Protective Levels of Diphtheria-Neutralizing Antibody Induced in Healthy Volunteers by Unilateral Priming-Boosting Intranasal Immunization Associated with Restricted Ipsilateral Mucosal Secretory Immunoglobulin A

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Subunit intranasal vaccines offer the prospect of inducing combined systemic-mucosal immunity against mucosally transmitted infections such as human immunodeficiency virus. However, although human studies have demonstrated the induction of active immunity, secretory immunoglobulin A (sIgA) responses are variable, and no study has demonstrated protection by accepted vaccine-licensing criteria as measured by direct toxin-neutralizing activity. Using the genetically inactivated mutant diphtheria toxoid CRM197 in a bioadhesive polyacationic polysaccharide chitosan delivery system, we found that a single nasal immunization was well tolerated and boosted antitoxin neutralizing activity in healthy volunteers, which could be further boosted by a second immunization. The neutralizing activity far exceeded accepted protective levels and was equivalent to that induced by standard intramuscular vaccine and significantly greater than intranasal immunization with CRM197 in the absence of chitosan. A striking but unexpected observation was that although unilateral intranasal immunization induced circulating antitoxin antibody-secreting cells, a nasal antitoxin sIgA response was seen only after the second immunization and only in the vaccinated nostril. If these data are reproduced in larger studies, an intranasal diphtheria vaccine based on CRM197-chitosan could be rapidly licensed for human use. However, a restricted sIgA response suggests that care must be taken in the priming-boosting strategy and clinical sampling techniques when evaluating such vaccines for the induction of local mucosal immunity.

In murine models of immunization, the nasal route appears to have the advantage of inducing both a systemic and a disseminated mucosal response, making it highly attractive for the delivery of vaccines against mucosally transmitted infections. Various human studies have reported nasal delivery of non-replicating antigens from Shigella (9), diphtheria and tetanus toxoids (1), cholaer toxin B subunit (4, 21), Neisseria meningitidis (10, 12), Pseudomonas (14), and Bordetella pertussis (5, 6). However, no study has reported the induction of immunity that would satisfy established vaccine-licensing criteria. Furthermore, reported secretory immunoglobulin A (sIgA) responses in mucosal secretions vary among subjects and anatomical sites (11), and intranasal diphtheria toxoid in alum induced significant side effects (1). Thus, a delivery system that can effectively induce both systemic and disseminated mucosal responses to nasally delivered nonreplicating antigens without inducing significant side effects remains a highly desirable goal.

The mutant diphtheria toxin CRM197 contains an inactivating glycineto-glutamic acid substitution at position 52 of the enzymatic A subunit and is widely used in humans as a licensed polysaccharide antigen carrier. CRM197 is inherently nontoxic and does not require chemical inactivation. However, low-dose (0.18%) formaldehyde treatment enhances its immunogenicity for intranasal immunization of mice and guinea pigs (17) while preserving its structural integrity and ability to induce highly active toxin-neutralizing antibodies (17). This is in contrast to higher concentrations of formaldehyde used to inactivate diphtheria toxin in the preparation of current vaccines, which has the unwanted effect of enhancing the reactogenicity of contaminants. The cationic polysaccharide chitosan is primarily a mucoadhesive agent, and it enhances systemic and mucosal immune responses in animal models of intranasal immunization with CRM197 (17) and influenza antigens (3). Chitosan enhances transepithelial transport of antigen to the nasal mucosal immune tissue through an effect on tight junctions and by decreasing mucociliary clearance (2). We report here the use of a combination of 0.18% formaldehyde-treated CRM197-chitosan for nasal immunization of healthy volunteers in an attempt to induce protective levels of serum antitoxin antibody, and we study the induction of systemic and local mucosal immune responses.
Subjects, immunization protocol, and sample collection. Twenty healthy adult volunteers (10 male and 10 female; mean age, 23.2 years) were randomly assigned to receive two intranasal immunizations either with 50 μg of CRM197, 7 mg of chitosan glutamate 213, and 2.5 mg of mannitol or with CRM197, with 9.5 mg of mannitol alone. CRM197, was obtained from Chiron Vaccines, Siena, Italy, and chitosan glutamate 213 was obtained from Pronova Biomedical AS (now FMC BioPolymer AS), Drammen, Norway. A further five subjects (two male and three female; mean age, 24.8 years) received a single intranasal immunization with a standard alum-adsorbed diphtheria toxoid vaccine [Dip/Vac/Ads(Adult); Farilimon, Ramford, United Kingdom]. The study was approved by the United Kingdom Medicines Control Agency and St Georges Local Research Ethics Committee. All of the subjects reported intramuscular diphtheria vaccination >5 years previously and provided written informed consent. Intranasal immunization was performed using a prefilled Valois single-use device to insufflate a dry powder into the right nostril on days 0 and 28. Nasal lavage was performed by inserting a Foley urinary catheter into the nostril, inflating the balloon gently to form a seal, and instilling normal saline, which was then aspirated after 5 min. Each nostril was sampled independently on days 7, 27, 42, and 84. The samples were held on ice before being frozen without delay at −80°C to prevent proteolysis of sIgA.

