Mucosal vaccination against diphtheria using starch microparticles as adjuvant for cross-reacting material (CRM197) of diphtheria toxin

Niclas Rydell, Ingvar Sjöholm*

Department of Pharmacy, Division of Pharmaceutics, University of Uppsala, P.O. Box 580, SE-751 23 Uppsala, Sweden

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Abstract

Mucosal vaccination has the advantage of eliciting a local mucosal immune response as well as a systemic response. In this investigation, polyacryl starch microparticles were conjugated to diphtheria toxin cross-reacting material (CRM197) as a mucosal adjuvant for oral or intranasal immunisation of mice. Various methods of stabilising CRM197 with formaldehyde were investigated. A good systemic and local mucosal immune response was attained with oral immunisation when CRM197 was treated with a relatively low formaldehyde concentration prior to conjugation to the microparticles. No immune response was seen after intranasal immunisation.

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1. Introduction

The total mucosal surface area of the respiratory apparatus, urogenital area and gastrointestinal tract is about 400 m² and these surfaces are all potential points of entrance for pathogens. Lymphoid tissues in these areas are collectively called mucosal-associated lymphoid tissue (MALT). In the gastrointestinal-associated lymphoid tissue (GALT), there are areas that are especially rich in microfold cells (M-cells) and immunocompetent cells (dendritic cells, macrophages and B- and T-cells). These areas, called Peyer’s patches (PP), have a very important role in the mounting of a mucosal immune response. Equivalent areas have been found in the nasal-associated lymphoid tissue (NALT) [1,2].

The original concept of a common mucosal immune system was based on the idea that the induction of an immune response at one mucosal site would also yield mucosal immunity at other sites [3,4]. The theory has been somewhat revised over the years and it now seems apparent that, although an immune response may be detected far from the site of induction, it is measurably stronger at the tissue targeted by the mucosal vaccine [5]. Interestingly, it has also become apparent that locally administered mucosal vaccines are often able to induce a systemic immune response in addition to the mucosal response. This is in contrast to parenterally administered vaccines, which are not often able to induce an effective mucosal immune response. In addition, parenteral vaccination is accompanied by various degrees of discomfort for the recipient, which adds to the attraction of oral vaccination methods.

Diphtheria is a bacterial disease that is spread like the common cold, i.e., via nasal secretions or saliva; consequently, it usually originates in mucosal areas of the respiratory tract. Cross-reacting material (CRM197) is a mutant non-toxic form of diphtheria toxin that has shown potential as a vaccine candidate. This molecule is derived from Corynebacterium diphtheriae strain C7(197), and contains only one mutation; the toxicity of the diphtheria toxin molecule is annulled by replacement of a glycine by glutamic acid in the catalytic domain [6–8]. Because of its innocuousness, CRM197 is an attractive alternative to diphtheria toxoid since the strict regulatory requirement to test for irreversibility of toxoid can be omitted (European Pharmacopoeia, fourth ed. 2002:0444).
CRM197 is more susceptible than the unmodified toxin to proteolytic attack, but it can be stabilised and therefore made more immunogenic by treatment with formaldehyde [7, 9, 10]. However, CRM197 is a poor antigen when administered mucosally, even after formaldehyde treatment some sort of protective delivery system is required. Several groups have investigated CRM197 as an experimental diphtheria vaccine, using both parenteral and mucosal administration routes [10–15].

This paper describes the use of biodegradable starch microparticles as a carrier for CRM197 in a mucosal vaccine against diphtheria. Several formulations, in which formaldehyde was added at different strengths and times during preparation, were administered to mice either orally or nasally. We monitored the systemic and mucosal immune responses to these formulations by analysing serum samples for diphtheria-specific IgG-IgM, IgG and IgE antibodies and secretory IgA (s-IgA) and compared the responses with those after vaccination with a commercial subcutaneous diphtheria vaccine.

2. Materials and methods

2.1. Materials

CRM197 was kindly supplied by Dr. Rino Rappuoli and Dr. Ulrike Fritzsche, Chiron Vaccines, Siena, Italy. The commercial vaccine against diphtheria (Difteri Vaccine) was obtained from Statens Serum Institut, Copenhagen, Denmark.

