Oral vaccination against diphtheria using polyacryl starch microparticles as adjuvant

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Abstract

Oral vaccination offers the advantage of eliciting both a mucosal and a systemic immune response. This study investigated the use of polyacryl starch microparticles as adjuvant for oral vaccination against diphtheria. Diphtheria toxin or cross-reacting material (CRM197) were covalently conjugated to the microparticles and fed to mice by oral gavage. Investigation of formaldehyde treatment as a means of either detoxifying (diphtheria toxin) or stabilising (CRM197) these formulations were also made. We show that all our formulations given orally or parenterally to mice induced a strong systemic immune response. Only formulations given orally induced a mucosal IgA-response. Furthermore, our formulations given parenterally or orally induced a strong diphtheria toxin-neutralising antibody response.

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1. Introduction

Diphtheria is an acute contagious, often fatal, disease caused by the Gram-positive Corynebacterium diphtheriae. C. diphtheriae, which is well adapted to air-borne transmission by way of nasopharyngeal secretions, produces diphtheria toxin, which in turn causes an inflammatory response in the host with subsequent formation of a greyish pseudomembrane on the respiratory mucosa. Diphtheria toxin, a 58,350 Da protein, is also absorbed into the circulatory system and distributed throughout the body, where it can destroy cardiac, kidney and nervous tissues by inhibiting protein synthesis[1]. Since most of the clinical symptoms of diphtheria are caused by this toxin, prevention focuses on induction of toxin-neutralising antibodies (antitoxin) by vaccination with non-toxic forms of diphtheria toxin. The currently available vaccines contain diphtheria toxin treated with formaldehyde (termed diphtheria toxoid) which renders the protein non-toxic but still immunogenic. Diphtheria toxoid is often adsorbed to aluminium salts and the vaccine is administered parenterally. Diphtheria toxoid is regarded as a safe antigen, which induces long-term immunity and can be given to infants [2]. Cross-reacting material (CRM197) is a non-toxic derivative of diphtheria toxin. A glycine to glutamic acid mutation at position 52 in the A subunit of the toxin molecule renders it enzymatically inactive and therefore—non-toxic [2]. CRM197 is well tolerated in humans and is used in vaccines as a carrier protein for carbohydrate antigens, e.g. from Haemophilus influenzae type b [3]. It has also been used in mucosal vaccination against diphtheria [4,5] and should be suitable, because of its natural lack of toxicity, as an antigen in new vaccines against diphtheria.

The possibility that vaccines may be administered mucosally has recently received a great deal of attention. Based on the concept of a common mucosal immune system, through which activated lymphocytes from one mucosal region are able to disseminate immunity to other mucosal and glandular tissues, vaccination at one mucosal site should be able to provide protection at other surfaces as well [6]. Polyacryl starch microparticles have previously been developed in our group as a drug carrier system [7]. We have shown that the microparticles function also as a strong adjuvant in oral vaccination [8,9], although naked particles were not immunogenic [10]. Furthermore, a model antigen (human serum albumin) covalently bound to the starch microparticles introduces a good humoral, long-lasting systemic response, as well as a strong IgA response and a cellular response of mixed Th1/Th2 nature when given orally to mice [10]. The response in mice after oral vaccination with Salmonella antigens bound to the starch microparticles was protective [9], which was also the case after parenteral vaccination with gp63 from Leishmania donovani [11].
The aim of this study was to evaluate the suitability of diphtheria toxin and CRM197 conjugated to polyacryl starch microparticles in different formulations as candidate vaccines for oral use. In investigating the immune response, the main focus was on the humoral immune response and the ability of the formulations to induce neutralising antibodies against diphtheria toxin. The effect of formaldehyde treatment of diphtheria toxin and CRM197 in these formulations was also investigated.

2. Materials and methods

2.1. Materials

CRM197 was kindly supplied by Dr. Rino Rappuoli, Chiron Vaccines, Siena; diphtheria toxin, diphtheria toxoid and diphtheria vaccine were obtained from SBL Vaccin AB, Stockholm.