ELISA and enzyme-linked immunospot (ELISPOT) assay. A modification of the method reported previously (15) was used to determine antitoxin IgG and IgA. Briefly, wells of 96-well high-binding immunosorbent assay (ELISA) plates (Costar, Cambridge, Mass.) received 1 μg of purified diphtheria toxin (Chiron Corporation) in carbonate-bicarbonate coating buffer, pH 9.6. After being blocked with 1% denatured casein, the wells received serial doubling dilutions of serum or nasal-lavage fluid. All antibodies were obtained from Sigma UK unless stated otherwise. Bound antitoxin antibody was detected by incubation with alkaline-phosphatase-conjugated goat anti-human IgG (γ-chain specific) or goat anti-human IgA (α-chain specific) antibody. Activity was measured by absorbance of 4-nitrophenyl phosphate substrate at 405 nm. Bound antibody activity was determined by the parallel-line method, relative to the reference sample, and was expressed as relative ELISA units (15, 16). Total IgA and albumin concentrations were measured by rate nephelometry in nasal-lavage samples of each subject at the time of peak sIgA response (Immage; Beckmann Coulter, High Wycombe, United Kingdom; international reference standard CRM470).

Antitoxin antibody-secreting cells (ASCs) were detected in an ELISPOT assay by coating and blocking 25-well Repli plates (Sterlin, Stone, United Kingdom) as described above and incubating peripheral blood mononuclear cells (PBMCs) separated by Ficol discontinuous-gradient centrifugation at a concentration of 5 × 10^5 cells/well for 21 h. After being washed, individual ASCs were detected by sequential incubation with goat anti-human IgA or IgG, rabbit anti-goat alkaline phosphatase-conjugate, and BCIP (5-bromo-4-chloro-3-indolylphosphate)-agarose substrate and were counted under low-power magnification as described previously (15).

Bead separation of PBMCs by surface expression of L-selectin. In seven subjects on a total of nine occasions after nasal immunization, PBMCs previously incubated with fluorescein isothiocyanate-labeled mouse anti-human L-selectin antibody (BD Pharmingen, San Diego, Calif.), were separated using MACS beads (Miltenyi Biotech, Auburn, Calif.) coated with goat anti-mouse IgG antibody. The purity of enrichment was confirmed to be >97% by fluorescence-activated cell sorter analysis. The number of L-selectin+ antitoxin ASCs was then determined by comparing separated and unseparated PBMCs in the ELISPOT assay, as reported elsewhere (19). Similar methodology was employed using an anti-ε-subunit integrin antibody; but a clear separation between labeled and unlaibered cells could not always be achieved, and those data are not shown.

Neutralizing-antibody assay. Serum samples were tested for specific antitoxin IgG neutralizing antibodies using a method similar to that of Miyamura et al. (18) as described previously (17). Briefly, serial twofold or threefold dilutions of serum or standard antitoxin for diphtheria (equine antiserum from the Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Md.) were added to wells of 96-well tissue culture plates. The plates were incubated at 37°C for 3 h with diphtheria toxin (Chiron Corporation). Following incubation, 10^6 Vero cells in M199 medium supplemented with 10% fetal calf serum were added to the plates. The neutralizing effects of antibodies versus diphtheria toxin were evaluated by analysis of the growth of the Vero cells after 3 days of incubation at 37°C. Supernatants from each well were removed, and viable cells, which remained adherent to the plates, were fixed and stained with crystal violet. Following solubilization of the dye, the cell density was determined by measurement of the absorbance at 540 nm. The levels of neutralizing antibodies in serum samples were expressed as international units per milliliter with reference to the absorbance values obtained for the standard antitoxin.

Statistical analysis. The unpaired Student’s t test was used to compare means between groups for ELISA and neutralizing-antibody assays, and the paired t test was used for left-right nasal-lavage antitoxin sIgA.

