Immunogenicity of Intranasally Administered Meningococcal Native Outer Membrane Vesicles in Mice

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Colonization of the human nasopharyngeal region by Neisseria meningitidis is believed to lead to natural immunity. Although the presence of bactericidal antibody in serum has been correlated with immunity to meningococcal disease, mucosal immunity at the portal of entry may also play an important role. This study was undertaken to examine in mice the possibility of safely using native outer membrane vesicles (NOMV) not exposed to detergent as an intranasal (i.n.) vaccine. The mucosal and systemic responses of mice to intranasal and intraperitoneal (i.p.) vaccination with NOMV were compared over a range of doses from 0.1 to 20 μg. Intranasal vaccination of mice with NOMV induced a strong systemic bactericidal antibody response, as well as a strong local immunoglobulin A immune response in the lungs as determined by assay of lung lavage fluid by enzyme-linked immunosorbent assay and lung antibody secreting cells by enzyme-linked immunospot assay. However, 8- to 10-fold-higher doses of NOMV were required i.n. compared to i.p. to elicit an equivalent bactericidal antibody response in serum. Some NOMV vaccine was aspirated into the lungs of mice during i.n. immunization and resulted in an acute inflammatory response that peaked at 1 to 2 days postimmunization and was cleared by day 7. These results indicate that i.n. delivery of meningococcal NOMV in mice is highly effective in eliciting the production of both a mucosal immune response and a systemic bactericidal antibody response.

Neisseria meningitidis frequently colonizes the human nasopharynx, which is its sole natural habitat, leading to the induction of natural immunity (11). In some cases, this colonization also initiates the pathogenic process that leads to invasive meningococcal disease. Serum bactericidal antibody, which develops after exposure to meningococcal antigen (10, 11), has been correlated with immunity to meningococcal disease, but mucosal immunity at the portal of entry may also play an important role.

There is currently much interest in the mucosal route of immunization to protect against various pathogens that gain entry into the host via mucosal tissues. Recent studies have shown that intranasal immunization can protect mice against challenge with a variety of organisms, including the bacterial pathogens Bordetella pertussis (4), Borrelia burgdorferi (17), Chlamydia trachomatis (27), streptococci (7), and Helicobacter felis (38). Importantly, meningococcal outer membrane proteins (OMPs) used as proteosomes have been successfully used as a mucosal delivery system in mice to present antigens such as staphylococcal enterotoxin B toxoid (18) and Shigella lipopolysaccharide (19, 26).

Several OMP-based vaccines for group B meningococcal disease have shown 50 to 80% efficacy in older children (43). However, efficacy in young children receiving the same vac-
MATERIALS AND METHODS

Mice. CD-1 outbred mice (Charles Rivers Laboratories, Wilmington, Maine) were used in all experiments. Mice were immunized at days 0 and 28 with NOMV with a dose of 25 μg of protein i.p. or 100 μg of protein i.n. in 25-μl volume. Dose-response experiments were conducted in which NOMV was given at i.p. or i.n. doses ranging from 0.01 to 10 μg or from 0.03 to 20 μg, respectively. The i.n. immunizations were given to unanesthetized (histology experiment) or anesthetized mice (0.3 mg ketamine HCl and 1.0 mg of xylazine [administered intramuscularly]) by using a micropipette. Bleeds were done via the retroorbital plexus at days 0 and 28 on anesthetized mice and by cardiac puncture at day 42 following euthanasia by CO₂ overdose. Lung lavage fluid was obtained on day 42 for both i.p. and i.n. immunizations by flushing 0.15 M NaCl, 0.05 M Tris-HCl, and 0.01 M EDTA at pH 7.5. The suspension was warmed at 56°C for 30 min and sheared in a Waring blender for 3 min. The suspension negative and unable to sialylate its LOS. The encapsulated parent strain 9162 was used as the target strain in bactericidal assays and in production of NOMV to use in enzyme-linked immunosorbent assays (ELISA) and enzyme-linked immunospot-forming assays (ELISPOT).

The vaccine consisted of NOMV, referred to in previous publications as native meningococcal O-antigen (NOM) or native meningococcal O-antigen (NOM), which was a case isolate obtained from a patient in Iquique, Chile, in October 1990. This isolate was representative of the epidemic strain prevalent in northern Chile from 1985 to 1994. This parent strain was genetically modified by partial deletion of synX (unpublished data) and replacement by a kanamycin resistance cassette. SynX, also called siaE (3), is essential for sialic acid biosynthesis (34), and the resulting mutant, 9162synX−, is capsule negative and unable to sialylate its LOS. The encapsulated parent strain 9162 was used as the target strain in bactericidal assays and in production of NOMV for use in enzyme-linked immunosorbent assays (ELISA) and enzyme-linked immunospot-forming assays (ELISPOT).