2.2. Preparation of polyacryl starch microparticles

The microparticles were prepared by polymerisation of acryloylated starch (maltodextrin, MD6, Stadex AB, Malmö, Sweden) in a water-in-oil emulsion, as previously described [16, 17]. Briefly, 400 mg of acryloylated starch was dissolved in 5 ml of a 0.2 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA. Ammonium persulfate (200 mM) was added to give a final concentration of 600 or 6000 μg/ml before conjugation to MP 3 μg protein were determined by the dry weight.

The mean diameter of the particles, based on the number distribution, was determined in a laser diffraction meter (LS 230, Coulter). The mean diameter was consistently <3 μm.

2.3. Conjugation of CRM197 to the polyacryl starch microparticles

CRM197, either after treatment with formaldehyde (0.06% or 0.6%) or un-treated, was coupled to the microparticles using the carbonyldiimidazol (CDI) method developed by Bethell et al. [18]. The microparticles were activated with CDI in dry N,N-dimethylformamide (DMF) for 1 h at room temperature. After several centrifugal washings with DMF to remove excess CDI, the microparticles were resuspended in coupling buffer (0.250 M boric acid with 0.15 M NaCl, pH 8.6) containing the CRM197. The container was rotated end over end at 4 °C for 72 h. The microparticles were then washed with sterile saline, pH 7.4, containing methylparaben (0.1% w/v), filtered through an 11 μm nylon filter and stored at 4 °C. The amount of CRM197 that had coupled to the microparticles was determined by amino acid analysis. The amount of microparticles/ml was determined by the dry weight.

The mean diameter of the particles, based on the number distribution, was determined in a laser diffraction meter (LS 230, Coulter). The mean diameter was consistently <3 μm.

The vaccine formulations and dosing regimens used are listed in Table 1.

<table>
<thead>
<tr>
<th>Vaccine formulation</th>
<th>Formaldehyde treatment</th>
<th>CRM197 content</th>
<th>Intranasal dose</th>
<th>Oral dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRM</td>
<td>None (un-treated)</td>
<td>Not formulated with MP</td>
<td>3 × 15 μg protein</td>
<td></td>
</tr>
<tr>
<td>CRM-0.06</td>
<td>600 μg/ml</td>
<td>Not formulated with MP</td>
<td>3 × 15 μg protein</td>
<td>9 × 150 μg protein</td>
</tr>
<tr>
<td>CRM-0.6</td>
<td>600 μg/ml</td>
<td>Not formulated with MP</td>
<td>3 × 15 μg protein</td>
<td>9 × 150 μg protein</td>
</tr>
<tr>
<td>MP</td>
<td>None (un-treated)</td>
<td>48 μg/mg MP</td>
<td>3 × 0.3 mg MP</td>
<td>9 × 3 mg MP</td>
</tr>
<tr>
<td>MP-0.06</td>
<td>600 μg/ml after conjugation to MP</td>
<td>47 μg/mg MP</td>
<td>3 × 0.3 mg MP</td>
<td>9 × 3 mg MP</td>
</tr>
<tr>
<td>MP-0.1</td>
<td>600 μg/ml after conjugation to MP</td>
<td>49 μg/mg MP</td>
<td>3 × 0.3 mg MP</td>
<td>9 × 3 mg MP</td>
</tr>
<tr>
<td>MP-0.06-Pce</td>
<td>600 μg/ml before conjugation to MP</td>
<td>39 μg/mg MP</td>
<td>3 × 0.3 mg MP</td>
<td>9 × 3 mg MP</td>
</tr>
<tr>
<td>MP-0.1-Pce</td>
<td>600 μg/ml before conjugation to MP</td>
<td>46 μg/mg MP</td>
<td>3 × 0.3 mg MP</td>
<td>9 × 3 mg MP</td>
</tr>
<tr>
<td>Comm</td>
<td>125 μg/ml (diphtheria toxoid)</td>
<td>Not formulated with MP</td>
<td>3 × 0.1 ml subcutaneously</td>
<td></td>
</tr>
</tbody>
</table>

CRM = commercial vaccine comprising formaldehyde-treated diphtheria toxin (toxoid) on alum (dose × 3 × 0.1 ml subcutaneously). MP = microparticles.
2.5. Immunisation of mice

The animals were kept in the animal house of The Medical Products Agency, Uppsala, according to the rules of Good Laboratory Practice. The animal experiments were approved by the regional Animal Ethics Committee and were carried out under the supervision of a licensed veterinarian.