2.2. Preparation of polyacryl starch microparticles

The microparticles were prepared by polymerisation of acryloylated starch (maltoedextrin, MD6, Stadex AB, Malmö, Sweden) in a water-in-oil emulsion, as previously described.[7,12] Briefly, 500 mg of acryloylated starch was dissolved in 5 ml of a 0.2 M sodium phosphate buffer, pH 7.5. 1 mM EDTA. Ammonium peroxide sulphate (200 μl) was added to give a final concentration of 0.8 M in the aqueous phase, which was homogenised in 300 ml of toluene: chloroform (4:1). N,N,N’,N’-Tetramethylethylenediamine (TEMED) was used to initiate the polymerisation.

2.3. Conjugation of diphtheria toxin and CRM197 to the polyacryl starch microparticles

The diphtheria antigens were coupled to microparticles using the carbodimidazol (CDI) method developed by Bethell et al.[13] 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 diphtheria toxin or CRM197. The mixture was rotated end over end at 4°C for 72 h. The microparticles were then washed with phosphate-buffered saline, pH 7.4 (PBS), filtered through an 11 μm nylon filter and stored at 4°C. The amount of diphtheria toxin or CRM197 coupled to the microparticles was determined by amino acid analysis. The amount of microparticles/ml was determined by the dry weight. The following nomenclature is used throughout the paper (for details concerning formaldehyde treatment, see Section 2.4); MP-DT: microparticles conjugated with diphtheria toxin (41 μg DT/mg microparticle); MP-DTxd: formaldehyde-treated MP-DT (41 μg DT/mg microparticle); MP-CRM: microparticles conjugated with CRM197 (49 μg/mg microparticle); MP-CRMxd: formaldehyde-treated MP-CRM (49 μg CRM197/mg microparticle).

The mean diameter of the particles, based on the number distribution, was determined in a laser diffractometer (LS 230, Coulter). The mean diameter was consistently <2.5 μm.

2.4. Formaldehyde treatment for detoxification of antigen-conjugated microparticles

A phosphate buffer (0.05 M, pH 7.5) containing 0.1 M glycine and 0.5% formaldehyde was prepared. Aliquots of diphtheria toxin-conjugated microparticles (MP-DT) were concentrated and added to the buffer as a suspension to give a protein concentration equivalent to 1000 μg DT/ml or 24.3 mg/ml of MP-DT. The mixture was rotated end over end at 37°C for 25 days. The suspension was washed four times with PBS (10 mM, pH 7.2) containing 0.1% (w/v) methyl paraben, rotated end over end for another 24 h and washed twice more. The resultant vaccine was termed MP-DTxd.

Similarly, aliquots of CRM197-conjugated microparticles (MP-CRM) were suspended in a phosphate buffer to a final concentration equivalent to 1000 μg CRM197/ml or 20.4 mg/ml of MP-CRM, but with a formaldehyde concentration of 0.7%, rotated end over end at room temperature for 7 days and washed as above (MP-CRMxd). The protein concentration of these formaldehyde-treated particles remained the same as after work up, i.e. MP-DTxd had a protein concentration of 41 μg/ml and MP-CRMxd 49 μg/ml.

2.5. Immunisation of mice

Female mice of the BALB/c Abom strain (Bomholtgård, Ry, Denmark), 8–15 weeks old, 3–12 mice per group, were fed a dose of the vaccines by oral gavage using a blunt-ended steel syringe on three consecutive days (0–2). Each dose given to the mice orally contained 3.0 mg microparticles suspended in 10 mM PBS containing 0.1% (w/v) methyl paraben. The protein concentration of the vaccines administered was for MP-DT and MP-DTxd 41 μg/mg MP and for MP-CRM and MP-CRMxd 49 μg/mg. Booster doses were given in the same manner on days 21–23 and 41–43 (except in Fig. 2 and 3, where the animals only received the first series of boosters on days 21, 22 and 23). Thus, each animal immunised orally was given a total of at least six doses if given only one booster, or nine doses if given two boosters. Mice immunised subcutaneously were given the same particles as above but 1 mg microparticles per dose in the SCC of the neck on days 0, 21 and 41. Thus each animal was given a total of three doses. Positive control mice were given a commercial vaccine (Vaccin mot difteri, SBL Vaccin AB, Stockholm) containing the equivalent of 3 μg diphtheria toxoid per injection on days 0, 21 and 41. Untreated mice were used as negative controls. The animals were kept...
in the animal house of The Medical Products Agency, Up-
psala, according to the rules of Good Laboratory Practice.
The animal experiments were approved by the regional An-
imal Ethics Committee.

