Antigen-Specific T-Cell Responses in Humans after Intranasal Immunization with a Meningococcal Serogroup B Outer Membrane Vesicle Vaccine

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We have studied the ability of the Norwegian group B meningococcal outer membrane vesicle (OMV) vaccine, when administered intranasally without adjuvant, to induce T-cell responses in humans. A group of 12 vaccinees was immunized with four doses of OMVs (250 μg of protein/dose) at weekly intervals, and a single booster dose was given 5 months later. In vitro T-cell proliferation in response to the OMV vaccine, purified PorA (class 1) protein, PorB (class 3) protein, and one unrelated control antigen (Mycobacterium bovis BCG) was measured by [3H]thymidine incorporation into peripheral blood mononuclear cells obtained from the vaccinees before and after the immunizations. The nasal OMV immunizations induced antigen-specific T-cell responses in the majority of the vaccinees when tested against OMVs (7 of 12) and the PorA antigen (11 of 12). None of the vaccinees showed a vaccine-induced T-cell response to the PorB antigen after the initial four doses. Although some individuals responded to all the vaccine antigens after the booster dose, this response was not significant when the vaccinees were analyzed as a group. We have also demonstrated that the PorA antigen-specific T-cell responses correlated with anti-OMV immunoglobulin A (IgA) levels in nasal secretions, with anti-OMV IgG levels in serum, and with serum bactericidal activity. In conclusion, we have shown that it is possible to induce antigen-specific T-cell responses in humans by intranasal administration of a meningococcal OMV vaccine without adjuvant.

Infections with Neisseria meningitidis represent a major health problem in several countries (12, 20, 27). Vaccines based on capsular polysaccharides have been developed against serogroup A and C meningococci (9). The serogroup B polysaccharides, however, are poorly immunogenic in humans (43). A protein based outer membrane vesicle (OMV) vaccine was therefore developed at the National Institute of Public Health in Norway (10) and proved to be protective against serogroup B meningococcal disease when given intramuscularly with Al(OH)3, as adjuvant (3). We have also used meningococcal OMVs as a model system to evaluate the prospects for developing future mucosal vaccines based on nonreplicating particulate antigens (8).

Mucosal delivery of vaccines may be advantageous, primarily due to simplified administration and induction of mucosal immune responses at the natural site of infection (22, 35). Such mucosal antibodies against meningococci might be important to block colonization and prevent systemic infection. In addition, mucosal vaccines may induce systemic immunity measured as both antibody and T-cell responses in peripheral blood (22, 35). It has been suggested that mucosal adjuvants should be added to such vaccines to enhance the immunogenic effect and avoid induction of tolerance (22, 35). However, we have demonstrated that it is possible to induce both mucosal and systemic antibody responses in mice by nasal immunization with OMVs without any mucosal adjuvant (8). Recently, we have also shown that OMVs given alone as a nasal vaccine to humans can induce local mucosal and systemic antibodies with strong bactericidal activity (15). Nonproliferating mucosal vaccines may thus be an alternative to systemic vaccines against bacterial diseases.

Whereas protection against extracellular bacterial infections is mediated mainly by antibodies, T cells also play an important role in this respect by regulating B-cell responses, e.g., by inducing immunoglobulin (Ig) class switching and affinity maturation and increasing the magnitude of the response (2). However, little is known about the induction of antigen-specific T-cell responses after mucosal immunizations in humans. In this work, we have extended the previous study with the nasal meningococcal OMV vaccine (15) by investigating antigen-specific T-cell responses to whole OMVs and purified meningococcal outer membrane proteins (OMPs). We have also compared such effects with the corresponding mucosal and systemic antibody responses (15). The aim of this work was to study cellular immune responses which might be useful for further understanding and monitoring of the immunogenicity of nonproliferating mucosal vaccines.

MATERIALS AND METHODS

Vaccine preparation. The nasal vaccine used in this study consisted of OMVs from the epidemic meningococcal strain 44/76 (B:15:P1.7,16.L3,7,9) (10). The OMVs were prepared by extraction of bacteria with 0.5% deoxycholate in 0.1 M Tris-HCl buffer (pH 8.6) containing 10 mM EDTA and purified by differential centrifugation (10). The nasal formulation of OMVs was given without Al(OH)3 as adjuvant. Each nasal dose consisted of 250 μg of OMVs (measured as protein)
in 0.5 ml of saline (15), which is 10 times the dose previously used for intramuscular injections (3).

