningitidis strain CU385 was “rat passaged”, to increase the virulence of the bacteria as previously described [24]. For challenge, the bacteria were grown overnight on BHI agar supplemented with 7% (v/v) defibrinated sheep blood (BHI-blood agar) at 37°C in 5% CO₂ subculture on a fresh BHI-blood agar plate and grown in the same conditions for 4 h. Then, the bacteria were collected from the plates in sterile PBS and the concentration was estimated by optical density (OD(600)). The number of viable bacteria in the inoculum was confirmed as colony forming units (cfu) by plating serial dilutions on BHI-blood agar. Challenge of mice immunized with OMV (as described above), and untreated age-matched control mice was performed by i.p. injection of ~5 × 10⁷ cfu (0.1 ml) together with 0.5 mg iron dextran per animal. To determine bacteremia, all groups of mice were bled by retro-
orbital puncture 4 h after challenge. Ten microliters of blood were taken from each mouse and diluted into sterile saline. Serial 10-fold dilutions from each sample were plated onto BHI-blood agar plates. The number of bacterial colonies was determined after 18 h incubation at 37 °C in 5% CO₂.

2.8. Analysis of antisera by Immunodot

_N. meningitidis_ strains used in the present investigation are shown in Table 1. Moreover, strains of _N. mucosa, N. flavescens_ and _Moraxella catharralis_, available in our institute collection, were included in this study. The _M. catharralis_ and the neisserial strains were grown overnight on BHI agar plates at 37 °C in a 5% CO₂ atmosphere, subcultured to a second plate and grown in the same conditions for 4 h. The _Streptococcus pneumoniae_ strain 191 (serotype 19F), isolated from a patient’s cerebro-spinal fluid, was kindly provided by the Institute of Tropical Medicine “Pedro Kourí”. The strain was grown in Tryptone Soy Agar (Oxoid) containing 7% defibrinated sheep blood, for 16 h, at 37 °C in a 5% CO₂ atmosphere, subcultured to a second plate and grown in the same conditions for 6 h.

To prepare a cell suspension from each strain, bacteria were collected from the plates in sterile PBS with 0.02% (w/v) sodium azide (Merck), and the absorbance of the suspensions was adjusted to OD₆₂₀ equal to 1.0. The cells contained in 1.11 ml of suspension were recovered by centrifugation. Each pellet was then suspended in 100 μl of PBS. Two microliters of each suspension were diluted up to 20 μl and were applied to the nitrocellulose membrane. After blocking with 5% (w/v) skimmed milk powder in PBS, the membranes were incubated with either pooled antisera (diluted 1:100) or a control monoclonal antibody. The immunoassay continued as previously described[40].

Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain</th>
<th>Serogroup</th>
<th>Serotype</th>
<th>Serosubtype</th>
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<tr>
<td>1</td>
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<td>B</td>
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<tr>
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<td>H355</td>
<td>B</td>
<td>15,19,15</td>
<td>MPMG</td>
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<tr>
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<td>IBN5421</td>
<td>B</td>
<td>4,16</td>
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</table>
| 7   | N3124  | B         | 4,7b,4,19,15 | PSH, the membranes were incubated with either pooled antisera (diluted 1:100) or a control monoclonal antibody. The immunoassay continued as previously described[40].

2.9. Statistical methods

For statistical analysis, the antibody titers and bacterial counts in blood were first log-transformed. The significance of differences in titers was determined either by Student’s t-test or by ANOVA followed by Newman-Keuls multiple comparison test, depending on the groups included in the experiment. The significance of differences in levels of bacteremia was determined by ANOVA followed by Dunnett’s multiple comparison test. The bacterial counts in blood determined in experimental groups were compared with the counts in a control group of non-immunized mice challenged in the same conditions. A P value of <0.05 was considered statistically significant. All the statistical analysis were done by using GraphPad Software, San Diego, USA.