2.6. Collection and preparation of blood samples

Blood samples were obtained from the tail arteries of the
mice, and serum was collected after centrifugation. Serum
from individual mice were used in all ELISA assays and
pooled sera from equal volumes were used for the Vero-cell
assays and skin rabbit tests. Negative serum was taken from
healthy non-immunised mice. Sera were kept at −20°C or
lower until use.

2.7. Collection and extraction of faecal samples

Six to 10 freshly voided faeces were collected on days
44–48, frozen and then freeze dried. The net dry weight
was determined and extracts of the faeces were prepared.
Briefly, ice-cold PBS with 5% non-fat dry milk, 0.1 mg soy-
bean trypsin inhibitor/ml and phenyl-methylsulfonylfluoride
(2 mM) was prepared and added to the faecal samples, 20 μl
solution/mg of dry faeces. Solid matter was suspended
by vigorous vortexing and separated by centrifugation at
18,000 × g for 15 min. The clear supernatants were frozen
at −20°C before further use. Non-immunised mice served
as negative controls.

2.8. Enzyme-linked-immunosorbent-assays (ELISA) of
antigen-specific antibodies (IgM–IgG and subclasses in
serum and IgA in faeces)

Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc
International, Rochester, NY, USA) were coated with diph-
theria toxoid (SBL Vaccin AB, Stockholm), 10 μg/ml,
100 μl per well, in PBS and incubated overnight in a moist
chamber at 4°C. No difference in antibody specificity
between coating with diphtheria toxoid or CRM197 was
observed (data not shown). Native diphtheria toxin was not
used during routine analysis due to the toxic hazards in a
normal laboratory. The plates were blocked with 1% BSA.
The antisera or extracts of sera were diluted to appropri-
ate concentrations in PBS with 0.05% Tween 20 (PBS-T)
and added to the plates in series of two-fold dilutions.
The following antibodies were used as secondary anti-
odies: alkaline phosphatase-conjugated goat-anti-mouse
IgM–IgG (Biosource, CA, USA), goat-anti-mouse IgA
(Sigma), rat-anti-mouse IgG2a, rat-anti-mouse IgG2b or
rat-anti-mouse IgG1 (Pharmpingen, San Diego, CA, USA).
4-Nitrophenylphosphate (Merck), 1 mg/ml in 10% di-
ethanolamine 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 microtitreplate spectrophotometer (Titertek Multiscan
MCC/340, Flow Laboratories).

A sandwich-type ELISA was developed to determine
IgG1, IgG2a and IgG2b in sera against relevant subclass
standard curves. Nine Immunoplate Maxisorb F96 plates
were coated with rat-anti-mouse IgG1, IgG2a or IgG2b
(Pharmpingen), respectively, and incubated overnight in
a moist chamber at 4°C. The plates were blocked with
1% OVA. The antisera were diluted to appropriate con-
centrations in PBS with 0.2% Tween 20 and added to
the plates in series of two-fold dilutions. The standard
mouse IgG1, IgG2a or IgG2b was diluted to appropriate
concentrations in PBS with 3% OVA to create a stan-
dard curve. The subsequent procedures were performed
as described above. In each sample, the final concentra-
tion of antigen-specific IgG1, IgG2a or IgG2b was deter-
mined from pooled standard curves by calculating a mean
concentration value from three different dilutions of a
sample.

When measuring IgM–IgG antibodies and subclasses,
pooled negative serum was added to each plate as a nega-
tive control. The negative serum absorbance values were
collected from all the plates and were found to lie below
0.1. Thus, 0.1 was set as the limiting value for a positive
result. Titres are given as −log2 (dilution 10×). A pos-
tive sample (from mice immunised subcutaneously with
a commercial vaccine) was included as a standard on all
plates and treated in the same way as the antisera to ensure
consistency between plates.

