Protective immunogenicity of two synthetic peptides selected from the amino acid sequence of Bordetella pertussis toxin subunit S1

(whooping cough/vaccine/ADP-ribosylation/leukocytosis)

PER ASKELÖF**, KARE RODMALM*, GÖRAN WRANGSELL*, ULF LARSSON*, STEFAN B. SVENSON*, JAMES L. COWELL†, ANDERS UNDÉN§, AND TAMAS BARTFAI§

*The National Bacteriological Laboratory, S-105 21 Stockholm, Sweden; †Praxis Biologies, Inc., Rochester, NY 14623-1493; and §Department of Biochemistry, Arhusenius Laboratory, University of Stockholm, 106 91 Stockholm, Sweden

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ABSTRACT Two peptides, corresponding to amino acids 1-17 and 169-186 of the amino acid sequence of pertussis toxin (PT) subunit S1, were synthesized and coupled to the diphtheria toxin cross-reactive mutant protein CRM 197 and evaluated for immunogenicity and protective capacity against PT challenge in vivo. The peptide-CRM conjugates induced high antibody titers against native toxin in mice (BALB/c, C57/Black, and outbred NMRI) as measured by ELISA. Upon PT challenge (0.5 µg of toxin) of the NMRI mice, the CRM conjugates of peptides 1-17 and 169-186 fully protected the mice from PT-induced leukocytosis. Immunization with the corresponding bovine serum albumin conjugates of these two peptides also fully protected mice. Rabbit antisera to the peptide 1-17-CRM conjugate was highly efficient in inhibiting the ADP-ribosylating activity of PT but did not neutralize the clumping effect of PT on Chinese hamster ovary cells. In contrast, the rabbit antisera raised against the peptide 169-186-CRM conjugate neutralized the clumping effect of PT on Chinese hamster ovary cells but did not inhibit the enzymatic activity of PT. Peptide 169-186-CRM conjugates mimic the immunoglobulin binding properties of PT and also cause clustering of Chinese hamster ovary cells. The CRM conjugates of these two peptides constitute a synthetic pertussis vaccine candidate with the ability to provide a chemically well-defined, safe, and efficient pertussis vaccine.

Most pertussis vaccines used today are of the whole cell type. Possible side effects of these vaccines, including serious effects on the central nervous system, have led to discontinuance of vaccination programs in some countries (1) and to drastically lowered compliance with vaccination programs in others (2, 3), resulting in pertussis epidemics in these countries. To eliminate side effects, acellular vaccines containing pertussis toxin (PT) inactivated with formalin (toxoid) and filamentous hemagglutinin were introduced in Japan (2). The protection rate was about 90% against severe disease for this type of vaccine as well as for a vaccine containing only PT during the follow-up of a phase 3 clinical trial in Sweden (4). The concept of pertussis as a toxin-mediated disease (5) is thus correct.

The S1 subunit of the oligomeric PT is immunodominant (6, 7) and also possesses the enzymatic ADP-ribosylating activity of PT (8). Therefore our peptide epitopes were selected from the S1 subunit. Upon cloning and sequencing the S1 subunit (9) we identified by use of synthetic peptides and convalescent serum several peptide sequences that contain linear B- and T-cell epitopes. We found five peptides (15-22 amino acids long) that contain B-cell epitopes for humans and animals as determined by their reaction in ELISA with antiserum raised against either whole cell vaccine or pertussis toxoid vaccine (10). Antibodies elicited in mice against each of these five peptides as well as against a sixth peptide from the S1 sequence bound to native PT (11). Here we report on the antigenic and protective capacity of a pertussis vaccine candidate based on two of these peptides conjugated to the diphtheria toxin cross-reactive mutant protein CRM 197 (12).

MATERIALS AND METHODS

Materials. Nontoxic mutant CRM diphtheria toxin was from Praxis (Rochester, NY). Crystalline pyrogen-free bovine serum albumin (BSA) (Pentex lot 23) was purchased from Miles. Bromoacetic acid N-hydroxysuccinimide ester was purchased from Sigma. All other chemicals were of pro analysis grade.

Synthesis of Peptides. Peptides were synthesized manually on a p-methylenbenzyldryamine resin (0.35 mmol/g). Protected tert-butycarbonyl (t-Boc) amino acids were aspartic acid [benzyl oxy carbonyl (OBzl)], glutamic acid (OBzl), serine [benzyl (Bzl)], threonine (Bzl), cysteine (4-Me-Bzl), arginine (tosyl), lysine (2-chlorobenzyloxycarbonyl), tyrosine (2-bromobenzylxoycarbonyl), methionine (sulfoxide), and histidine (dimtrophenyl). Asparagine, glutamine, arginine, and methionine were coupled as active hydroxybenzotriazol esters. All other amino acids were activated by dicyclohexylcarbodiimide in a molar ratio of 1:1. The protocol for stepwise solid-phase synthesis using the t-Boc-benzyl protective group strategy has been published elsewhere (13). Deprotection was carried out by low trifluromethanesulfonic acetic acid/dimethyl sulfide (14); the resin was washed with trifluoroacetic acid (two times 1 min), dichloromethane (three times 1 min), dimethyl formamide (three times 1 min), ethanol (two times 1 min), and diethyl ether (one time 1 min). The resin was dried under vacuum followed by final deprotection by HF containing 5% p-cresol and 5% p-thiocresol at 0°C for 40 min. Purification of the cleaved peptides was carried out by HPLC on a C18 reversed-phase column.