3. Results

3.1. Immunogenicity of the outer membrane vesicles in neonatal mice

We tested the immunogenic capacity of meningococcal OMV in a murine infant immunization model that resembles the immune state of human newborns. The immunogen was initially administered to 1-week-old mice. A second and a third dose were administered 1 and 5 weeks, respectively, after primary immunization. Mice were bled 3, 4, 6, 7 and 8 weeks after birth, for measurement of anti-OMV antibodies in serum. The dynamics of OMV-specific serum antibodies, as detected by ELISA, are shown in Fig. 1. When we analyzed the humoral immune response against OMV, elicited after the first two doses of OMV systematically administered,
we observed that 80% of the mice had an IgG antibody titer higher than 1:10,000. The OMV-specific antibodies continued increasing after the third dose of antigen, delivered by the same route, and reached the highest value 2 weeks after the third immunization, when the last blood samples were taken. When OMV were given by a mucosal route, the magnitude of the humoral response elicited in neonatal mice was significantly lower. Only 30% of the mice that received two i.n. doses of OMV had IgG antibody titers higher than 1:100 on day 21. When a third dose was applied by the same route, a booster response was observed, producing an increase in the antibody titers that was significant if compared to the titers detected before (week 7 versus week 6, \( P < 0.001 \)). Eight weeks after birth, mice immunized by the intranasal route were divided into two groups. Lung homogenates were prepared from half of the mice and lung washes were obtained from the other half. When IgA antibodies were determined by ELISA, we detected the induction of a mucosal antibody response in both kinds of samples. The IgA antibody titer in lung homogenates and lung washes was 1:560 ± 238 and 1:98 ± 66, respectively (geometric mean ± standard deviation).

The functional activity of the antibodies found in the serum of mice immunized during early life is summarized in Table 2. We detected higher bactericidal antibody titers in pooled sera of mice immunized by i.p. route than in pooled sera of mice intranasally immunized with OMV.

### 3.2. Protection against bacteremia conferred by immunization of neonatal mice with outer membrane vesicles

To assess correlation between humoral immune responses in neonatal mice and a protective capacity against meningococcal bacteremia, a second immunization schedule was accomplished. Mice received either two doses (10 \( \mu \)g each) of OMV absorbed onto 400 \( \mu \)g of aluminium hydroxide intraperitoneally, or two doses (50 \( \mu \)g each) of OMV diluted in PBS intranasally. The doses were given when mice were 1 and 2 weeks old, respectively. Blood sampling was done 1 week after the second dose, when mice were 21 days old. The same day, mice were challenged with meningococci. All the animals immunized by i.p. route developed anti-OMV antibody titers equal or higher than 1:10,000 (data not shown), but most of the mice immunized intranasally failed to mount a significant antibody response (geometric mean titer 1:87), confirming the results obtained in the first immunization experiment. The administration of two doses of OMV through the i.p. route was highly effective (\( P < 0.01 \)) in conferring protection against bacteremia to 21-day-old sensitized mice challenged with live meningococci (Fig. 2). Animals intranasally immunized with OMV were not protected, since the bacterial counts in blood detected 4 h after the i.p. challenge with meningococci did not differ from the counts determined for untreated mice (\( P > 0.05 \)).