2.9. ELISA of total IgA in faecal samples

A sandwich-type ELISA was developed to determine the
total amount of IgA in faecal samples. It was performed as
described above for antigen-specific IgA, with the plates
coated with goat-anti-mouse IgA (0.5 μg per well) (Sigma).
The standard IgA was diluted to appropriate concentra-
tions 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 by calculating
a mean concentration value from three different dilutions
of the sample. Positive and negative samples were included
on all plates and treated in the same way as the unknown
samples. The negative faeces absorbance values were col-
llected from all the plates and were found to lie below
0.1. Thus, 0.1 was set as the limiting value for a positive
result.

2.10. Analysis of diphtheria toxin neutralisation efficacy in
rabbits

A neutralisation test was performed according to a mod-
ification of the method developed by Jensen [14] at SBL-
Vaccin AB (Stockholm, Sweden). Briefly, equal volumes of
sera were pooled from each of the three different treatment
groups of mice, i.e. MP-DT-or, MP-DT subcutaneously
Briefly, the vaccine in test group 1 (MP-DT) was stored at
microparticles itself rendered the vaccine non-toxic and stable.
jugation of the diphtheria toxin to the polyacril starch mi-
(4th Ed., 2002). This test clarifies whether the covalent con-
European Pharmacopoeia the toxicity test described in the
2.11. Test for absence of diphtheria toxin activity using the
guinea pig test
The absence of diphtheria toxin was tested according to
the toxicity test described in the European Pharmacopoeia
(4th Ed., 2002). This test clarifies whether the covalent con-
jugation of the diphtheria toxin to the polyacryl starch mi-
croparticles itself rendered the vaccine non-toxic and stable.
Briefly, the vaccine in test group 1 (MP-DT) was stored at
4°C for 6 weeks in sterile glass bottles, the vaccine in test
group 2 (MP-DT) was incubated at 37°C for 25 days) was
incubated at 37°C for 6 weeks just before the test. All vac-
cines tested were suspended in PBS containing 0.1% (w/v)
methyl paraben. The study was performed in 17 female al-
bino guinea pigs of the strain Dunkin Hartley, from M&B, at
Scantox, Lille Skensved, Ejby, DK-4623. Two guinea pigs
in the control group were each injected subcutaneously with
1 ml buffer containing 0.1% methyl paraben as a negative
control. The guinea pigs were observed for signs of toxaemia
for a 42-day period.

2.12. Vero-cell assay for detection of neutralising
antibodies
The diphtheria antitoxin concentration in the mouse sera
was assessed using the microculture neutralisation test in
Vero cells [15]. Equal volumes of sera previously collected
on day 56 were pooled (n = 3–4 mice per group) and
used in the Vero-cell assay. The collected sera from group
MP-CRMxd-or, were not enough to perform the Vero-cell
assay and we therefore used sera pooled from equal volumes
in Section 2. Mean antibody titres are given as −log₂ (dilution 10ⁿ⁺) ±
SD (n = 6). MP-DT, diphtheria toxin conjugated to microparticles; or,
oraly; s.c., subcutaneously.

3. Results

3.1. Diphtheria toxin covalently coupled to starch
microparticles as adjuvant in oral immunisation induces
a systemic humoral immune response similar to that
obtained with a commercial parenteral diphtheria vaccine
We hypothesised that the covalent conjugation of the na-
tive diphtheria toxin to the polyacryl starch microparticles
would be sufficient to detoxify diphtheria toxin. As shown
in Fig. 1, MP-DT given orally (or) or subcutaneously gave
rise to a strong humoral response in mice, similar to that
induced by the commercial diphtheria vaccine given s.c.
Corresponding amounts of free diphtheria toxoid in phys-
iological saline given orally only induced low amounts of
detectable antibodies. Specific antibody titres were detected
already after the primary dose and the levels increased after
the first booster for all three groups; the mice receiving s.c.
vaccination had a slightly faster onset of antibody response
than mice immunised orally.