The vaccine of NOMV, referred to in previous publications as native cell wall complex or outer membrane complex (40, 41). The vesicles were extracted from whole cells of strain 9162synX− with the use of detergents or denaturing agents (41). Briefly, packed cells were suspended in a buffer containing 0.15 M NaCl, 0.05 M Tris-HCl, and 0.01 M EDTA at pH 7.5. The suspension was warmed at 56°C for 30 min and sheared in a Waring blender for 3 min. The resulting suspension was centrifuged at 23,500 × g for 20 min to remove cells and cell debris. The supernatant was retained, and the cells were extracted a second time with a volume of distilled water equal to half the volume of buffer used in the first extraction and with the heating step omitted. Combined supernatants were centrifuged at 23,500 × g for 1 h to pellet the outer membrane vesicles. The pellets of NOMV were washed once by repelleting from distilled water and stored at 4°C until spots were counted with a stereomicroscope. The number of viable cells was ascertained by trypan blue dye exclusion.

Histopathology. Unanesthetized mice were immunized i.n. with 20 μg of NOMV in a 25-μl volume (n = 12) or sham immunized with 25 μl of saline at day 0 (n = 5, 3 mice from each group). Three mice from each group were sacrificed at days 7, and the lungs were inflated with and fixed in 10% phosphate-buffered formalin, then sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

ELISPOT. An ELISA was performed as previously described (31) except that mouse antibodies were detected. Briefly, flat-bottom high-binding 96-well plates (Costar Corp., Cambridge, Mass.) were coated with NOMV for 2 h at 37°C, incubated with 200 μl of blocking solution for 1 h at 37°C, and washed twice with PBS buffer. Serial twofold dilutions made in separate plates were added and incubated overnight at room temperature (RT). Plates were washed with PBS, and alkaline phosphatase-labeled goat anti-mouse IgG, IgA, or IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added. The plates were incubated right at RT for 1 h. Plates were washed with PBS, and NOMV was used for 30 min with nitrophenylphosphate (Sigma 104; Sigma, St. Louis, Mo.). The development reaction was stopped by the addition of 3 N NaOH. Absorbance values were read at 405 nm. Quantitation of immunoglobulin values in serum was done by running standard plates through a 60-mesh screen with the plunger from a 3-ml syringe. Cells were suspended in RPMI 1640 (Life Technologies, Inc., Gaithersburg, Md.) and pelleted by centrifugation at 200 × g for 10 min. Lysis of erythrocytes was accomplished by resuspending cells in RT NH₄Cl-Tris (1 ml per 1 ml of packed cells; Sigma) for 3 min, resuspending them in 12 ml of RPMI 1640, and washing them four times with RPMI 1640 (200 × g for 10 min). The number of viable cells was ascertained by trypan blue dye exclusion. Cells were resuspended in culture medium (RPMI 1640 with 10% heat-inactivated fetal bovine serum, 50 μg of gentamicin per ml, and 2 mM L-glutamine) and counted. Cells were added to 8 to 12 wells of the antigen-coated plates at the desired concentration and then incubated overnight at 37°C in 5% CO₂. Plates were washed with PBS five times, and 100 μl of a 1-μg/ml concentration of phosphate-antibody-labeled antibody (Kirkegaard & Perry) of the appropriate specificity was added to each well. Plates were incubated for 1 h at 37°C in a humidified chamber and washed four times with PBS. Development was accomplished by adding 2 ml of 5-bromo-4-chloro-3-indolyl-phosphate toluidin blue solution (BCIP; Kirkegaard & Perry) to 1 ml of 0.7% agarose in 0.1 M Tris and adding 100 μl per well. After the agarose solidified, the plates were wrapped in plastic wrap and stored at 4°C until spots were counted with a stereomicroscope. The number of isotype-specific antibody-secreting cells (ASCs) was then calculated. (i) and expressed in terms of the number of ASCs per 10⁶ lymphocytes plated.

RESULTS

NOM stimulation of systemic and mucosal antibody responses. High bactericidal antibody levels in serum were observed at day 42 for mice immunized with NOMV regardless of the immunization scheme (Fig. 1A), although significantly higher levels were observed for the i.p./i.p. group (P < 0.01) or i.n./i.n. (P < 0.01). An initial i.n. immunization at day 0 induced bactericidal levels in serum at day 28 comparable to those with the i.p. immunization. However, the i.n. boost at day 28 only increased the day 42 bactericidal titer slightly (2-fold) for the i.p./i.n. and i.n./i.n. groups, whereas the i.p. boost at day 28 increased the day 42 bactericidal titer by almost 14-fold for the i.p./i.p. group.

