Intranasal Administration of a Meningococcal Outer Membrane Vesicle Vaccine Induces Persistent Local Mucosal Antibodies and Serum Antibodies with Strong Bactericidal Activity in Humans

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A nasal vaccine, consisting of outer membrane vesicles (OMVs) from group B Neisseria meningitidis, was given to 12 volunteers in the form of nose drops or nasal spray four times at weekly intervals, with a fifth dose 5 months later. Each nasal dose consisted of 250 µg of protein, equivalent to 10 times the intramuscular dose that was administered twice with a 6-week interval to 11 other volunteers. All individuals given the nasal vaccine developed immunoglobulin A (IgA) antibody responses to OMVs in nasal secretions, and eight developed salivary IgA antibodies which persisted for at least 5 months. Intramuscular immunizations did not lead to antibody responses in the secretions. Modest increases in serum IgG antibodies were obtained in 5 volunteers who had been immunized intranasally, while 10 individuals responded strongly to the intramuscular vaccine. Both the serum and secretory antibody responses reached a maximum after two to three doses of the nasal vaccine, with no significant booster effect of the fifth dose. The pattern of serum antibody specificities against the different OMV components after intranasal immunizations was largely similar to that obtained with the intramuscular vaccine. Five and eight vaccinees in the nasal group developed persistent increases in serum bactericidal titers to the homologous meningococcal vaccine strain expressing low and high levels, respectively, of the outer membrane protein Opc. Our results indicate that meningococcal OMVs possess the structures necessary to initiate systemic as well as local mucosal immune responses when presented as a nasal vaccine. Although the serum antibody levels were less conspicuous than those after intramuscular vaccinations, the demonstration of substantial bactericidal activity indicates that a nonproliferating nasal vaccine might induce antibodies of high functional quality.

Vaccines administered directly onto mucosal surfaces may induce local mucosal as well as systemic immune responses (23, 24). Even nonproliferating mucosal vaccines may thus offer a challenging alternative to traditional parenteral vaccines, as has been shown for an oral cholera vaccine (15). It is required, however, that induction of tolerance to antigenic components of such vaccines be abrogated or that so-called mucosal adjuvants be added (10, 24).

We have shown that in mice, the nasal mucosa is the preferred site for presentation of a vaccine consisting of whole killed pneumococci in suspension, with cholera toxin (CT) added as mucosal adjuvant (1). Even for intestinal immune responses, as measured by antibodies in feces, nasal immunizations were superior to administering the antigen by both the oral and gastric routes. Subsequently, we found that outer membrane vesicles (OMVs) from group B meningococci were also immunogenic in mice when given nasally (8). The antibody responses to OMVs in these experiments were largely independent of adding CT; i.e., the vesicles themselves possessed the necessary structures for induction of mucosal and systemic immune responses after application on mucosal surfaces.

Outer membrane proteins from group B meningococci are clearly immunogenic in humans (31), and the OMVs which we used as a mucosal vaccine in mice were originally developed to be the main component of a parenteral vaccine against group B meningococcal disease (12). In a large-scale study of adolescents, this OMV vaccine was shown to protect against disease when given intramuscularly with aluminum hydroxide as adjuvant (6). In the present study, we used OMVs, suspended in saline without aluminum hydroxide, as a mucosal vaccine in the form of nasal drops or spray to human volunteers. The demonstration by others of M cells in the human nasopharyngeal area (30) forms the basis for an effect of such a vaccine when applied intranasally (19). Other researchers have recently also demonstrated that intranasal immunizations with either live influenza virus (18), the B subunit of CT (CTB) (4), or diphtheria-tetanus vaccines (2) can induce specific immune responses in humans. The results with our nasal OMV vaccine against meningococcal disease were compared with those obtained in another group of volunteers who received two intramuscular doses of the parenteral OMV vaccine formulation with aluminum hydroxide. The aim was to determine whether intranasal delivery of such particles might also induce immune responses in humans, and that they might serve as a model system for creating alternative mucosal vaccines against other bacterial diseases.