3.2. Oral immunisation with diphtheria toxin conjugated
to microparticles induces a strong mucosal immune response
The amount of antigen-specific IgA in relation to the total
amount of IgA in faeces was analysed on days 45 and 48
(the second and fifth day after the second booster). On day
45, there was 3.9 ± 1.1 μg of antigen-specific IgA/mg of
total IgA found in faeces and, on day 48, there was 6.8 ±
1.4 μg/mg of total IgA after oral administration. There was
no antigen-specific IgA in the faeces detectable in the mice
immunised s.c. or the mice immunised with soluble free
diphtheria toxoid or the commercial vaccine. The limit of
quantification was 15 ng/ml.

Fig. 1. Serum diphtheria toxoid (DTxd)-specific IgM-IgG. Arrows indicate
immunisations and boosters given on days 0–2, 21–23, 41–43 as discussed
in Section 2. Mean antibody titres are given as −log₂ (dilution 10ⁿ⁺) ±
SD (n = 6). MP-DT, diphtheria toxin conjugated to microparticles; or,
oraly; s.c., subcutaneously.
3.3. Sera obtained after oral or parenteral immunisation with antigen-conjugated starch microparticles show excellent protection against diphtheria toxin in rabbit tests

In order to correlate the antibody titres with an actual toxin-neutralising effect in the intradermal test in rabbits according to Jensen et al., sera were pooled from 3 groups of mice (MP-DT-or, MP-DT-s.c. and commercial vaccine-s.c.). Four mice per group were bled on day 85 and equal volumes of sera were pooled to yield sufficient for the test to be performed.

The commercial vaccine served as a positive control. The groups vaccinated with the starch particle adjuvant (MP-DT-or and MP-DT-s.c.) both resulted in 16–32 IU/ml of diphtheria-specific neutralising antibodies and the commercial vaccine yielded a response of 16 IU/ml. These results suggest that MP-DT is a very potent inducer of neutralising antibodies.

3.4. Formaldehyde treatment of diphtheria toxin-coupled starch microparticles slightly decreases the immunogenicity

The present regulatory requirements for diphtheria vaccines as specified in the European Pharmacopoeia (4th Ed., 2002) presume that the vaccines are treated for detoxification with formaldehyde. Consequently, tests for the absence of toxin activity and the irreversibility of the toxoid are included in the monograph (No. 444). The diphtheria toxin-conjugated microparticles were therefore treated with formaldehyde (0.5% (v/v), 25 days at 37 °C) and an immunogenicity study in mice was undertaken. As seen in Fig. 2, oral administration of the formaldehyde-treated MP-DT (MP-DTxd-or) seemed to result in somewhat lower immunogenicity than oral administration of particles not treated with formaldehyde (MP-DT).

Diphtheria specific IgA in faeces was only detected after a second oral booster and was therefore not analysed.

3.5. Diphtheria toxin-conjugated microparticles yield a mixed Th1/Th2 response

The subclass profile of the humoral IgG response analysed with ELISA (Fig. 3) was further analysed to assess the Th1/Th2 response. In this analysis, IgG1 represents a Th2-type response and IgG2a + IgG2b represent a Th1-type response. It is concluded that the immune response was a Th1/Th2 mix.

3.6. Diphtheria toxin-conjugated microparticles are toxic

The MP-DT stored at 4 °C for 6 weeks were all found dead on the second day after treatment (1000 µg per animal), as were the five animals treated with the MP-DT stored at 37 °C for 6 weeks (1000 µg per animal). The five animals treated with MP-DTxd that had been stored for 6 weeks at 37 °C and the two control animals receiving only buffer (PBS + methyl paraben 0.1% (v/v)) all survived to the end of the scheduled observation period of 42 days. No signs of ill health were observed.

Fig. 2. Comparison of serum diphtheria toxoid-specific IgM-IgG levels after oral (or) or subcutaneous (s.c.) vaccination with either formaldehyde-treated diphtheria toxin conjugated microparticles (MP-DTxd) or non-treated MP-DT. Arrows indicate immunisations and boosters given on days 0–2, 21–23, according to the methods section. Mean antibody titres are given as −log (dilution 10^n) ± S.D. (n = 8–12 for days 21–41 and n = 4–6 for days 56–85).

Fig. 3. The diphtheria toxoid-specific subclass profile in serum at day 56. The bars represent mean µg/ml ± standard error of the mean (n = 4–6). MP-DT, diphtheria toxoid conjugated to microparticles; MP-DTxd, formaldehyde-treated MP-DT; or, orally; s.c., subcutaneously.
in these animals during the macroscopic examination at necropsy.