Isotype-specific immunoglobulin responses as measured by ELISA showed the mean day 42 IgG level in serum for the i.p./i.n. group was significantly higher than for the i.p./i.n. group (P < 0.05) but was not significantly different from the i.n./i.n. group (Fig. 1B). No significant differences in IgM levels in serum were observed between groups at day 28 or 42 (Fig. 1C). No detectable meningeococcus-specific serum IgA was found 28 days after an initial i.p. immunization, and only 1 of 10 mice immunized i.p./i.p. had a detectable serum IgA response at day 42. In contrast, two i.n. immunizations induced specific serum IgA in 100% of mice at day 42 (Fig. 1D), and a significantly higher response was observed in the i.n./i.n. group compared to the i.p./i.p. group (P < 0.01).

Lung lavage IgA was detected on day 42 in 80% of the mice immunized i.p./i.p., and 100% of the mice immunized i.n./i.n., but none was detected in mice immunized i.p./i.p. (Fig. 1E).

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of IgG ASCs was present in the lungs of mice immunized i.p./i.p. (Table 1). A threefold-higher number of splenic IgG, IgA, and IgM ASCs were observed in the lungs from any group. Virtually no IgM ASCs were observed in the lungs from any group.

**Stimulation of local ASCs.** Mice were immunized at days 0 and 28, and lung and spleen tissues were harvested at day 33 and used in an ELISPOT assay to determine the number of meningococcus-specific IgA, IgG, and IgM ASCs present. Two i.n. immunizations induced a strong mucosal response, as evidenced by the more than 500 IgA ASCs per 10^6 lymphocytes in the lungs, whereas no IgA ASCs were observed in the lungs of mice immunized i.p./i.p. (Table 1). A threefold-higher number of IgG ASCs was present in the lungs of mice immunized i.n./i.n. than in those immunized i.p./i.p. Virtually no IgM ASCs were observed in the lungs from any group.

Mice immunized i.n./i.n. had significantly higher numbers of splenic IgA ASCs (Table 1) than the i.p./i.p. or the i.p./i.n. group (P < 0.05). Similar levels of splenic IgG ASCs were observed in both the i.p./i.p. and the i.n./i.n. groups, although significantly higher numbers of splenic IgA ASCs were observed in i.p./i.p. compared to i.n./i.n. mice (P < 0.05).

**Dose-response testing.** Mean bactericidal activity in serum and lung lavage fluid for mice immunized with meningococcal NOMV. Animals were immunized in groups of 10 (i.p. [IP] dosage = 1 μg; i.n. [IN] dosage = 20 μg) (i) i.n. twice (IN/IN), (ii) i.p. at day 0 and i.n. at day 28 (IP/IN), or i.p. twice (IP/IP). Serum was collected on days 28 and 42, and lung lavage fluid was collected on day 42. A fourth group was sham immunized i.n. and i.p. with saline; all values for this group were negligible and are not shown. Boxplots show median, minimum, and maximum values and the 25th and 75th percentiles. Panels: A, serum bactericidal antibody; B, serum IgG; C, serum IgM; D, serum IgA; E, lung lavage IgA; F, lung lavage IgG. Percentages above the maximum values in panel D represent percentage of mice with detectable specific IgA antibody.

**TABLE 1. Results of ELISPOT assay on mouse spleen and lung lymphocytes after immunization with meningococcal NOMV**

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>Route of immunization</th>
<th>Mean ASCs/10^6 lymphocytes ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Spleen</td>
<td>i.p./i.p.</td>
<td>104.7 ± 21.0</td>
</tr>
<tr>
<td></td>
<td>i.p./n.</td>
<td>42.3 ± 9.9</td>
</tr>
<tr>
<td></td>
<td>i.n./i.n.</td>
<td>91.4 ± 9.4</td>
</tr>
<tr>
<td></td>
<td>Saline i.n. and i.p.</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>i.p./i.p.</td>
<td>75.1</td>
</tr>
<tr>
<td></td>
<td>i.p./n.</td>
<td>133.3</td>
</tr>
<tr>
<td></td>
<td>i.n./i.n.</td>
<td>311.7</td>
</tr>
<tr>
<td></td>
<td>Saline i.n. and i.p.</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a Mice (five per group) were immunized at days 0 and 28 with either 1 μg of NOMV i.p. (i.p./i.p.), 20 μg of NOMV i.n. (i.n./i.n.), or 1 μg of NOMV i.p. at day 0 followed by 20 μg of NOMV i.n. at day 28. Lungs and spleens were harvested at day 33; lymphocytes were plated onto NOMV-coated microtiter plates, and ELISPOT assays were conducted.

b Lung lymphocytes were pooled from five mice prior to plating.