### 3.3. Comparison of the immune response elicited following different immunization schedules

Fig. 3 shows the antibody levels obtained when we compared three immunization schedules. Neonatal mice received 10 \( \mu \)g of OMV absorbed onto 400 \( \mu \)g of aluminium hydroxide by i.p. route. One week later, the immunized animals were divided into three groups to proceed with the immunization schedule. Mice in group 1 received a second dose of OMV, by the same route (i.p.). Mice in group 2 received 50 \( \mu \)g of OMV diluted in PBS, intranasally, and those in group 3 received 50 \( \mu \)l of PBS, without antigen. All the animals were bled and challenged 1 week later, on day 21 after birth. The highest antibody titers were detected in the group that received two i.p. doses of OMV. When mice received only one i.p. dose of OMV, administered on day 7, the antibody titers were lower, with statistical significance (\( P < 0.001 \)). The administration of the second dose by i.n. route did not
Fig. 3. Anti-OMV antibody levels developed in 21-day-old mice (n = 8) after the administration of the same antigen following three different schedules. An intraperitoneal dose was administered on day 7 to all the animals. According to the schedule, on day 14 mice received either a second intraperitoneal dose (i.p. 2×), an intranasal dose (i.p., i.n.), or no dose at all (i.p. 1×). The results shown are mean (plus standard deviation) of the antibody titers determined for the individual animals in each group. (***) Denotes a significant statistical difference (P < 0.001) between the antibody levels in the indicated groups, according to an ANOVA followed by a Newman–Keuls multiple comparison test.

improve the serum OMV-specific antibody response detected in mice, compared to the group that received only one dose (P < 0.001). Only mice immunized with a second dose of OMV showed a detectable serum bactericidal activity in vitro (bactericidal antibody titer = 8). The other two groups of mice had an undetectable bactericidal activity in their sera, like the pre-immune serum (bactericidal antibody titer <4). The difference in the antibody levels was reflected in the grade of protection afforded against the meningococcal challenge (Fig. 4). Only mice immunized with two i.p. doses had significantly lower bacterial counts in blood (P < 0.01) compared to the control group (untreated mice), and were considered protected against bacteremia. Animals that received the second dose by i.n. route, or did not receive a second dose at all, were not protected against bacteremia after the challenge with the homologous meningococcal strain (P > 0.05).

3.4. Cross-reactivity of the antibodies generated after intraperitoneal immunization

The antibodies generated after early life immunization with OMV cross-reacted with meningococcal antigens present in strains of diverse serological classification, as detected by Immunodot experiments performed with pooled murine sera (Fig. 5(I)). The antibodies elicited after two i.p. doses of OMV strongly recognized antigens present in strains that belong to the same serosubtype of the strain CU385 (serosubtype P1.15), and reacted also with strains of the same serotype. In addition, they reacted with strains such as H44/76 and Z4181, which express major antigens from a different serotype and serosubtype, and with antigens present

Fig. 4. Bacteremia of mice vaccinated with OMV according to three immunization schedules, and unimmunized mice challenged on day 21 after birth (n = 8). An intraperitoneal dose was administered on day 7 to all the animals. According to the schedule, on day 14 mice received either a second intraperitoneal dose (i.p. 2×), an intranasal dose (i.p., i.n.), or no dose at all (i.p. 1×). Immunized and untreated age-matched control mice were challenged with 5 × 10⁷ meningococci plus 0.5 mg iron dextran per animal. The results shown are mean (plus standard deviation) of the level of bacteremia of individual animals within each group. (**) Indicates a significant statistical difference (P < 0.01) between the bacterial counts in the blood of animals immunized with two intraperitoneal doses and the blood of unimmunized mice, according to an ANOVA followed by a Dunnett’s multiple comparison test.

Fig. 5. Recognition of the antigens present in several meningococcal strains determined by Immunodot. The membranes were probed with: (I) pooled sera of OMV vaccinated mice (1:100), (II) pooled sera of unimmunized mice (1:100), (III) murine monoclonal antibody specific to meningococcal PorA, subtype 15 (1 µg/ml). The strains employed are the following: CU385 (A1), BSN6157 (A2), Z159 (A3), H155 (A4), BSN385 (A5), BSN421 (A6), NZ224 (A7), IH1660 (A8), H44/76 (A9), M982 (B1), MC51 (B2), Z1127 (B3), Z4181 (B4), S. cinerea (B5), N. mucosa (B6), N. flavescens (B7), M. catarrhalis (B8), S. pneumoniae (B9). The classification of the meningococcal strains employed for the Immunodot is shown in Table 1.
in the strain of *N. cinerea* employed in this study. Sera taken from control mice that remained untreated did not react with the bacterial antigens applied in the Immunodot (Fig. 5 (II)). The specificity of the assay was checked by using a monoclonal antibody specific to PorA, which only reacted with antigens present in meningococcal strains of serosubtype 15 (Fig. 5 (III)).