3.7. Starch microparticles conjugated with cross-reacting material and treated with formaldehyde (MP-CRMxdt) give a good humoral immune response

The MP-DTxd vaccine had potential as a candidate for a new oral vaccine against diphtheria. However, extensive irreversibility tests, both time-consuming and very costly, need to be performed for a vaccine based on a potent toxin such as native diphtheria toxin. Therefore, we turned our attention towards the non-toxic mutant form of diphtheria toxin, CRM197. Irreversibility evaluations are not needed with this material. CRM197 is, however, known to be a poor immunogen, because of its sensitivity to proteases. We therefore coupled CRM197 to the polyacryl starch microparticles and gave them to mice parenterally and orally either untreated (MP-CRM) or after formaldehyde treatment (MP-CRMxdt).

As seen in Fig. 4, formaldehyde treatment in relatively high concentrations (0.7% (v/v), 7 days at room temperature) enhanced the immunogenicity of CRM197-coupled starch microparticles in the oral group. Treatment with formaldehyde seemed to have little effect on the immunogenicity of the vaccines administered parenterally compared to the non-formalised CRM197-coupled microparticles, indicating that the gastrointestinal acids and enzymes are degrading the protein faster than proteases in muscle. MP-DT had previously been seen to be highly immunogenic in oral vaccination and served as a positive control for the kinetics of the humoral immune response after oral immunisation.

3.8. CRM197-conjugated starch microparticles induce a mixed Th1/Th2 response in mice

The subclass profile of the humoral IgG response analysed with ELISA (Fig. 5) was used to assess the Th1/Th2 response. It is concluded that the immune response is a mixed Th1/Th2 response.

3.9. Formaldehyde-treated CRM197-conjugated polyacryl starch microparticles induce a strong intestinal mucosal response

Only the MP-CRMxdt and MP-DT vaccines induced a diphtheria-specific IgA response. The response was presented as the ratio of diphtheria-toxoid-specific IgA (µg) over the total amount of IgA (mg) in faeces. The response in the group given MP-CRMxdt-or was 4.5 ± 1.1 and 3.0 ± 0.88 µg/mg on days 48 and 49, respectively. The response in the group given MP-DT-or was 1.6 ± 0.70 and 1.1 ± 0.74 µg/mg on days 48 and 49, respectively.

3.10. Neutralising antibodies

Table 1 shows the levels of neutralising antibodies produced by immunisation with the different diphtheria vaccine formulations.

In general, the mice immunised subcutaneously had a greater response of neutralising antibodies compared with the animals immunised orally. Formaldehyde treatment of CRM197 conjugated to MP resulted in a more efficient
neutralising antibody than its non-treated counterpart, whereas the result was the opposite for MPs conjugated with diphtheria toxin.

4. Discussion

The present study focused on the development of a new oral vaccine against diphtheria, using the biodegradable starch microparticles as adjuvant. Good immune responses were obtained in mice for each of the diphtheria antigens conjugated to the microparticles. The aims of the study included monitoring the humoral immune response generally and, specifically, to monitor the total amount of diphtheria toxin-specific IgG-IgM antibodies. Currently, vaccination against infection by C. diphtheriae is focused on the induction of specific neutralising antibodies against the toxin produced by the bacterium. National vaccination programmes aim at attaining antibody levels of 0.01–0.1 IU/ml, which are considered to be protective. However, a post-vaccination titre of ≥0.1 IU/ml is often quoted as a goal to be achieved. On the other hand, it is clear that previously well-immunised individuals with antitoxin levels below the minimal protective level have an immunological memory and can mount a rapid increase in antitoxin levels upon subsequent antigen challenge, either by natural infection or by vaccination.[16,17]

Since the introduction of routine immunisation against diphtheria in the 1940s and 1950s, the incidence of diphtheria has steadily declined in industrialised countries, leading to almost complete eradication of the disease. However, despite successful early childhood vaccination programmes, several studies in west-European countries have shown low levels of protecting antibodies against diphtheria in adults[18–21]. As a result of this generally poor immunity to the disease, there was a resurgence of diphtheria in some European countries in the mid-1980s and an epidemic in the former Soviet Union in the mid-1990s.[22–24]. This suggests that there is a great need for re-establishing good herd immunity in the population using booster vaccinations.