RESULTS

Safety and tolerability of the nasal vaccine. Intranasal immunization using the Valois single-use device was well tolerated, with only transient and mild-to-moderate symptoms. Table 1 gives the frequency of the most severe symptom scored by a subject in the 7 days after each immunization. No subjects withdrew from the study or refused to have the second intranasal immunization.

Serum antibody responses. Systemic protective immunity against Corynebacterium diphtheriae toxin is defined for vaccine-licensing purposes as >0.01 international neutralizing-antibody units/ml of serum. A single nasal immunization with CRM197, with or without chitosan, induced serum antitoxin IgG and IgA (Fig. 1) and protective levels of toxin-neutralizing antibody (14.8 and 5.4 IU/ml, respectively) (Fig. 2) as effectively as intramuscular immunization (6.3 IU/ml) (Fig. 2).
SECOND INTRANASAL IMMUNIZATION BOOSTED NEUTRALIZING ACTIVITY, WHICH WAS SIGNIFICANTLY HIGHER IN THE CHITOSAN DELIVERY GROUP (PEAK, 20 IU/ml).

**sIgA Responses.** Whereas all volunteers had evidence of pre-existing antitoxin serum IgG and, interestingly, detectable levels of serum IgA from previous intramuscular vaccinations, no antitoxin sIgA was detected in nasal-lavage fluids at recruitment or following intramuscular immunization (Fig. 3a). Unilateral intranasal priming-boosting did induce a highly significant local antitoxin sIgA response in nasal-lavage fluid, but only after the second immunization. Chitosan significantly increased the sIgA response by >10-fold in comparison with the mannitol group (Fig. 3a). An unexpected and striking observation was that the sIgA response occurred almost exclusively in the vaccinated nostril. Even when correction was made for the flow rate of nasal secretions (by dividing specific IgA ELISA units by the total IgA), subjects either did not mount a sIgA response or did so only in the immunized nostril (Fig. 3b). The difference was greater for the chitosan delivery group due to the higher level of antitoxin sIgA induced; however, a similar and significant left-right difference was seen with the mannitol delivery group. No difference in the albumin concentration was observed between groups or between left and right nostrils, indicating that alterations in passive transudation of proteins (e.g., as a result of nasal inflammation) was not responsible for the observed differences in antitoxin sIgA levels. Similarly, no differences in total IgA concentrations were observed between groups or between left and right nostrils, again indicating that differences in nasal-secretion flow rates or variable dilution of the nasal secretions during washout did not account for the observed differences in specific antitoxin IgA levels in the lavage fluid.

**Induction of ASCs.** Circulating antitoxin ASCs were enumerated by ELISPOT assay (15) before and after immunomagnetic bead separation on the basis of L-selectin surface expression (Table 2). Intramuscular and intranasal immunizations both induced circulating IgG antitoxin ASCs (Fig. 4), as might be expected from previous studies of intranasal immunization (9, 12, 20, 21). However, only the chitosan-delivered immunization induced a significant circulating IgA ASC response, and then only after a second immunization.

**Discussion**

Various studies have confirmed the potential for intranasal immunization of humans with nonreplicating vaccines against a wide range of bacterial pathogens (5, 6, 9, 10, 12, 14), including *C. diphtheriae* (1). Furthermore, intranasal immunization appears to offer the advantage of inducing combined systemic and secretory immunity, including at diverse mucosal sites,
such as the lung and genital tract (4, 11, 21, 22). This ability to induce a mixed immune response and the potential for T-cell immunity (6, 17) make intranasal immunization very attractive for sexually transmitted infections, as well as those mediated by toxins produced by mucosally acquired pathogens such as *C. diphtheriae*. However, in order to gain acceptance by vaccine-regulatory authorities, such novel vaccination strategies will have to demonstrate efficacies at least equivalent to currently accepted licensing standards. We believe this to be the first study to report the use of nasal immunization in humans to induce protective immunity that exceeds current regulatory-authority criteria for vaccine licensing as measured by direct toxin-neutralizing activity.

The inherent immunogenicity of CRM197 and the structural integrity of the molecule after only mild formaldehyde treatment may partly account for the high-quality, functional toxin-neutralizing antibody induced via the nasal route in this study (Fig. 2). Chitosan is thought to act as a bioadhesive (2) and may transiently reduce mucociliary action, which may localize the antigen within the nose and allow for prolonged uptake. Furthermore, by transiently opening tight junctions, chitosan may enhance direct transepithelial transport of CRM197 to the NALT, both enhancing the immune response (Fig. 2) and localizing it within the mucosa (Fig. 3). The ability of chitosan to deliver structurally intact CRM197 to the immune system is especially evident in the toxin-neutralizing assay, where the activity after one immunization was significantly greater than that in the mannitol delivery group, and it could be further boosted by a second intranasal immunization (Fig. 2). Chitosan does not induce histological changes in human studies of nasal delivery (2) or ex vivo studies of direct application to tissue explants (2). Our subjects tolerated intranasal delivery of chitosan-CRM197 on two occasions extremely well, with only transient and mild-to-moderate symptoms. This contrasts significantly with a previous study of intranasal delivery of diphtheria toxoid with alum adjuvant in humans, which resulted in signif-