### 3.5. Priming capacity of the outer membrane vesicles in neonatal mice

Early life immunization of mice with OMV sensitized them for a memory immune response, as determined in a fourth immunization experiment. Neonatal mice were divided into two groups. Mice in group 1 received 10 μg of OMV absorbed onto 400 μg of aluminium hydroxide by i.p. route, and those in group 2 received 400 μg of aluminium hydroxide without antigen, intraperitoneally. Seven days later, the animals received a second dose of the same immunogen in the first immunization. All the mice had a third immunization when they were 6 weeks old, and the immunogen consisted in 10 μg of OMV absorbed onto 400 μg of aluminium hydroxide (i.p.) for both groups. Mice were bled on weeks 3, 6 and 7. On week 7, we found significantly higher antibody titers (P < 0.001) in the animals that received the antigen early after birth compared to the mice that received it only on week 6 (Fig. 6(A)). Moreover, when mice immunized as neonates were given a booster immunization at 6 weeks of age, and the AI was determined 1 week later, we found that early life priming with OMV generated antibodies with higher avidity (AI = 87.1%), compared to the one achieved in those mice that remained unprimed until 6 weeks of life (AI = 22.14%). We also detected higher bactericidal antibody titers in pooled sera of mice immunized in the neonatal period than in pooled sera of animals immunized only in the adult period (Fig. 6(B)).

### 4. Discussion

Among the antigens present in the outer membrane of *N. meningitidis*, the major outer membrane proteins have called the attention of researchers and vaccine producers, due to their protective capacity [41]. These proteins are included in OMV vaccines [8], which are safe and immunogenic, as indicated by a number of clinical studies conducted in the past [17–19]. However, little is known about the immunogenicity of meningococcal OMV in animal models of early life immunity. Hence, in the present study, we evaluated the immunogenic capacity of this antigen in a murine infant immunization model that resembles the immune state of human newborns [29].

We found that two doses of OMV, given at 7 and 14 days after birth, by i.p. route, were enough to induce a significant IgG antibody response, as detected in serum samples taken when mice were 3 weeks old, which best correspond to the state of immune maturation of human infants [2]. This is in agreement with reports that provide evidence about the immunogenicity of meningococcal antigens in young children, studied both after meningococcal infection [42], and after vaccination with OMV vaccines [32,43]. Pollard et al. [42] examined the nature of humoral immune responses in children, following infection with *N. meningitidis*, in relation to age. They found that infants produced levels of total IgG and IgG subclass antibodies similar to those in older children, as detected by a meningococcal ELISA. The immunogenicity in infants of a serogroup B meningococcal outer membrane protein vaccine (VA-MENGOC-BC®, Instituto Finlay, Cuba) has been investigated [32]. It was found that the vaccine induced a demonstrable immune response in infants against both the serogroup B vaccine strain and against a serogroup C strain, since the vaccine comprises antigens from both serogroups. Similarly, Cartwright et al. [43] assessed the immunogenicity in infants of a novel meningococcal
vesicle vaccine containing multiple PorA outer membrane proteins. They reported that this hexavalent PorA meningococcal vaccine evoked encouraging immune responses in infants.