The availability of suitable, easily administered formulations of a diphtheria vaccine for this purpose would substantially contribute to the successful realisation of such programmes. Undoubtedly, an oral vaccine that induces a sufficient antitoxin response, with small or negligible adverse reaction potential and a high potential for compliance, would be an attractive alternative.

In this paper, we have shown that native MP-DT is a strongly immunogenic formulation. A strong systemic IgG-IgM response following oral vaccination was observed, as well as significant induction of a mucosal immune response in the form of s-IgA. The toxin-neutralising skin test performed in the rabbit according to a modification of Jensen[14] showed that the starch microparticles induced neutralising antibodies with the same potency as the non-formalinised vaccine. This could, however, be explained by the fact that mice given MP-DTbxd only received one booster, while mice given MP-DT received two. Treatment of the same vaccine with formaldehyde (MP-DTxd) on the contrary, rendered it non-toxic throughout the whole study period (42 days), with no signs of ill health in the guinea pigs. However, formaldehyde treatment somewhat decreased the amount of specifically induced IgG-IgM. In the Vero-cell assay[15] for detection of diphtheria toxin-specific neutralising antibodies, MP-DTbxd failed to induce as high a level of neutralising antibodies, 6–8 weeks after primary immunisation, as the non-formalised vaccine. This could, however, be explained by the fact that mice given MP-DTbxd only received one booster, while mice given MP-DT received two.

Because of the toxic potency of diphtheria toxin and the many regulatory requirements to avoid the risks for reversion to the toxic state when it is treated with formaldehyde, and because of the slightly lower immunogenicity of
MP-DTxd compared to MP-DT, our attention was drawn to the recombinant, genetically detoxified protein CRM197. Polyclaryl starch microparticles covalently conjugated with this protein (MP-CRM) and administered orally to mice induced specific IgG-IgM antibodies to a lower extent than the commercial diphtheria toxin vaccine given subcutaneously. CRM197 in its native form is described as rather poorly immunogenic compared to conventional diphtheria toxoid [25]. Consequently, the vaccine was treated with formaldehyde (MP-CRMxd) in order to stabilise it against potential degradation of gastric juices and enzymes. The MP-CRMxd vaccine gave rise to a substantially stronger humoral immune response and, furthermore, also induced specific mucosal s-IgA antibodies as well as toxin-neutralising antibodies at levels >0.1 IU/ml according to the Vero-cell assay, which the non-formalised MP-CRM vaccine failed to do. Several studies have also indicated that formaldehyde treatment stabilises the protein against proteolytic degradation [5,25–28]. Formaldehyde concentrations ranging from 0.06% (McNeela et al. [5]) to 0.7% (Porro et al. [27]) have been used to treat CRM197; these formaldehyde treated products have induced the same levels of antibodies after parenteral injection as conventional diphtheria toxoid. However, experience tells that formaldehyde treatment of diphtheria toxoid is a reaction that is not easily controlled, and which requires careful lot-to-lot evaluation of potency, toxicity and irreversibility. Formaldehyde treatment may furthermore alter immunogenic epitopes on the protein, resulting in a decrease of antigenicity [28]. The reaction is apparently delicate and obviously dependent on the type of protein to be formalinised and probably also on the adjuvant to be used. Further investigation of this matter is therefore needed before optimal reaction properties can be established for the formaldehyde treatment of CRM197-conjugated microparticles. Such studies have thus been initiated.