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**FIG. 3.** Nasal-wash anti-diphtheria toxin (DT) sIgA response after intramuscular and intranasal immunizations. Subjects were immunized twice (open arrows) intranasally with CRM197-chitosan (▲) or CRM197-mannitol (●) or once (thin arrow) intramuscularly (IM) (□). (a) Geometric mean nasal-wash IgA anti-diphtheria toxin activity measured by ELISA in the left and right nostrils. The error bars indicate standard errors of the mean (capped lines for the CRM197-chitosan and CRM197-mannitol groups; uncapped lines for the intramuscular group) and are cropped where overlaps occur. (b) Paired left- and right-nasalwash anti-diphtheria toxin sIgA response [log(IgA anti-diphtheria toxin activity)/total sIgA concentration (in milligrams per milliliter)] for each subject on the day of peak response. The error bars indicate standard errors of the mean. *, *P* < 0.05, and **, *P* < 0.01 between CRM197-chitosan or CRM197-mannitol on day 42 and (intramuscular) day 27 and left-to-right corrected nasal-lavage sIgA.
The discordance between serum IgG and nasal-lavage sIgA responses is in keeping with the concept that systemic and mucosal immune responses may occur independently (7) and the relatively short duration of mucosal sIgA responses. Thus, although all subjects reported previous intramuscular diphtheria immunization and had measurable preimmunization antitoxin IgG and low levels of toxin-neutralizing activity, no preimmunization antitoxin sIgA was detected in lavage fluid. In addition, only intranasal immunization induced sIgA responses, with no antitoxin sIgA appearing in lavage fluid after intramuscular immunization, as had been described previously (10). Similarly, prior intramuscular immunization did not appear to prime the nasal-associated lymphoid tissue (NALT) for sIgA response, as unilateral intranasal priming-boosting induced a local antitoxin sIgA response in nasal-lavage fluid and circulating IgA antitoxin ASCs only after the second immunization. This implies that nasal priming is required for a nasal response using this delivery system and is in keeping with other studies of NALT immunization (20).

The unexpected and striking observation that nasal priming was essentially restricted to the vaccinated nostril (Fig. 3b) is, we believe, the first report of this finding. We were unable to find any previous human study in which unilateral intranasal priming-boosting was accompanied by the lavage of the left and right nostrils independently. Most studies employ liquid preparations that may reach other induction sites, such as adenoids and tonsils, and from which antigen is cleared much more rapidly than chitosan-admixed CRM197 powder (24) or that have been delivered bilaterally, or they do not report the priming-boosting strategy. Our observations are analogous to those in a seminal human study of direct intratonsilar injection of cholera toxin B subunit, in which an IgA response restricted to the injected tonsil was observed (20) and subsequent boosting again led to a higher response in the primed tonsil. The trafficking of immunoblasts within a “common mucosal immune system” regulated by specific binding of lymphocyte ad-Addressins such as a4ß7 to venule endothelial receptors such as MadCAM-1 is well established (19). In this paradigm, a degree of compartmentalization occurs, with certain mucosal inductive sites preferentially populating other, distant mucosal sites with memory cells. Studies of intranasal immunization have demonstrated the trafficking of primed lymphocytes and priming of distant sites, such as the genital tract and gut, as described above (4, 11, 21, 22). However, within the mucosal immune system there appears to be further subcompartmentalization, especially within the structures of Waldeyer’s ring (20). Direct intratonsilar immunization appears to induce two populations of plasmablasts—one that recirculates and homes to distant sites and another that matures in situ to preferentially prime for an ipsilateral immune response, probably regulated by the different addressin phenotype of primed cells (20). Whether chitosan-delivered intranasal immunization induces a similar response and primes distant mucosal sites remains to be elucidated.