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MATERIALS AND METHODS

Vaccines. Twelve healthy volunteers, nine women and three men at 25 to 61 (median, 46) years of age, were included in the nasal vaccine study regardless of their prevaccination antibody levels. They had not previously received a meningococcal vaccine. Of the volunteers, seven women and four men at 24 to 49 (median, 38) years of age, were immunized intramuscularly with the regular vaccine formulation and served as controls for the nasally immunized volunteers. This group of volunteers was selected on the basis of low serum immunoglobulin G (IgG) antibody levels to meningococcal OMVs. The reason for different selection criteria in the two groups of volunteers is that the study was originally planned as two separate experiments. Even so, it happened that the prevaccination serum antibody levels of the nasally and intramuscular vaccine groups were not significantly different (P > 0.2), with median (range) levels of 12.4 (7.3 to 77.4) and 12.5 (3.7 to 30.8) kU/mL, respectively (see below for antibody measurements).

Collection of samples. Sera, separated from freshly drawn whole blood, oral secretions, and nasal fluid were obtained before each immunization and at 1 day, 2, 4, and 8 weeks after the final dose. Subsequently, sera obtained at various times were determined by the two-tailed Mann-Whitney U test and correlation coefficients were calculated with use of StatView 512+ for Macintosh computers.

Quantification of antibodies and immunoglobulins. Levels of IgA, IgG, and IgM antibodies to OMVs, and total IgA, IgG, and IgM concentrations, were determined by ELISA using Neurospora crassa (strain 11) as test antigen. Standards were prepared as described previously (26, 28).

RESULTS

Nasal vaccine induced strong mucosal ELISA antibody responses. After the first or two doses of nasal vaccine, at least twofold increases in IgA antibody levels to OMVs were observed in nasal secretions of 9 of the 12 volunteers. The mean levels of such antibodies, which reached about 10 times the prevaccination levels, remained high until at least 3 months after the start of immunizations (Fig. 1). This was markedly different from the constant low levels of nasal IgA antibodies in the group of individuals receiving the intramuscular vaccine.
Although the concentrations of nasal antibodies in four of those who received the nasal vaccine were still at least double the prevaccination levels after 6 months, the mean level was then not significantly raised. In some individuals, increases in nasal mucosal IgA antibodies were found after the fifth nasal dose, given at 6 months from the start, but this difference was likewise not significant. We could therefore not demonstrate any local mucosal booster effect of the nasal vaccine.

Increased levels of IgA antibodies to OMVs, although less pronounced than in nasal secretions, were also observed in saliva after nasal immunizations. The mean postvaccination concentrations reached almost three times the prevaccination levels (Fig. 1), and unlike the findings in nasal fluid, saliva IgA antibodies after 6 months were significantly higher than the prevaccination levels ($P < 0.05$). We have thus demonstrated that a mucosal vaccine can initiate a continuous production of mucosal antibodies for that period of time.

From comparison of IgA antibody levels in nasal fluid before and 3 months after the start of immunizations, it was evident that all vaccinees had responded with at least twofold increases (Fig. 2). The magnitude of these responses was approximately the same in those with high preexisting local mucosal antibodies as in those with low antibody levels, and it did not seem to be influenced by the way the vaccine had been administered, i.e., as nasal spray or as drops.

Concerning the antibodies in saliva, however, only 8 of the 12 vaccinees were considered responders with at least twofold increases at 3 months from the start of vaccinations (Fig. 2). In saliva, moreover, none of those who had relatively high preimmunization antibody levels were considered responders. On the other hand, these nonresponders were also among those who had received the nasal vaccine as spray and not as drops.

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Since the antibody responses in nasal fluid did not seem to have been influenced by preexisting local mucosal antibodies, the relatively poor salivary antibody responses might be due to the way the nasal vaccine had been administered. However, this difference in saliva antibody responses after immunizations with spray rather than drops was not significant ($P = 0.2$).