In the present study, i.n. immunization of newborn mice with OMV was less effective than i.p. administration, in terms of IgG antibody levels. The difference found in the magnitude of the antibody response between the groups that received OMV intraperitoneally and intranasally was expected, since the administration route determines which immune organs and cell populations are involved in the response [44]. Over the past few decades the intranasal route has received considerable attention for its potential for vaccine delivery [45]. In meningococcal disease, where invasion of the host is via the nasal epithelium, the production of secretory IgA may be important. Direct application of vaccine antigens to the nasal mucosa is an appealing possibility [20]. It is encouraging that even in the absence of an adjuvant, the OMV were able of eliciting a serum antibody response after mucosal administration in this animal model, characterized by a marked immaturity of the immune system. It could be due to the nature of the antigen, a proteoliposome, with an average diameter of 10–100 nm [31], that has demonstrated immunogenicity in adult mice by i.n. route [46]. Moreover, it is documented that neisserial porins induce immune stimulation, dependent on toll-like receptor 2 and MyD88 [47], up-regulating the expression of the co-stimulatory molecule B7-2 on the surface of B cells and other antigen presenting cells [48].

Typically, for the induction of an effective immune response after mucosal immunization, higher doses of antigen are required compared with systemic immunization. High doses of antigen administered intranasally are likely to reach the intestinal tract or be drained directly by the posterior cervical lymph nodes instead of the superficial cervical lymph nodes [45]. In our study, we administered intranasally 50 μg of OMV, in a total volume of 10 μl, divided into two portions (5 μl/nare). Although we intended to employ a volume as low as possible, it should be considered that it is very unlikely that 10 μl would be retained and subsequently absorbed within the nasopharynx.

When we looked at the functional activity of the antibodies generated after two immunizations with OMV, given early in murine life, we found low or undetectable bactericidal antibody titers in this model (sera taken on day 21 after birth). After a third immunization administered later in life, the bactericidal titer in the sera raised considerably, even in the group that received the OMV by intranasal route. This is in agreement with a previous study where bactericidal titers were examined in young children following immunization with an OMV vaccine. HexaMen® [49], a recombinant hexavalent PorA OMV vaccine administered in infants at 2–4 months of age, showed good bactericidal titers only after a fourth dose, which was administered at 12–18 months of age [49]. Moreover, in the study performed by Pollard et al. [42], where the humoral immune responses were examined in children, it was found a striking age dependency of bactericidal antibody response following infection. They reported that serum bactericidal activity was poor in children under 12 months of age despite recent infection with N. meningitidis. Whether titers of anti-serogroup B meningococcal bactericidal antibody correlate with protection from meningococcal disease is unknown, and it has been suggested that protection against group B infection may also be due to opsonic antibodies and to innate immune responses [50,51]. Protective mechanisms different from the complement-mediated bactericidal killing can be elicited following the administration of OMV vaccines. The antigen-specific immune response developed after immunization with the OMV vaccine VA-MENGO-BC was investigated, and the cellular response was measured by in vivo delayed type hypersensitivity (DTH) in 50 healthy nursing babies that received two doses of this vaccine, at 3.5 months and 5 months of age, respectively [52]. A strong DTH response was observed against the OMV in all nursing babies after vaccination, and it was negative before immunization.

Murine neonates have been previously employed in meningococcal research. The intranasal infection of infant mice with N. meningitidis was first reported by Saltt and Tomsaly [53], and later by Mackinnon et al. [54]. Nonetheless, the model was not used for active immunization, but those groups investigated either the virulence of several meningococcal strains or murine host factors important in this model. Saukkonen [24] developed an infant rat model for meningococcal infection. However, it is only useful for passive protection studies [26,55]. Fukasawa et al. [28] tested formulations of polysaccharide C-OMV conjugate vaccines in the neonatal mice model, using adjuvants which are either licensed or in clinical trials for human use. They presented evidence that the inclusion of MF59 enhanced the immune response against OMV in newborn mice, but animals were bled when they were 5 weeks old. In addition, these authors corroborated the functional activity of the antibodies by an in vitro serum bactericidal assay. The animals received the immunizations by i.p. route, like in our study, but mice were not infected with meningococci. In the present work, we found that active immunization of neonatal mice with meningococcal OMV protected them from bacteremia when mice were as young as 3 weeks old. It is worth noting that this result was achieved with the antigen combined with aluminum hydroxide, one of the few adjuvants approved by the regulatory authorities to be used in humans [56].