**Purified vaccine antigens and controls.** The PorA (class 1) and PorB (class 3) OMP antigens used in proliferation assays in vitro were purified from the mutant variants of strain 44/76, H55 and HII5, which lack the class 1 and 3 OMPs, respectively. Both variants were devoid of the RmpM (class 4) protein. The porins were solubilized by the detergent Zwittergent, purified by chromatography, and reconstituted as proteosomes devoid of potentially lymphotoxic determinant (4, 13, 14, 39). There was no contamination by other OMPs, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Lipopolysaccharide contamination was less than 0.01%, as judged by gel electrophoresis and silver staining (32). Throughout the study, *Mycobacterium bovis* BCG (Statens Serum Institut, Copenhagen, Denmark) and phytohemagglutinin (PHA) (Sigma, St. Louis, Mo.) were used as the positive control antigen and mitogen, respectively.

**Vaccines and immunization schedule.** Twelve healthy volunteers, nine women and three men, 25 to 61 (median, 46) years of age, were included in the study (Phase II-M1). The vaccine was given four times at weekly intervals (initial immunizations), and a fifth dose was given 5 months later (booster). Six of the volunteers received the vaccine as nasal drops, and the other six received it as a nasal spray, as previously described (15). None of the vaccinees were found to be meningococcal carriers as determined by nasopharyngeal cultures taken immediately before and during the study. This study was approved by the Norwegian Medicines Control Authority as well as the Regional Committee of Medical Research Ethics in Norway.

**In vitro T-cell proliferation assays.** Blood samples, collected in ACD Vacutainer tubes (Becton Dickinson, Rutherford, N.J.), were obtained before vaccination and at weeks 1, 2, 3, 4, 5, 7, 11, 24, 25, and 26 of the vaccination schedule. Peripheral blood mononuclear cells, freshly isolated from whole blood by density centrifugation (Lymphoprep; Nycomed, Oslo, Norway), were assayed by the [3H]thymidine incorporation method for proliferative responses to whole OMVs, PorA-antigen, and PorB-antigen, as well as to *M. bovis* BCG and PHA for control purposes. Antigen in triplicate (concentrations given below) and 100,000 PBMC per well, suspended in RPMI 1640 (Biowhittaker, Verviers, Belgium) (containing 10% pooled human AB serum), were plated in flat-bottom 96-well microtiter plates (Costar, Cambridge, Mass.) at a final volume of 200 µl well. After 6 days of incubation in 5% CO2 at 37°C, the cells were pulsed with [3H]thymidine (1.3 µCi/well) (Amersham, Little Chalfont, United Kingdom) for 6 h and harvested (Packard FilterMate), and incorporated [3H]thymidine was determined by liquid scintillation counting (Packard Top-Counter). The CD4+ phenotype of proliferating cells on day 6 was confirmed by flow cytometry. In addition, it was possible to obtain a dose-dependent inhibition of proliferation by adding blocking anti HLA-DR antibodies (B8.11) to the assay (results not shown).

To determine the optimal concentrations of the antigens used in the proliferation assays, the following titrations were performed. OMVs were obtained by centrifugation (Lymphoprep; Nycomed) and were lyophilized and reconstituted in saline (15). Determination of serum bactericidal activity was described previously (15, 16). Immunizations were given intranasally on days 0 and 28 of the study schedule. The results presented in this study were based on means of triplicate cpm values in the presence (cpm cpm) of the optimal concentrations of the antigens used: OMVs, 0.8 µg/ml; PorA protein, 5 µg/ml; PorB protein, 5 µg/ml; BCG, 20 or 4 µg/ml; and PHA, 5 µg/ml.

Proliferative T-cell responses are expressed as cpm values calculated in the following way: the mean of triplicate cpm values in the absence of antigen (cpm medium) was subtracted from the mean of triplicate cpm values in the presence of antigen (cpm Ag). A proliferative response exceeding 2,000 cpm (background level) was defined as a positive T-cell response. Participants at anytime point reaching a twofold increase (compared to prevaccination levels) in their proliferative response as well as exceeding 2,000 cpm point were considered responders.