**Histopathology.** Light microscopic examination of the lungs of the saline control mice were within normal limits at all time
points (Fig. 4a). The lungs of mice immunized i.n. with meningococcal NOMV and sacrificed on days 1 and 2 PI revealed an acute inflammatory response characterized by neutrophils centered around airways and extending into the surrounding alveoli (Fig. 4b). The neutrophilic response was decreased in severity on day 4 (Fig. 4c) and was absent by day 7 (Fig. 4d). Two of the three mice immunized with NOMV had evidence of active antigenic stimulation distinguished by mononuclear infiltration and lymphocytic aggregates (interpreted as bronchiolar associated lymphoid tissue) which contained morphologically activated lymphocytes and plasma cells. The remaining day 7 mouse had essentially normal lung tissue.

DISCUSSION

The importance of mucosal IgA in preventing group B meningococcal disease is unknown, but it may be a factor in preventing colonization (1) or in preventing the progression of carriage to invasive disease (12). Nasopharyngeal carriage of meningococci typically results in natural immunization in people by inducing bactericidal antibodies in serum which are believed to be required for protection (10). Meningococcal and influenza vaccines delivered i.n. have been shown to be immunogenic in humans (14, 23, 25). Further, human challenge studies following i.n. immunization with a purified group A Streptococcus M protein vaccine have shown a reduction in colonization, as well as protection from clinical disease among the vaccinees (6, 29).

Meningococcal NOMV is a good mucosal immunogen, as evidenced by the results presented in this study. Mice immunized i.n. with NOMV responded with bactericidal antibody titers in serum at levels approaching those in mice immunized i.p. The level of the mucosal and systemic response obtained by immunization with NOMV was significant, particularly when compared to that obtained with a vaccine consisting of OMPs purified by using the detergent Empigen BB and complexed to alkaline-detoxified LOS. Mice immunized i.n. with NOMV responded with bactericidal antibody titers in serum approximately 2 logs higher than mice immunized i.n. with the OMP-LOS vaccine (unpublished data). Leaving the phospholipid and LOS intact in the NOMV ensures that the OMPs have...
surface exposure and conformation similar to that on the whole organism. This serves to direct the immune response toward those epitopes that can induce protective antibodies. The native conformation of NOMV proteins, as well as the adjuvant effect of the LOS present, may be responsible for the higher antibody responses that NOMV elicits in animals. It has been previously noted that purified group B OMPs have adjuvant activity in the mucosal immunization of both mice and guinea pigs (36).

The i.n. route of immunization has several desirable characteristics over intramuscular immunization, not the least being the fact that the i.n. route of delivery was preferred by human volunteers over intramuscular injection for an influenza vaccine (13). Furthermore, meningococcus-specific IgA has been detected in the nasal secretions of patients convalescent from the disease (39) and in volunteers who have been immunized i.n. with meningococcal antigens (14; unpublished data). It has been suggested that host IgA is disadvantageous to the survival of meningococci and that a specific IgA1 protease produced by pathogenic Neisseria species may be a virulence factor (16, 21). However, antibody to IgA1 protease also develops after nasopharyngeal carriage and disease, but whether this is important in preventing meningococcal disease is unknown (3).

The vaccine strain used for the production of the NOMV vaccine was chosen in part because it had good expression of Opa proteins that could potentially mediate the binding of the NOMV to the mucosal surfaces of the nose and throat and possibly induce antibodies that could block adherence (37). The vaccine strain was genetically modified to block sialic acid synthesis, which resulted in the lack of a capsule and the lack of sialylation of the LOS (unpublished data). Use of a sialic acid-negative mutant was thought to have several advantages for a mucosal vaccine. First, several studies have shown that the sialic acid capsule inhibits adherence of meningococci to epithelial cells (33, 37). Thus, capsular polysaccharide present on the NOMV might reduce interaction of the vaccine with the epithelial cells and result in reduced uptake (immunogenicity) of the vaccine. Second, both the group B capsule and sialylated LOS have been shown to be molecular mimics and are therefore both undesirable as vaccine components and are poor immunogens (9, 20). Third, both the capsule and the sialylated LOS appear to be virulence factors (15, 21). Eliminating them results in a much safer vaccine strain with which to work.