Preparation of Peptide-Protein Conjugates. (i) Activation of carrier proteins with bromoacetic acid N-hydroxysucinimide ester: Fifteen milligrams of bromoacetic acid N-hydroxysuccinimide ester was added to 20 mg of CRM or BSA in 4 ml of 10 mM phosphate buffer (pH 7.0) containing 0.11 M NaCl, and the reaction mixture was agitated gently for 1 hr at 20°C. The reaction mixture was then chromatographed on a Bio-Gel P2 column (1.5 cm x 20 cm) irrigated with 0.1 M NaHCO3 (pH 8.0) buffer, and the fractions containing the modified protein were pooled and immediately used for coupling of the peptides. (ii) Coupling of cysteine-containing...

Abbreviations: PT, pertussis toxin; CRM, diphtheria toxin cross-reactive mutant 197; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

†To whom reprint requests should be addressed.
peptides to activated carrier proteins: The peptide was dissolved in dimethyl sulfoxide/water (1:1) under a gentle stream of N₂. The peptide solution was then, under N₂, added slowly to the activated carrier protein solution, which previously had been gently bubbled with N₂, and the reaction was allowed to proceed under gentle agitation for 2 hr at room temperature. The reaction mixture was then extensively dialyzed against 10 mM phosphate buffer with 0.14 M NaCl (PBS) containing 0.05% 1-butanol and finally lyophilized.

**Immunization of Rabbits.** New Zealand White rabbits were immunized at days 0, 14, and 28 in the popliteal lymph nodes with 50 μg of the respective peptide-CRM conjugate per lymph node. The conjugates were suspended in Freund's complete adjuvant (1:1). Blood samples were drawn at days 0 and 28.

**Immunization of Mice.** Groups of five NMRI (C57/Black, BALB/c) mice were immunized i.p. at days 0, 14, and 28 with 10 μg of the respective peptide conjugate suspended in Freund's complete adjuvant (1:1). Mice were bled at days 1 and 35.

**ELISA.** The ELISA was performed essentially as described (10). Briefly, microtiter plates (Nunc) were coated with PT (2 μg/ml). When antibodies against peptide 1-17 and the carrier protein CRM were analyzed, a concentration of 10 μg of respective antigen per ml was used for coating of the plates. Alkaline phosphatase-labeled goat anti-rabbit IgG and goat anti-mouse IgG (Sigma) were used for developing the ELISA. The enzyme conjugates were used in a 1:500 dilution. ELISA titers are expressed as the 405-nm absorbance value of the serum dilution, giving an absorbance between 0.2 and 0.7 after 60 min multiplied by the dilution factor.

**ADP-Ribosylation Assay.** The assay was carried out with purified bovine transducin as substrate. It was performed essentially as described by Manning et al. (15). The incubation mixture (0.1 ml) contained PT (50 ng/ml), preactivated by incubation for 20 min at 37°C in 20 mM dithiothreitol, dithiothreitol (1 mM), [³²P]NAD (10 μCi) (28 Ci/mmole; 1 Ci = 37 GBq) (NEN), sodium acetate (0.1 M), ATP (1.0 mM), GTP (1.0 mM), lysophosphatidylcholine (0.1 mg/ml), and transducin (10 μg/ml). After incubation for 80 min at 37°C the reaction was stopped by the addition of 10 μl of trichloroacetic acid. The precipitate was collected and dissolved in SDS and separated in SDS/PAGE. The rabbit antisera to peptide conjugates (10 μl of a 1:5 dilution) were incubated with PT for 60 min at 37°C prior to the start of the ADP-ribosylation assay. The ability of antisera to neutralize the ADP-ribosyltransferase activity was detected by a decrease in absorbance of the radiolabeled protein band corresponding to [³²P]ADP-ribosylated α subunit of transducin in the autoradiography.

**PT Challenge.** The immunized NMRI mice were injected i.p. with 0.5 μg of PT (1/10 of LD₅₀) dissolved in 0.2 ml of PBS. On day 3 blood was drawn and the number of circulating leukocytes was counted.

**Chinese Hamster Ovary (CHO) Cell Culture Assay.** The inhibitory effect of antisera to peptide-CRM and peptide-BSA conjugates on the in vitro clustering effect of PT on CHO cells was determined as described (16).