**OMV-specific antibodies and serum bactericidal activity.** The vaccinees were previously tested for OMV-specific IgG antibodies in serum and IgA antibodies in nasal secretions by enzyme-linked immunosorbent assay and the results are expressed as arbitrary units (15). The IgA levels in nasal secretions were corrected by reference to total IgA in the samples and expressed as the ratios between specific antibodies and total IgA concentrations (U per microgram) (15). Determination of serum bactericidal activity was performed in triplicate (15, 16).

**Statistical analyses.** The Wilcoxon signed-rank test and Spearman correlation coefficients were determined by the use of PRISM Software (GraphPad Software, San Diego, Calif.).

**RESULTS**

**T-cell proliferation induced by OMVs.** All participants, except one, were considered negative to OMVs at the start of the study (cpm < 2,000) (Fig. 1). After nasal vaccination, 7 of the 12 participants were found to be responders to OMVs (Fig. 1), since they showed at least a twofold increase in proliferative responses and exceeded the background level (2,000 cpm). Four of these responders reached maximum response levels more than four times their prevaccination levels, and two of them showed 156- and 35-fold increases. When the group was analyzed on the basis of the maximum increases reached by each individual, the OMV-specific T-cell response was significant ($P < 0.001$). The results did not seem to be influenced by
the way the vaccine had been given; four and three of the OMV responders received drops and spray, respectively, whereas five and six of the PorA responders received this treatment.

The kinetics of the cellular immune responses to OMVs during the complete vaccination experiment are shown in Fig. 2 in comparison with the results obtained with the purified PorA and PorB antigens (median cpm). The maximum median response to OMVs was reached after the second dose (week 2), and the response then declined toward week 7. The vaccine-induced increase in proliferation to OMVs measured at week 2 was also significant \( (P = 0.0093) \). The single vaccine dose given 5 months after the initial four doses did not result in a statistically significant increase in the OMV-specific T-cell responses, even though four vaccinees responded to this dose (4- to 20-fold increases) (Fig. 3). The response of one of these vaccinees was enhanced compared to the level observed after the initial immunizations.

T-cell proliferation induced by PorA and PorB proteins. At the start of the study, five of the participants showed a negative response to the PorA antigen (<2,000 cpm) whereas seven participants showed a positive response (Fig. 1). After the intranasal immunizations, 11 of 12 vaccinees were found to be vaccine responders when tested against the PorA antigen (Fig. 1). Moreover, a majority of the responders (6 of 11) exceeded a fourfold increase in their response to this antigen, with the best responder reaching a postimmunization level 16-fold higher than before vaccination. When the group was analyzed on the basis of the maximum increases reached by each individual, the PorA antigen T-cell response was significant \( (P < 0.001) \). Although the individual maximum responses against the PorA antigen were reached at different time points, the median peak response was obtained simultaneously with the corresponding peak of the OMV response (week 2) (Fig. 2). The vaccine-induced increase in the PorA antigen-specific T-cell response at this time point was also significant \( (P < 0.001) \). Similar to the results obtained with OMVs, the median T-cell response to the PorB antigen declined toward the prevaccination level at week 7 (Fig. 2). The fifth vaccine dose did not result in a significant response when the vaccinees were considered as a group. However, four of the vaccinees responded to this last vaccine dose, and one of them showed an enhanced response compared to the initial immunizations.

The kinetics of the PorB antigen T-cell response is included in Fig. 2 for comparison with the results obtained with the OMV and PorA antigens. None of the vaccinees were considered responders to the PorB antigen during the four initial immunizations (Fig. 1), but three of them nevertheless responded to the fifth dose (Fig. 3). These vaccinees were among...

FIG. 2. Kinetics of the OMV (0.8 \( \mu g/ml \)), PorA (5 \( \mu g/ml \)), and PorB (5 \( \mu g/ml \)) antigen-specific T-cell responses expressed as median cpm in 12 volunteers after intranasal immunizations with a meningococcal OMV vaccine without adjuvant. The immunizations are indicated by arrows.