Some discrepancies between ELISA, Vero-cell assay and rabbit skin test results for the serum levels of diphtheria toxin-specific IgG-IgM antibodies were found. Generally, the ELISAs showed a correlation with the rabbit skin test in vivo assay. However, the correlation between the ELISA and Vero-cell assays, both with diphtheria toxin- and CRM197-conjugated microparticles, treated with formaldehyde or untreated, was poorer in the sense that the high titres measured with ELISA did not give the expected high level of neutralising antibodies in the Vero-cell assay. The reason for the differences is not apparent but, in the light of the methodological differences between the methods, only results obtained using the same method should be compared. Therefore, ELISA shows that MP-DT or is a more immunogenic vaccine compared to its formaldehyde-treated counterpart. When CRM197 was used, the relationship with its formaldehyde-treated counterpart was the opposite. The Vero-cell assay, on the other hand, showed a low level of neutralising antibodies, which was not at all expected in light of the high titres of the ELISA assay. However, the relative amounts of neutralising antibodies correlated well with results from ELISA, i.e. MP-CRMxd yielded a higher IgG-IgM response than untreated MP-CRM and, consequently, according to the Vero-cell assay, MP-CRMxd induced higher levels of neutralising antibodies. Recently published results on tetanus toxin regarding the correlation between ELISA and the Vero-cell assay, suggest that the lower values seen in the Vero-cell assay might be due to the presence of asymmetrical antibodies with uneven glycosylation of the Fab-region, which thereby should display different affinities for the two paratopes towards tetanus toxin [29]. This may also be the case with diphtheria toxin.

Regardless of asymmetry, the ELISA method counts every IgG and IgM antibody. (This, however, does not account for the discrepancy between the Vero-cell assay and the rabbit skin test. Discussion of this discrepancy is interesting but unfortunately beyond the scope of this article.) The Vero-cell assay is the preferred method for determining the efficacy of a vaccine against diphtheria and ultimately the results from this assay should be considered when debating the capacity of the different formulations to induce a sufficiently high neutralising antibody response. Nevertheless, the rabbit skin test and the ELISA performed here still gave good indications as to which formulation to proceed with.

Of note, a specific feature of the microparticles used orally was the high s-IgA titres identified in faeces of mice receiving either diphtheria toxin or CRM197 as antigens. No s-IgA was detected after s.c. injection. Similar results were obtained when human serum albumin was conjugated to the microparticles [8]. The relative specific IgA content obtained with MP-CRMxd corresponded to about 3–4.5 µg/mg total IgA. The relative role of such a mucosal antibody response in protection against a diphtheria infection is, unfortunately, not yet possible to evaluate. All regulatory requirements in the evaluation of the potency of diphtheria vaccines are focused on the diphtheria toxin-neutralising activity either in guinea pigs after parenteral injection or by the in vitro surrogate method based on the Vero cells. This situation may have arisen partly because of a lack of understanding of the pathogenicity of the diphtheria bacterium, in particular the uptake in vivo, and partly because of the focus on the toxicity of diphtheria toxin. Regrettably, this is a problem which is not unique for diphtheria vaccines but is applicable for the evaluation of the efficacy of most new vaccines, and particularly for those based on microparticles as carrier and adjuvant [30].

While our results with the oral MP-DT and MP-CRM vaccines in mice appear promising, the suitability of this new formulation can only be proven in formal clinical trials in humans. When selecting a formulation for a candidate vaccine, it is important to keep a favourable balance between efficacy, measured by the induction of toxin-neutralising antibodies, and any potential adverse effects.

From the results of the present study, the formaldehyde-treated polyclaryl starch microparticles conjugated with native diphtheria toxin seem to be an attractive option for a future oral vaccine. However, the consequences of the reg-
ulatory requirements for the future approval of this formulation as a medicinal product require attention. Strict, time-consuming (more than 3 months) and therefore expensive tests are required nowadays to show that the inactivation process of the diphtheria toxin is effective and stable. If these tests can be avoided by using an already genetically detoxified and highly reproducible protein of high purity, the time and costs involved in manufacturing such a vaccine would decrease significantly. The results obtained in mice are promising, with diphtheria toxin and CRM197 eliciting comparable immune responses. Considering the air-borne transmission of diphtheria, it may even be that the good specific mucosal IgA response, induced by the orally administered vaccines, may effectively prevent the invasion and dissemination of the bacteria in vivo and decrease the requirement for a humoral systemic response. Considering all these factors, the formulation based on formaldehyde-treated CRM197 conjugated to polyacryl starch microparticles seems to be the best candidate for future clinical trials. However, the kinetics of the formaldehyde treatment of CRM197 require thorough investigation such trials have been initiated in our laboratory.

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