2.5.1. Controls

Untreated mice were used as negative controls. Positive control mice were given 0.1 ml (equivalent to 12.5 μg diptheria toxoid) of the commercial vaccine subcutaneously in the scruff of the neck on days 0, 21 and 41.

2.5.2. Oral administration

Female mice of the BALB/c AnCrl strain (Charles River, Germany), 10–11-week-old, 7–10 mice/group, were given a dose of vaccine by oral gavage using a blunt-ended steel syringe on three consecutive days (days 0–2). The mice were deprived of food 2 h prior to oral immunisation and 1 h after. Each dose (0.2 ml) contained 3.0 mg microparticles suspended in equal parts of 0.1 ml saline containing 0.1% (w/v) methylparaben and 0.1 ml water containing 20 mg/ml NaHCO3. Booster doses were given in the same manner on days 21–23 and 41–43. Mice given non-conjugated, formaldehyde-treated CRM197 (CRM-0.06 and CRM-0.6) received the doses in the same manner and with the same amount of protein as the mice given the microparticle formulations, i.e., each dose contained about 150 μg of protein suspended in 0.2 ml saline with 0.1% (w/v) methylparaben.

2.5.3. Intranasal administration

Mice immunised nasally were lightly anaesthetised with isoflurane (but conscious) and given 10 μl of the vaccines (30 mg/ml) suspended in sterile saline containing 0.1% (w/v) methylparaben on days 0, 21 and 41. Mice given the formulations without microparticles received the same treatment and the same amount of protein, i.e., 15 μg of protein per dose, suspended in 10 μl of saline with 0.1% (w/v) methylparaben.

2.6. Collection and preparation of blood and faecal samples

Blood samples were obtained from the tail arteries of the mice, and serum was separated by centrifugation. Serum samples from individual mice were used in all enzyme-linked immunosorbent assays (ELISA) and in the Vero cell assays. Negative serum was taken from healthy non-immunised mice, and serum was separated by centrifugation. Serum samples from individual mice were used in all enzyme-linked immunosorbent assays (ELISA) and in the Vero cell assays. Serum samples were kept at −20°C until use.

Six to ten freshly voided faeces per mouse were collected on day 48, frozen and then freeze-dried. The net dry weight was determined on extracts of the faeces were prepared. Briefly, ice-cold PBS containing 5% non-fat dry milk, 0.1 mg soybean trypsin inhibitor/ml and 2 mM phenylmethylsulfonylfluoride was added to the faecal samples (20 μl solution/mg of dry faeces). Solid matter was suspended by rigorous vortexing and was then separated by centrifugation at 13,000 rpm for 15 min. The clear supernatant was frozen at −20°C until further use. Non-immunised mice served as negative controls.

2.7. ELISA of antigen-specific antibodies (IgM-IgG, IgG in serum and IgA in faeces)

Briefly, Immunomol 2HB 96-well plates (Thermo Lab-systems, Franklin, MA, USA) were coated with 10 μg/ml diphtheria toxoid (SBL Vaccin AB, Stockholm) in PBS, 100 μl per well, and incubated overnight in a moist chamber at 4°C. The plates were blocked with 1% bovine serum albumin (BSA) for IgG-IgM and 1% ovalbumin (OVA) for IgA. The antisera or extracts of faeces were diluted to appropriate concentrations in PBS with 0.05% Tween 20 (PBS-T) and added to the plates in series of two-fold dilutions. The following secondary antibodies were used: alkaline phosphatase-conjugated goat-anti-mouse IgM-IgG, alkaline phosphatase-conjugated rabbit-anti-mouse IgG (Nordic Biosite, Stockholm) and goat-anti-mouse IgA (Sigma). 4-Nitrophenylphosphate (Merck), 1 mg/ml in 10% diethanolamine buffer, pH 9.6, with 0.5 mM MgCl2, was used as substrate for the alkaline phosphatase-conjugated antibodies. The absorbance was measured at 405 nm in a microtitre plate spectrophotometer (Titer tek Multiscan MCC/340, Flow Laboratories). Titres are given as −log2 (dilution × 10). For further details, see previous publication [14].