The discordance between serum IgA and sIgA responses is interesting. The function of serum IgA remains elusive, but the ability of both intranasal and parenteral immunization to induce serum IgA is well documented (1, 4, 9–11, 14, 20–22). The discordance between the presence of a serum IgA response and the highly localized presence of an sIgA response in only one nostril was especially striking in this study. This confirms the independence of systemic and mucosal immune responses, the unique character of sIgA, and its specific transport across the mucosa by the polymeric IgA receptor (8). In one model of

**TABLE 2. Frequency of antitoxin IgA ASCs in l-selectin**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Day</th>
<th>Frequency of antitoxin IgA ASCs*</th>
<th>l-selectin*</th>
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<td>35</td>
<td>14</td>
<td>23.2 (46.2)</td>
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<tr>
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<td>7</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
<tr>
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<td>3</td>
<td>8</td>
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<td>7</td>
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*Expressed as number of ASCs per 10^6 PBMCs.

**FIG. 4.** Circulating antitoxin ASC response after intramuscular and intranasal immunizations. Subjects were immunized twice (open arrows) intranasally with CRM197/chitosan (▲) or CRM197/mannitol (●) or once (thin arrow) intramuscularly (○). The mean IgG and IgA anti-diphtheria toxin (DT) ASC responses 7 days after each immunization are expressed as the number of specific ASCs/10^6 PBMCs tested. The error bars indicate standard errors of the mean.
antigen uptake by rodent NALT, a difference between particulate and soluble antigens was observed (13, 23). Whereas particulate antigens are taken up by M cells and presented to B and T cells within the NALT which drain to the posterior cervical lymph nodes and induce both local and systemic immunity, soluble antigen is taken directly to the superficial cervical lymph nodes, where tolerance rather than immunity is induced. The ability of the dry-powder formulation to enter the former pathway, which is further enhanced by chitosan, may account for the ability to induce significant local and systemic immunity, as well as explaining the localization of the slgA response within the NALT. The importance of nasal priming in the induction of a local slgA response observed here and elsewhere (20) and the unilateral nature of the subsequent response have critical implications for the design of priming-boosting strategies with bioadhesive or powder formulations. Furthermore, studies which sample nasal secretions should pay close attention to the sampling procedure to avoid bias when left and right samples are pooled or nostrils are randomly sampled independently.

The frequency of antitoxin-specific ASCs of 20 to 60 per 10⁶ PBMCs, with more IgG than IgA, is in keeping with other studies of human intranasal immunization (9, 12, 20, 21). In contrast to oral immunization, which induces circulating lymphocytes with a mucosal phenotype characterized by expression of α4β7 integrin receptor, and parenteral immunization, which induces a predominantly systemic phenotype characterized by expression of t-selectin (CD62L) receptor, intranasal immunization is associated with a mixed mucosal-systemic phenotype of circulating lymphocytes expressing α4β7 integrin and t-selectin receptors (19). We also found that 46% of antitoxin ASCs were t-selectin positive (Table 2), and this was in keeping with the mixed IgG-IgA ASC response induced by intranasal immunization in our study (Fig. 4) and others (4, 9, 12, 20, 21). The ability to induce a mixed systemic-mucosal immune response, especially in the genital tract, is very attractive for vaccines against mucosally acquired systemic infections, such as those with human immunodeficiency virus, where a combined immune response will be crucial to effective vaccination strategies. The homing destinations of the IgA ASCs observed in this study were not addressed, but the association of IgA ASCs appearing in the blood in significant numbers and the maximal nasal slgA response being seen only in the intranasal-chitosan group suggests they may be homing to NALT. However, the circulation of IgA ASCs and the absence of a slgA response in the unimmunized nostril remains paradoxical. Future investigations should determine whether intranasal immunization with chitosan-delivered dry-powder formulations is capable of protecting distant mucosal sites such as the genital tract, as has been shown in other studies with enterotoxin antigens (4, 11, 21, 22), or whether the slgA response remains highly localized. In the case of diphtheria, a localized NALT slgA response in addition to a systemic IgG response would be highly attractive.

In conclusion, this clinical study provides proof of principle in humans that the technology is available to make mucosal vaccines that are acceptable according to existing requirements of regulatory agencies and that these vaccines, in addition to inducing the required systemic immunity, also induce a local mucosal response. The study not only opens the way for the immediate development in humans of mucosal vaccines against diphtheria, and potentially other subunit antigens, it has consequences for the design of a systemic-priming–mucosal-boosting strategy for the development of effective vaccines against human immunodeficiency virus infection and other diseases where mucosal immunity would be desirable. If these data are reproduced in larger studies, an intranasal diphtheria vaccine based on CRM197-chitosan could be rapidly licensed for human use. However, a restricted slgA response suggests that care must be taken in the priming-boosting strategy and clinical sampling techniques when evaluating such vaccines for the induction of local mucosal immunity.

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