Only low levels of IgG or IgM antibodies to OMVs could be demonstrated in nasal secretions and saliva from either group of vaccinees (results not shown). Generally, the absolute concentrations in nasal secretions of these antibody isotypes were only $1$ to $3\%$ of the corresponding serum concentrations, with exceptional values reaching $9$ and $8\%$ of serum IgG and IgM antibodies, respectively. The IgG antibodies in nasal secretions were thus the most likely result of passive leakage from serum and not the result of local production in the mucosa.

Nasal vaccine induced modest serum ELISA antibody responses. Antibody responses in serum after nasal immunizations were much less pronounced than in secretions. Approximately twofold increases in the mean levels of IgG antibodies to OMVs were attained after two to three doses of the nasal vaccine (Fig. 3). The corresponding mean IgA antibody levels increased almost threefold, whereas no significant increase in serum IgM antibodies was observed. This was markedly different from the responses in those who received the intramuscular vaccine, with maximal 20-, 10-, and 3-fold increases in mean levels of IgG, IgA, and IgM, respectively. However, after the nasal vaccine, the mean IgG antibody levels remained constant up to 6 months after the start of the experiment. As in secretions, we did not observe any significant booster effect of the fifth dose intranasally.

Analyses of serum antibody concentrations 3 months after the start of immunizations showed that only 5 of 12 individuals...
responded to the nasal vaccine with at least twofold increases in IgG antibodies, whereas 10 of 11 individuals responded in this way to the intramuscular vaccine (Fig. 4). Intramuscular vaccinations also induced marked increases in serum IgA antibodies, and the increases were more pronounced than after nasal immunizations. The vaccinees receiving the nasal vaccine were not selected on the basis of their prevaccination serum antibody levels, which also included high values. Serum IgG responses, however, were seen in vaccinees with high as well as low prevaccination levels. Moreover, since there were three responders among those who had received the nasal vaccine as drops and two responders after the spray vaccine, we could reach no conclusion as to whether the systemic antibody responses depended on the way the nasal vaccine had been given.

**Nasal vaccine induced a mucosal antibody pattern partially different from that of serum.** On immunoblots, serum IgG antibody responses to the nasal vaccine were mainly directed against the class 1 (PorA) and class 5 (including Opc) outer membrane proteins, as well as lipopolysaccharide (LPS) and higher-molecular-mass (70- to 80-kDa) proteins (Fig. 5), which are also the main immunogens after intramuscular vaccinations (22). In nasal fluid and saliva, however, no reaction of antibodies to LPS or to the high-molecular-mass components was observed after intranasal immunizations (Fig. 5). The IgA antibodies in the secretions were mainly directed against the class 1 and class 5 proteins (antibodies to the class 5 protein were less distinct on the picture), whereas the binding to the class 4 protein did not appear to increase after immunizations.

Some individuals who had received the nasal vaccine responded with antibodies in secretions directed against the same antigens as the serum antibodies, e.g., against class 1 protein in vaccinee 011M (Fig. 5). In others, as in vaccinee 005M, antibodies against class 1 and 4 proteins were found in secretions, whereas class 1 and 3 protein antibodies were demonstrated in serum. Nasal immunizations may thus induce mucosal immune responses somewhat different from systemic responses.

**Nasal vaccine induced serum antibodies with strong bactericidal activity.** Increases of in vitro bactericidal activity were demonstrated in sera from several individuals who received the nasal vaccine (Fig. 6). This was evident both with the meningococcal SL strain that had been used for the vaccine production and with the homologous strain expressing higher levels of the Opc outer membrane protein (at the time of vaccine production, there was little knowledge about the possible role of Opc as an antigen). The sera with the highest bactericidal titers all had distinct IgG responses on blots against class 1, class 5 (including Opc), and/or LPS antigens.
Three months after start of the study, 5 of 11 vaccinees immunized nasally (one vaccinee was excluded because of antibiotic therapy) had at least fourfold increases in serum bactericidal titers against the SL strain, whereas all 10 who had been immunized intramuscularly (one vaccinee had left the study) had similar increases (Fig. 7). When the strain expressing higher levels of the Opc protein was used in the assay, 8 of 11 in the nasal group and all 10 in the intramuscular group had similar bactericidal activities. Sera from those who responded to the nasal vaccine attained levels of bactericidal activity in the same range of magnitude as in vaccinees given the intramuscular vaccine.