2.8. ELISA of total IgA in faecal samples

A sandwich-type ELISA was developed to determine the total amount of IgA in the faecal samples. It was performed as described above for antigen-specific IgA, with the plates coated with goat-anti-mouse IgA (0.5 μg/ml well) (Sigma). The standard IgA was diluted to appropriate concentrations in PBS with 1% OVA to create a standard curve and the extracts from the faecal samples were diluted in PBS with 0.05% Tween 20 and added to the plates in series of two-fold dilutions. The concentrations of specific and total IgA were determined from the standard curve.

2.9. ELISA of antigen-specific and total IgE in serum samples

Immunomol plates were coated with 10 μg/ml diphtheria toxoid in PBS, 100 μl per well, or with capture antibody anti-mouse IgE (BD-Pharmingen) for total IgE determination. The plates were incubated overnight in a moist chamber at 4°C and blocked with 10% foetal calf serum (FCS) in PBS. Individual serum samples collected on day 56 were diluted to appropriate concentrations in PBS-T and added to the plates in series of two-fold dilutions. Standard mouse IgE (BD-Pharmingen) was diluted in PBS with 10% FCS
to create a standard curve. Biotinylated rat anti-mouse IgE (Sigma) was used as secondary antibody. After incubation and several washings, avidin-horseradish peroxidase (HRP) (Sigma) in PBS and 10% FCS were added. 3-, 3′-, 5-, 5′- Tetramethylbenzidine (Sigma) was used as substrate and absorbance was measured at 450 nm. The concentrations of specific and total IgE were determined from the standard curve.

2.10. Vero cell assay for detection of neutralising antibodies

The concentration of diphtheria antitoxin in the mouse serum samples was assessed using the microculture neutralisation test in Vero cells [19]. Individual serum samples collected on day 56 from mice immunised with MP-0.06-Pre (see Table 1 for formulation abbreviations), MP-0.6 or the commercial vaccine were used in the Vero cell assay. The assay was performed at the Department of Clinical Microbiology, Karolinska Hospital, Stockholm, Sweden. The results are given as international units (IU) per ml after comparison with a WHO international standard for diphtheria antitoxin (DI-01).

2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism® version 3 (GraphPad Software, San Diego, CA). One-way ANOVA was used to compare and evaluate mean values from treatment groups. A level of significance of 95% was chosen for all tests. The following abbreviations are used: S.D. = standard deviation; S.E.M. = standard error of the mean.

3. Results

3.1. Treatment of CRM197 with low-concentration formaldehyde prior to its conjugation to microparticles resulted in a strong systemic immune response after oral immunisation

CRM197 was treated with formaldehyde either before or after conjugation of the toxin to the microparticles. In both cases, two concentrations of formaldehyde were used (600 or 6000 μg/ml), resulting in four unique vaccine formulations for oral use. Fig. 1a indicates that treatment of CRM197 with low-concentration formaldehyde prior to conjugation to the microparticles (MP-0.06-Pre) gave the most effective formulation for oral use. One-way ANOVA with Dunnett’s post-test was used to compare the response in the MP-0.06-Pre group with that in all oral groups. MP-0.06-Pre induced a statistically higher serum IgG-IgM response than the other oral vaccination groups ($P < 0.05$ versus MP-0.06 and $P < 0.01$ versus the other groups, except for MP-0.6 which was not statistically different from MP-0.06-Pre). The titres induced by MP-0.06-Pre were significantly lower than...
Fig. 2. Serum diphtheria toxoid-specific IgE (white bars) and total IgE (grey bars) expressed as µg/ml ± S.D. On day 56 (n = 7–10). For abbreviations and immunisation programme, see Table 1.

3.2. CRM197-conjugated polyacryl starch microparticles induced an intestinal mucosal immune response

In Table 2, the mucosal immune response is given as the ratio of antigen-specific IgA divided by the total amount of IgA detected in faeces extracts. MP-0.06-Pre induced a statistically stronger mucosal immune response in terms of the ratio of antigen-specific IgA/total IgA than all other groups (P < 0.05) except MP-0.6 (no statistical difference). Diphtheria-specific IgA was only detected in the faecal extracts from mice immunised orally with CRM197 conjugated to microparticles. Neither free CRM197 given orally nor the commercial vaccine given s.c. induced detectable amounts of antigen-specific IgA.

3.3. The commercial vaccine induced the highest concentration of specific serum IgE

All formulations gave rise to a systemic IgE response (Fig. 2). Of the total IgE, 4–47% was anti-diphtheria toxoid IgE. There were no significant differences in specific IgE concentrations among the groups immunised orally. However, the amount of both specific and total IgE was at least ten times higher in the mice immunised subcutaneously with the commercial vaccine than in the oral groups. Also, there were no statistical differences in the ratio of specific IgE to total IgE among the groups.