To our knowledge, this is the first time that intranasal immunization with a meningococcal antigen is performed in neonatal animals. Previously, Jakobsen et al. [57] explored the immune response induced in newborn mice after the intranasal immunization with several pneumococcal antigens. They reported that immunization by intranasal route with pneumococcal conjugate vaccines and the adjuvant LT-K63, a nontoxic mutant of heat-labile enterotoxin, rapidly induced protective immunity against lethal pneumococcal infections in a neonatal model and elicited a strong salivary IgA response in neonatal and infant mice. Mucosal immu-
iron, which is required for bacterial growth [59]. To obtain transferrin receptors are specific for human transferrin and in mice is mainly due to the fact that the meningococcal investigation.

vants in the neonatal murine immunization, is presently under investigation.

nimmune response against OMV, by using new mucosal adjuvants under investigation [58]. The improvement in the mucosal administration of subsequent vaccine doses is essential for protection, even for hepatitis B vaccine which is often mentioned as an exception to this general observation [61].

The antibodies elicited after early life immunization with OMV, by i.p. route, recognized antigens present in strains that belong to serosubtype P1.15, but also cross-reacted with meningococcal antigens present in strains of diverse serological classification. The cross-reactivity found by Immunodot could be due to conserved regions within the major outer membrane proteins PorA and PorB [62] and/or to minor antigens that are widely expressed across strains of diverse classification [41]. The level of cross-reactivity found for the elicited antibodies does not indicate that cross-protection can be achieved by neonatal immunization of mice with OMV prepared from a single strain. Further protection experiments conducted by immunizing neonatal mice with OMV and challenged with heterologous meningococcal strains could make clear this point.

Reviews of preclinical and human vaccine studies indicate that, although neonatal immunization does not generally lead to early and strong antibody responses, it may result in efficient immunological priming, which can serve as an excellent basis for future responses [2]. An OMV-specific memory response was demonstrated when mice immunized as neonates were given a booster immunization at 6 weeks of age. It is known that B cell priming to vaccine antigens can be induced very early in life [29]. Our experimental data suggest that meningococcal OMV behave like certain vaccine antigens that appear to induce B cell activation and differentiation into memory B cells already following early life administration [63,64], and is consistent with a report made by Cartwright et al. [43]. When they investigated the immunogenicity in infants of a meningococcal hexavalent PorA OMV vaccine, infants received the vaccine at ages 2, 3 and 4 months, with a fourth dose at 12–18 months. Following the fourth dose, larger bactericidal responses to all six meningococcal strains expressing PorA proteins contained in the vaccine were observed. It suggested that the initial course had primed memory lymphocytes and revaccination stimulated a booster response.

Passively transferred maternal antibodies protect infants against meningococcal infections during the first few months of life, whereas high incidence rates are recorded in the age group 6–12 months [20]. In the present study, we addressed issues regarding the maturity of the immune system during early life. We do not explore the potential influence of pre-existing maternally-derived antibodies.

In summary, we investigated here the immunogenicity of meningococcal OMV administered to neonatal mice. Two doses of this bacterial antigen, given at 7 and 14 days after birth, by i.p. route, induced a significant antibody response, and conferred protection against bacteremia to 21-day-old mice challenged with meningococci. Moreover, neonatal priming elicited a significant increase in antibody levels to a subsequent antigen dose, compared to unprimed mice. This mouse model of early immunity could be employed, during the research and development process, to evaluate the immunogenicity of vaccine candidates against meningococcal disease.

Acknowledgements

The excellent technical assistance of Yusleydis de la C. Pefuez Fuentes is gratefully acknowledged. We thank Dr. Andrew Gorringe (Health Protection Agency, Porton Down, UK) for his critical reading of the manuscript and valuable suggestions.

References