In the following months, serum bactericidal activity after nasal vaccinations seemed well preserved (Fig. 6). All vaccinees who responded to the nasal vaccine at 3 months were still considered as responders before the fifth vaccine dose at approximately 6 months from the start of the experiment; i.e., 5 and 8 of 11 nasal vaccinees had persistent bactericidal activity against the SL and Opc strains, respectively. After the fifth vaccine dose, however, there was no consistent increase in serum bactericidal activity (Fig. 6). This result confirmed our observations with antibody levels, as measured in ELISA, that a nasal vaccine might not easily induce a detectable booster effect.

Surprisingly, only two of the five individuals who had responded to the nasal vaccine with bactericidal activity to the SL strain 3 months after start of the study were also serum IgG responders as determined by at least twofold increases in ELISA. Even so, there was a linear correlation \( r = 0.68, P = 0.02 \) between bactericidal activity and IgG antibodies in ELISA (Fig. 8). A stronger correlation, however, was found for the bactericidal activity against the strain expressing high levels of the Opc protein and serum IgG antibody levels \( r = 0.86, P = 0.0008 \). The Opc protein may thus be important for induction of immunity via the mucous membranes.
the Opc protein ($r = 0.80, P = 0.003$). However, the increases in serum IgA antibodies coincided with those of IgG antibodies ($r = 0.80, P = 0.002$). A possible negative influence of IgA antibodies on the bactericidal activity could therefore not be ascertained.

**DISCUSSION**

In addition to the simplicity of administration, the commonly recognized advantage with vaccines applied directly onto mucosal surfaces is their ability to induce mucosal antibodies which might act as a barrier to the invasion of pathogenic microorganisms through the mucosal membranes (7). In this study, we have demonstrated that OMVs from group B meningococci, suspended in saline and given as nasal drops or spray were indeed able to initiate mucosal immunity with transfer into nasal secretions and saliva of specific IgA antibodies. The most marked effect, however, was seen in the local mucosal area which had been exposed to the vaccine, i.e., in secretions from the nasal mucosal area as opposed to saliva or secretions obtained from the adjacent oral cavity. Although others have found that nasal immunizations with CTB lead to antibody responses in vaginal secretions (4), we did not study a possible induction by the nasal OMV vaccine of antibodies at distant mucosal sites.

As opposed to concentrations of serum antibodies, which depend on a balance between production and degradation, the persistence of antibodies in secretions depends more on the ability of the local mucosal immune system to keep up a de novo synthesis of antibodies which are continuously secreted (7). It is not expected, therefore, that antibodies in secretions will persist in the same way as antibodies in serum. Our demonstration of elevated antibody levels to meningococcal OMVs in secretions for up to 6 months after the start of nasal immunizations indicated that nonproliferating nasal vaccines might eventually be made to induce a protective barrier for a prolonged time.

The demonstration in this study of only low levels of IgG antibodies in secretions, which seemed to mirror serum antibodies, contrasts with the findings by others of relatively high IgG antibody concentrations in nasal secretions after intranasal immunizations with live attenuated influenza vaccine (18) or with CTB (4). Possibly this discrepancy can be explained by differences in effects on the mucosa by the antigens used. Also, the different methods for sampling of secretions from the mucosal surfaces may have influenced the results.

From previous experience, we know that intramuscular administration of the aluminum-adsorbed OMV vaccine induces high levels of serum IgG antibodies (22). In comparison OMVs administered intranasally without any adjuvant induced only low levels of serum IgG antibodies in our volunteers. But despite the fact that we in this study on humans used the same nasal doses as some of us previously used in mice (8), we obtained significant increases in serum IgG and IgA antibodies. Moreover, the modestly raised serum antibodies were persistent for the whole observation period. Similar to the continued transfer of antibodies into secretions, this finding suggests that OMVs presented as a nasal vaccine can lead to prolonged systemic immune stimulation.