3.4. Neutralising antibodies

Three of the formulations discussed above were tested for diphtheria toxin-neutralising antibodies with the Vero cell assay. Sera from day 56 were used. MP-0.06-Pre, the most promising formulation for oral administration, induced 0.05 ± 0.019 IU/ml (mean ± S.E.M., n = 10). Induced levels were below 0.006 IU/ml in mice immunised orally with MP-0.6 (n = 8) and were 3 ± 0.64 IU/ml (mean ± S.E.M.) in

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio specific/total, µg/µg ± S.E.M.</th>
<th>Specific IgA (µg/ml) ± S.E.M.</th>
<th>Total IgA (µg/ml) ± S.E.M.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-0.06</td>
<td>0.048 ± 0.038</td>
<td>6.68 ± 5.80</td>
<td>115 ± 9.15</td>
<td>8</td>
</tr>
<tr>
<td>MP-0.6</td>
<td>0.57 ± 0.44</td>
<td>26.5 ± 21.7</td>
<td>68.0 ± 12.6</td>
<td>8</td>
</tr>
<tr>
<td>MP-0.06-Pre</td>
<td>1.5 ± 0.55</td>
<td>86.2 ± 25.2</td>
<td>73.0 ± 6.54</td>
<td>10</td>
</tr>
<tr>
<td>MP-0.6-Pre</td>
<td>0.18 ± 0.089</td>
<td>21.9 ± 12.1</td>
<td>110 ± 4.27</td>
<td>10</td>
</tr>
<tr>
<td>CRM-0.06</td>
<td>–</td>
<td>n.d.</td>
<td>77.6 ± 15.8</td>
<td>10</td>
</tr>
<tr>
<td>CRM-0.6</td>
<td>–</td>
<td>n.d.</td>
<td>144 ± 20.0</td>
<td>7</td>
</tr>
<tr>
<td>Comm (s.c.)</td>
<td>–</td>
<td>n.d.</td>
<td>100 ± 18.2</td>
<td>7</td>
</tr>
</tbody>
</table>

S-IgA content in faeces on day 48 after oral immunisation. The immune response was measured as the ratio of diphtheria toxoid-specific IgA/total IgA in faeces. MP-0.06-Pre induced a statistically stronger immune response (P < 0.05) than that in all groups except that receiving MP-0.6 (no statistical difference). For abbreviations and immunisation programme, see Table 1.
3.5. Nasal vaccination with microparticles induced a poor systemic immune response

The systemic immune response after nasal immunisation was low (see Fig. 3). Neither formaldehyde treatment nor conjugation of CRM197 to microparticles had much effect. No significant differences were seen among any of the formulations on day 56.

4. Discussion

It is well-established that formaldehyde treatment of CRM197 in free form as well as conjugated to starch microparticles increases both its stability against proteolytic degradation and its immunogenicity [7,9–12,14,15,20]. This study clearly shows that the immunogenicity of a formulation with pre-treated CRM197 is superior to a formulation treated with formaldehyde after conjugation of CRM197 to the starch microparticles.

The structure of the final vaccine formulation may be affected by the timing of the formaldehyde treatment: before (MP-0.6-Pre, MP-0.06-Pre) or after (MP-0.6 and MP-0.06) conjugation of CRM197 to the polyacryl starch microparticles. In the coupling process, the protein is not only conjugated at the surface of the microparticles, but is also evenly distributed within the particles by covalent binding [21]. Formaldehyde primarily reacts with the primary amino groups of the protein, which are also required for the CDI coupling process to the starch microparticles as described [18,22]. Indeed, Metz and co-workers showed that a reduction of up to 60% of the primary amino groups may occur when using industrial formaldehyde treatment protocols to detoxify diphtheria toxin [22]. In our study, we found that when CRM197 was treated with formaldehyde before conjugation to the microparticles, 30–60% more CRM197 was required in order to obtain the same protein-to-microparticle ratio as when the protein was treated after conjugation. Possibly, the pre-treated CRM197 may find fewer points of conjugation to the starch matrix due to its lower number of unblocked primary amino groups compared to non-treated CRM197 and will then be more easily released from the matrix during the antigen processing in the antigen-presenting cell (APC).