FIG. 3. Comparisons of T-cell proliferation (cpm) in response to OMVs (0.8 \( \mu g/ml \)), the PorA antigen (5 \( \mu g/ml \)), and the PorB antigen (5 \( \mu g/ml \)) in 12 volunteers before and after intranasal immunization with a fifth dose of a meningococcal OMV vaccine without adjuvant. Each symbol represents the prebooster cpm \( (x \text{ value}) \) and the maximum postbooster cpm \( (y \text{ value}) \) obtained for one vaccinee. The diagonal solid line in each graph indicates the same post- as prebooster values, and the two broken lines indicate twofold increases and decreases. The dotted horizontal lines define postbooster levels of 2,000 cpm, considered to be the limit of background responses. Values less than 100 cpm are approximated to 100. A vaccinee is considered to be a responder to the antigen tested if the symbol is above the doubling level (diagonal broken line) as well as above the background level (2,000 cpm; horizontal line).
the same individuals as those who responded to OMVs and among the four who responded to the PorA protein after the fifth dose. However, an enhanced effect of the fifth dose detected with all the three antigens was found in only one vaccinee.

T-cell proliferation induced by BCG and PHA. Since all participants in the study previously had been vaccinated with BCG, this antigen was included as a positive control in the in vitro T-cell proliferation assays. The individual responses to BCG varied considerably (10,000 to 150,000 cpm) and in all cases exceeded the response levels to the meningococcal antigens. The results demonstrated that the proliferative T-cell response to BCG did not show any significant changes during the immunizations (Fig. 4). Likewise, the mitogen (PHA)-induced proliferation observed was fairly constant for each individual throughout the vaccination experiment (Fig. 4).

Correlations between T-cell proliferation and antibody responses. Based on pre- and maximum postimmunization levels, nonparametric correlation analyses were used to test for relationships between the vaccine-induced cellular immune responses and the previously reported antibody responses (15). We obtained a positive correlation between the PorA antigen-specific T-cell responses and the corresponding anti-OMV IgA levels in nasal secretions ($r = 0.71$, $P < 0.0001$) (Fig. 5). A positive correlation was also found between the same T-cell responses and the concentrations of anti-OMV IgG in serum ($r = 0.67$, $P = 0.017$) (Fig. 6). Preimmunization values were not included in this analysis, because IgG levels in sera from some individuals were elevated compared to mucosal IgA antibody levels prior to vaccination (15). When both the pre- and post-vaccination data were included, no correlation was obtained. In addition, we observed a positive correlation between the PorA antigen-specific T-cell responses and serum bactericidal activity against the 44/76 meningococcal vaccine strain ($r = 0.59$, $P < 0.005$) (Fig. 6).

**DISCUSSION**

Several candidate vaccines against serogroup B meningococcal disease are based on membrane vesicles with different OMPs as potential protective antigens (3, 6, 10, 26, 31). Since the Norwegian OMV vaccine contains antigens capable of inducing protective immunity (3) and is of a particulate nature (10), it represents an attractive candidate antigen for investigating immune responses induced to meningococci by mucosal delivery. In addition, the OMV vaccine might serve as a model system for evaluating the potential of nonreplicating mucosal vaccines to prevent bacterial diseases in general. By applying the same mucosal immunization procedure as was successfully used to obtain anti-OMV antibody responses in mice (8), we have now investigated the ability of the OMV vaccine to induce T-cell responses in humans when it is given intranasally without additional adjuvant. Previous analysis of the humoral immune responses observed in the same vaccinees has demonstrated that this vaccine induces local mucosal antibodies to OMVs as well as systemic antibodies with bactericidal activity (15).

Although the protective effector functions against extracellular bacterial infections are believed to be mediated by antibodies, T cells also play an important role by promoting the systemic as well as the mucosal B-cell response in terms of Ig class switching, affinity maturation, and magnitude of responses (2). By monitoring T-cell proliferation against defined vaccine antigens in vitro in the present study, we have shown...
that intranasal immunizations with OMVs also induced cellular immune responses in humans. The majority of the participants showed a vaccine-induced T-cell response to both OMVs (7 of 12) and the PorA antigen (11 of 12). In contrast, none of the vaccinees showed an increase in their T-cell response to the PorB protein during the four initial immunizations. Although potential mitogenic effects of neisserial porins have been reported (23, 38), these or any other mitogenic compounds will eventually represent only a minor and constant contribution to the proliferation observed, since the vaccine-induced responses were considerably higher than before the immunizations. Thus, the results obtained showed that intranasal OMV vaccination can induce cellular immune responses in humans, measured as T-cell proliferation in response to OMVs and the PorA antigen.