The pattern of antibody responses after nasal immunizations, as revealed by immunoblots, showed that serum IgG antibodies were largely directed against the same antigens (70- to 80-kDa high-molecular-mass proteins, class 1 and 5 proteins, and LPS) as were immunogenic by intramuscular administration of the OMV vaccine (22, 29). This finding might indicate that the nasal OMV vaccine is able to induce serum antibodies with at least some protective power. However, the IgA antibody pattern in secretions was more restricted than in serum, as no activity against LPS and the high-molecular-mass components was observed. It was also demonstrated that antibodies specific for a meningococcal immunogen can be induced in secretions and not in serum of that same individual. This finding seems to confirm previous observations that the mucosal immune system can operate independently of the systemic one (7). Antibodies in secretions, with specificities which are not found in serum, might also add to the potential beneficial systemic effects of nasal vaccines.

Clinical studies with the OMV vaccine, given intramuscularly with aluminum hydroxide, have shown that the serum bactericidal activity may represent a reasonable in vitro correlate to protection against invasive meningococcal disease (20). Since we found that the nasal OMV vaccine in many of the vaccinees induced serum bactericidal activity in the same range of magnitude as after intramuscular immunizations, it seems likely that this vaccine would also confer protection.

Similarly to the modest levels of serum ELISA antibodies which were induced by the nasal vaccine, the bactericidal activity was also remarkably persistent over the whole observation period. Thus, the findings so far indicate that outer membrane particles, without an added adjuvant, possess the antigens and conformation necessary to initiate sustained and strong local mucosal as well as systemic immune responses. Studies in animals have shown that this might also be the case with several airway pathogens presented intranasally as whole heat-inactivated bacteria in suspension (1, 5, 16).

The discrepancy between the low IgG antibody responses and the high bactericidal activity in sera after nasal immunizations made us question the identity of the factor responsible...
for this bactericidal activity. Others, who observed a similar difference between low levels of specific antibodies and high degree of protection against infection after mucosal immunizations with a live rotavirus vaccine, suggested that the protective effect might be ascribed to a hitherto unknown factor (25). However, the positive correlation that we observed between serum IgG antibody levels to OMVs and the bactericidal activity, especially to the 44/76 meningococcal strain expressing high levels of the Opc protein, indicated that the bactericidal activity was probably conferred by the antibodies. It is likely, therefore, that the antibodies measured by ELISA after nasal immunizations were of higher functional quality than those initiated by intramuscular immunizations.

It has been claimed that serum IgA antibodies, which do not normally bind complement, may bind to the microbial antigens and thus inhibit the complement-dependent bactericidal activity (17). Compared to the results after intramuscular immunizations, however, the nasal vaccine in the present study induced only negligible serum IgA antibody increases. Moreover, the demonstration of a positive correlation between serum bactericidal activity and IgA (as well as IgG) antibody levels after nasal immunizations does not support the notion that a nasal vaccine might be more of a hazard in this respect than a vaccine given parenterally.

Our observations that neither the local nor the serum antibody responses to the nasal OMV vaccine increased with the third to fourth doses could indicate that further responses are hampered by the presence of local mucosal antibodies. This could also explain the lack of a booster effect, or even a primary response, to the single fifth nasal dose given months later when local mucosal antibodies were still present. If that is the case, our success in animals with OMVs as a presumed vaccine carrier or mucosal adjuvant for killed influenza virus given nasally (9) may have only limited applicability. The limitations of potent immunogens as mucosal adjuvants have also been addressed by others (3). Anyhow, the present results indicate that with improved formulations or methods of delivery, efficient nonproliferating mucosal vaccines may soon be a reality.

To further study the functional effects of such experimental vaccines, however, there is a need for good animal models or in vitro correlates to protection.

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