Formaldehyde treatment of CRM197 after conjugation to the particles might lead to extra cross-links between protein molecules within the matrix of the particles, making these more rigid and less degradable. In a previous paper [14], we showed that non-formaldehyde treated CRM197-conjugated polyacryl starch microparticles (MP-CRM) were less effective when given orally than formaldehyde-treated conjugates. The immunogenicity of MP-CRM is poor also after intranasal administration (Fig. 3).

Fig. 1 shows that pre-treatment of CRM197 with a low concentration of formaldehyde (MP-0.06-Pre) potentiates the vaccine most effectively when given orally to mice if the total amount of diphtheria toxoid-specific IgG-IgM antibodies is used as a measure of vaccine efficacy. It was interesting to note, however, that high-dose formaldehyde treatment after conjugation of CRM197 to the microparticles (MP-0.6) was more effective than low-dose treatment after conjugation (MP-0.06). This indicates, as hypothesised, that the formulation properties are affected by the timing of the formaldehyde treatment.

The relative amounts of IgG (Fig. 1b) correlated well with the total amount of IgG-IgM, when comparing the best candidate (MP-0.06-Pre) with the commercial vaccine, suggesting the potential of the oral vaccine to induce neutralising antibodies.

We know from earlier work that the vaccine antigens are protected by the porous structure of the microparticles during transport through the alimentary canal to the Peyer’s patches and that, after uptake through the mucous membranes, the microparticles are metabolised in the follicle-associated antigen-presenting cells in such a way that an immune response is evoked. Soluble antigens administered orally do not induce nowhere near as strong an immune response as when conjugated to the microparticles. We have also shown in previous publications that starch microparticles conjugated with ovalbumin or human serum albumin are taken up in pig respiratory nasal mucosa in vitro [21] and by mouse Peyer’s patches, respectively [23]. Moreover, it is obvious that the balance between the particle stability during transport and uptake and subsequent degradation in the APCs is crucial for the production of a good immune re-
The stability of the particle may thus be affected by the timing of the formaldehyde treatment of the antigen (before or after conjugation to the particles). We suggest that low-concentration formaldehyde treatment prior to conjugation results in a more reproducible reaction and that the resulting vaccine formulation is less difficult for APCs to metabolise and process for display of the antigen. The formaldehyde treatment of CRM197 conjugated to starch microparticles may be less consistent than pre-treatment of CRM197. However, in species other than mice, a protein-particle conjugate with a different degree of cross-links might be more favourable because of different composition and volume of intestinal fluids, transport time through the gut and concentration of digesting enzymes.

Only the microparticle conjugate formulations administered orally induced a diphtheria toxoid-specific mucosal immune response in the form of s-IgA in faeces. Neither the commercial vaccine given subcutaneously nor the antigens given in free form induced s-IgA in detectable amounts. The fact that the formulation resulting in the strongest s-IgA response also gave rise to the strongest IgG-IgM response when given orally may indicate that the local response is linked in some way with the systemic immune reaction. Nevertheless, this was apparently not the case for the commercial vaccine; the subcutaneous route of administration may be relevant here. This offers an indirect proof that the IgA detected in faeces was produced mucosally at the site of vaccine uptake rather than by circulating systemic IgA being excreted in bile. Indeed, our group has earlier proved, using an ELISPOT assay, that mucosal s-IgA detected in faeces is produced in the intestinal mucosa [24].

Although IgG is the only antibody isotype that can neutralise diphtheria toxin [25], the induction of IgA after oral but not parenteral vaccination gives some indication of the potential of a mucosal vaccine. The clinical symptoms of diphtheria infection are almost entirely caused by the diphtheria toxin. C. diphtheriae is not an invasive bacterium and the purpose of diphtheria vaccination is solely to induce diphtheria toxin-specific antibodies [8,26]. In most cases, however, pathogens enter the body via mucosal surfaces and the presence of a strong mucosal s-IgA response should improve the immunological defence against such pathogens.

There were no significant differences in the IgE responses induced by the different oral vaccines in this study (Fig. 2). The subcutaneous commercial vaccine, however, induced a much greater IgE response than the oral vaccines. This difference might be a reflection of the difference in IgG-IgM titres between the groups, but it could also be the result of differences in types of adjuvant and routes of administration. Further studies of these systematic differences in IgE response are required for evaluation of potential allergic side effects.