The kinetics of the vaccine-induced cellular immune responses to both OMVs and the PorA antigen appeared similar, with median cpm responses peaking 1 week after the second dose and then declining to prevaccination levels toward week 7. Although the relative increases in median cpm seen with these antigens were approximately of the same magnitude, the in vitro responses to the PorA antigen were superior to those to OMVs with respect to both the pre- and maximum postimmunization levels (Fig. 1 and 2). Since the optimal amount of each antigen was used in the study, this difference was not due to a concentration effect. However, any difference in antigen processing and presentation occurring between purified and OMV-bound proteins might have influenced the T-cell responses.

Although single individuals responded to the booster dose, only one vaccinee showed an enhanced response to all three antigens after this dose. However, when analyzed as a group, the vaccinees did not show a significant response to any of the antigens tested after the booster dose. The same observation was made with respect to both the corresponding mucosal and systemic antibody responses (15). The lack of an observed booster effect on the cellular as well as the humoral immune responses might thus be due to an insufficient immunological memory induced by the initial immunizations or to clearance of OMVs at the mucosal level by the previously induced IgA antibodies. In addition, the question of tolerance induction cannot at present be excluded. However, it should be noted that the interpretation of results based on peripheral blood mononuclear cells in this context may be difficult, because memory T cells induced by mucosal immunizations are probably homing to other compartments (42). We have therefore initiated a study designed to test for tolerance induction in mice by using a similar immunization procedure.

Bactericidal IgG antibodies in serum appear to be of primary importance for protection against systemic meningococcal disease (5, 11, 25, 28). In addition, induction of mucosal IgA antibodies is believed to play a role in local protection against invasion by pathogenic bacteria through the mucosal membranes (22, 35). We have previously demonstrated both local IgA responses in nasal secretions and persisting IgG responses in serum with bactericidal activity in the same vaccinees as were studied here (15). The demonstration of a correlation between cellular immune responses and secretory IgA levels, IgG levels in serum, and bactericidal activities suggested that the T-cell responses described here are of putative importance for induction of both mucosal and systemic antibody responses. These observations are in agreement with the notion that cytokine-mediated T-cell help is required for B cells to differentiate and produce antibodies of the relevant isotypes (1, 2).

The results of a similar study of the cellular immune responses induced by intramuscular vaccinations with OMVs (25 μg of protein per dose), given in three doses with Al(OH)3 as adjuvant (24), were compared with the results obtained in the present study. After intramuscular OMV immunizations (24), the T-cell responses to OMVs were of the same magnitude as those to PorA, but these responses were significantly higher (1 log unit difference) than those obtained by intranasal immunizations. However, the importance of this difference with respect to protection is difficult to assess.

The observation that the vaccine-induced T-cell response to the PorA antigen was significantly higher than the PorB responses, after both mucosal (see above) and systemic vaccinations (24), is in accordance with other studies of T-cell responses against meningococcal porins (41), and may be due to differences in epitope density (40, 41) or antigen processing and presentation. With respect to the humoral response, serum bactericidal activity, which is considered important for protection, correlates with the presence of PorA antibodies after systemic OMV vaccination (29, 33, 34, 36, 44). Although monoclonal antibodies to the PorB antigen show low bacteri-

![Graph showing correlation between T-cell proliferation to PorA (cpm) and concentration of IgG antibodies to OMVs](http://iai.asm.org/)
cidal activity in animal models (30). OMV-vaccinated patients surviving systemic meningococcal disease have been reported to show higher IgG levels against PorB than against PorA (13, 14, 37). A possible role of the PorB antigen in relation to vaccine protection can therefore at present not be excluded. However, it is noteworthy that the most pronounced T-cell response seen here was directed toward the PorA antigen, which is considered to be important with respect to protection against meningococcal disease.

Potential intrinsic mucosal adjuvant properties of OMVs have previously been shown in mice by our group, with the demonstration that immune responses to influenza virus can be enhanced by intranasal coadministration of inactivated influenza virus with OMVs (7). Consistent with this, the results obtained in this vaccination study showed that mucosal application of OMVs in humans can induce B-cell (15) and T-cell responses without the use of any additional adjuvant. These observations are in accordance with the potential adjuvant effect of neisserial porins previously described by others (38). Neisserial porin proteoliposomes have also been shown to function as mucosal adjuvants, as demonstrated for different model antigens delivered intraanasally (17, 18, 19, 21). In this context, our findings suggest that meningococcal OMVs may be used as a vaccine vehicle for mucosal immunizations against heterologous antigens as well.

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