A potential drawback in using NOMV as a vaccine is its high native endotoxin content, which might lead to unacceptably high reactogenicity in humans. However, we have given high doses (>400 µg of protein and 90 µg of LOS) of NOMV i.n. to both rabbits and humans without eliciting a pyrogenic response (unpublished data), indicating that relatively large amounts of antigen containing native endotoxin can be safely administered by the i.n. route. In mice, NOMV (20 µg of protein and 5 µg of LOS) given i.n. appeared to be more toxic, as evidenced by acute weight loss, than when given i.p.

We performed an experiment with unanesthetized mice to determine the body weight changes over time at various doses of NOMV i.n. (Fig. 3). The results showed that the body weight decreased as the dose of NOMV increased, with the highest decrease observed at the highest dose of 10 mg of protein and 5 mg of LOS. This trend was consistent with the results from the dose response experiment (Table 2), where the highest decrease in body weight was observed at the highest dose of NOMV i.n.

**TABLE 2. Serum and lung lavage IgA values at day 42 for mice immunized i.n. or i.p. with meningococcal NOMV at days 0 and 28**

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>i.n. groups</th>
<th>Mean serum IgA (µg/ml) ± SEM</th>
<th>Mean lung lavage IgA (µg/ml) ± SEM</th>
<th>i.p. Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. with detectable serum IgA (%)</td>
<td>No. with detectable lung lavage IgA (%)</td>
<td>No. with detectable serum IgA (%)</td>
<td>No. with detectable lung lavage IgA (%)</td>
</tr>
<tr>
<td>0.01</td>
<td>3/5 (60)</td>
<td>3.7 ± 1.2</td>
<td>0/5 (0)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>0.03</td>
<td>4/5 (80)</td>
<td>6.3 ± 3.1</td>
<td>4/5 (80)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>0.10</td>
<td>5/5 (100)</td>
<td>13.1 ± 6</td>
<td>5/5 (100)</td>
<td>3/5 (60)</td>
</tr>
<tr>
<td>3.0</td>
<td>5/5 (100)</td>
<td>24.0 ± 5.9</td>
<td>5/5 (100)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>10</td>
<td>5/5 (100)</td>
<td>64.9 ± 18.9</td>
<td>5/5 (100)</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>20</td>
<td>5/5 (100)</td>
<td>33.1 ± 19.1</td>
<td>5/5 (100)</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

* Lung lavages were not taken from mice immunized i.p. in this experiment; however, in an identical experiment with mice immunized i.p. with meningococcal NOMV, no IgA was detected in lung lavage samples.
* Calculated for just those mice with detectable IgA.

**FIG. 3.** Body weight differences (median, minimum, and maximum values) from day 0 of mice (five per group) at day 1 (A), day 3 (B), day 7 (C) after i.n. immunization with various doses of meningococcal NOMV.
determine if alert, responsive mice could successfully swallow excess vaccine, thus avoiding the aspiration of endotoxin. We observed the aspiration of the vaccine regardless of anesthesia, as determined by histopathology and by the gasping response of some mice after vaccination. We believe this was due to the relatively large volume (25 μl) of NOMV used to immunize the mice, which was aspirated into the lungs, resulting in an acute inflammatory response, the timing of which coincided with weight loss. The acute inflammatory response we observed in the lungs is similar to a previous report of acute lung injury in mice after i.n. administration of lipopolysaccharide, where peak lung injury occurred at 24 to 48 h (35). The administration of a smaller volume of vaccine would eliminate the involvement of the lungs and bronchus-associated lymphoid tissue (38). We do not believe it would be necessary or desirable for an i.n. NOMV vaccine to reach the lungs in humans since the palatine tonsils and adenoids serve as mucosal induction sites (30). Further investigation with a larger animal model, the rabbit, to exclude lung involvement has been undertaken in our laboratory.

The goal of this study was to examine the local and systemic antibody response of mice given meningococcal antigens i.n. We found this route of presentation effective in both respects. Since the meningococcus is strictly a human pathogen, a good animal challenge model that correlates with efficacy in humans does not exist. The anti-meningococcal response in mice is not highly predictive of the human response, but the development of bactericidal antibodies and protection in animal models is the best available correlate of the potential of inducing a protective response in humans (28). Further investigations of the immune response in the mouse model will be used to compare modifications in NOMV production, immunization schedules, and dose responsiveness.

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