The toxin-neutralising capacity of the serum antibodies, as assessed by the Vero cell assay, was surprisingly low after oral vaccination. Our best vaccine candidate (MP-0.06-Pre) induced a neutralising antibody response of 0.05 IU/ml on day 56, which just barely qualifies according to the often quoted limits of 0.1–0.01 IU/ml [27,28]. The response obtained with our second best candidate (MP-0.6) was very low (<0.006 IU/ml); induced levels were =0.001 IU/ml. The difference in IgG titres (Fig. 1b) between the commercial vaccine and MP-0.06-Pre was 3.4 titres (log2), i.e., a 10-fold dilution difference, but if neutralising antibody results are taken into account there was really a 60-fold difference. Similar findings have been reported by other groups. For instance, McNeele et al. [12] used chitosan as a carrier for CRM197 in mucosal (nasal) immunisation of guinea pigs. IgG titres of 3.85 (log10) were induced, but the neutralising antibody response was only 0.081 IU/ml. However, McNeele et al. also observed that although the specific IgG titre in guinea pigs immunised subcutaneously with formaldehyde-treated CRM197 and alum was only slightly higher, 3.99 (log10), there was a much higher neutralising antibody response of 5.49 IU/ml (67 times greater than that in the nasal group). The difference in toxin-neutralising efficacy appears to lie in the specificity of the antibodies produced after mucosal or parenteral priming. The commercial positive control vaccine used in our study comprised formaldehyde-treated diphtheria toxoid adsorbed to alum, which might partly explain the higher production of induced neutralising antibodies; for whatever reason, parenteral vaccination is known to induce a much more effective neutralising antibody defence than oral vaccination.

The different levels of efficacy in inducing toxin-neutralising antibodies are probably related to differences in antigen presentation as a result of different immunisation routes. The particles given orally may be more affected by the digestive environment in the gastrointestinal tract than antigens administered parenterally. It is possible that the antigenic epitopes crucial for the development of toxin-neutralising antibodies are degraded to such an extent in the gastrointestinal tract that they fail to be presented correctly by APCs. The conjugation via the primary amino groups of the antigen to the particles may impair the presentation of the required epitopes on the surface of the antigen. In addition, alum used in the parenteral vaccine is known to form persistent areas of high antigen concentration which might allow the immune system to interact more effectively for a more prolonged period of time with the antigen than after mucosal uptake [29].

Only low levels of IgG-IgM were induced after nasal immunisation (Fig. 3). The fact that there was no statistical difference in efficacy between the groups immunised with the microparticle formulations and the groups immunised with the antigens in free form suggests that the particles were not taken up through the nasal mucosa to any significant extent. Our previous studies with pig nasal mucosa in vitro also indicated that only a small number of particles are taken up [21]. We have also shown that no protein is loosely entrapped, as the particles did not release protein into a saline supernatant over a 6-month period (unpublished data). Instead, even if the nasal mucosa has a much lower proteolytic capacity than the gastrointestinal tract, it is probable that a small fraction of...
the particles has been degraded and released CRM197, which induced the systemic IgG-IgM titres detected.

Nonetheless, several groups have suggested that some sort of enhancement is needed to provide sufficient uptake of particles or proteins. Aggerbeck et al. [30] used surfactants to prolong the duration of antigen in the mucosa and suggest that the hydrophilic-lipophilic balance is very important for nasal uptake. McNeela et al. [11,12] and Mills et al. [15] used chitosan, which affects tight junctions and decreases mucooeiliary clearance. Illum et al. [31] used bioadhesive surfactant-like enhancer molecules in combination with starch particles. In all these studies, the antigen load after nasal immunisation was about 30 times lower than that after oral immunisation. This study has shown that significant levels of IgG and neutralising antibodies can be induced by oral CRM197-conjugated starch microparticles. Formaldehyde treatment in combination with conjugation to polyacryl starch microparticles was found to be a crucial step when optimising the vaccine formulation. Treating CRM197 with a low concentration of formaldehyde (0.06%) prior to conjugation to microparticles was found to be a crucial step when optimising the vaccine formulation. Formaldehyde treatment induces the systemic IgG-IgM titres detected. VERO cell assays. Karolinska Hospital, Stockholm, Sweden, for help with theing CRM197 and Ms Carina Bengtsson and Prof. Marta

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