**RESULTS**

**Synthesis of Peptide-CRM and -BSA Conjugates.** Two peptides corresponding to amino acid residues 1-17 (Asp-Asp-Pro-Pro-Ala-Thr-Val-Tyr-Arg-Tyr-Asp-Pro-Pro-Asp-As) and 169-186 (Gln-Gln-Thr-Arg-Ala-Asp-Pro-Asp-Pro-Tyr-Thr-Arg-Ser-Arg-Ser-Val-Ala-Val) were used in the sequence of the S1 subunit of PT were coupled to CRM and to BSA. The apparent molecular weights of the conjugates show that 5-25 μg of peptide could be coupled to each carrier molecule as determined by SDS/PAGE (data not shown). The devised coupling method allows control of the number of peptides being attached to each carrier molecule and avoids polymerization of the peptide and of the carrier.

**Immunochromatography Characterization of Peptide-CRM and -BSA Conjugates.** The coating curves obtained in ELISA using the peptide-protein conjugates and PT were compared with a rabbit hyperimmune serum raised against pertussis toxoid. Peptide 1-17-CRM (and BSA) conjugate was 10 times more effective in weight basis in binding antibodies than the purified PT itself (Fig. 1). The peptide 169-186-CRM and -BSA conjugates bind the goat anti-rabbit enzyme conjugate used for developing the ELISA, and the exclusion of the rabbit hyperimmune serum in the ELISA and titration against the enzyme conjugate alone result in indistinguishable coating curves (Fig. 1). The peptide 169-186 conjugates bind strongly to various immunoglobulins at a site(s) distinct from the antigen binding site, mimicking the immunoglobulin binding property of PT (data not shown).

![Fig. 1. Coating curves in ELISA for peptide-CRM and peptide-BSA conjugates and PT. The peptide conjugates and PT were evaluated with a 1:500 dilution of a rabbit hyperimmune serum raised against pertussis toxoid. P1 and P2 denote peptides 1-17 and 169-186, respectively. OAb depicts the reaction obtained in the absence of the rabbit hyperimmune serum between peptide conjugate and the alkaline phosphatase-labeled goat anti-rabbit IgG used for developing the ELISA. The enzyme conjugate was used in a 1:500 dilution and the absorbance was read after 30 min.](image-url)
Immunological Characterization of Peptide-CRM Conjugates in Mice. The antibody response in mice immunized with the peptide-protein conjugates was evaluated against native PT, and the respective peptides and carrier proteins were evaluated by ELISA. Both peptide-CRM conjugates were highly immunogenic in each individual in all tested strains of mice: C57/Black, BALB/c, and outbred NMRI. The highest titers were found in the outbred NMRI mice (Table 1).

Immunological Characterization of Peptide-CRM Conjugates in Rabbits. The peptide 1–17-CRM and the peptide 169–186-CRM conjugates were evaluated for immunogenicity in New Zealand White rabbits. The rabbits responded with high titers (Table 1). For comparison, the titers obtained with serum from a rabbit hyperimmunized with pertussis toxoid are included.

Rabbit Anti-Peptide-1–17 Serum Blocks the Enzymatic Activity of PT. The rabbit antiserum against peptide 1–17-CRM conjugate (dilution 1:50) completely inhibited the ADP-ribosylation of bovine transducin by PT (50 ng/ml) (Fig. 2). The rabbit antiserum against peptide 169–186-CRM conjugate did not affect the catalytic activity of PT in the ADP-ribosylation assay with transducin as substrate (data not shown).

Rabbit Anti-Peptide-169–186 Serum Blocks the CHO-Cell Clustering by PT. The rabbit antiserum against peptide 169–186-CRM conjugate in dilution 1:8 completely neutralized the CHO-cell clustering caused by PT (50 ng/ml). In contrast, the antiserum against peptide 1–17-CRM conjugate did not neutralize this effect of PT (data not shown).

Protection Against PT Challenge in Mice. In vivo challenge with 0.5 μg of purified PT administered i.p. was carried out in NMRI mice immunized three times with CRM conjugate of peptide 1–17 or with CRM conjugate of peptide 169–186 (10 μg). The leukocyte counts taken 3 days after challenge showed that each of the peptide conjugates afforded full protection of mice against leukocytosis (Fig. 3). The BSA conjugates of peptide 1–17 and of peptide 169–186 also protected NMRI mice against PT challenge (0.5 μg) (data not shown).

Table 1. Antibody response against PT, peptides, and carrier protein in mice and rabbits immunized with peptide-CRM conjugates or pertussis toxoid as measured by ELISA or neutralization of diphtheria toxin

<table>
<thead>
<tr>
<th>Peptide-CRM conjugate†</th>
<th>Coating antigen*</th>
<th>Mouse serum</th>
<th>PT</th>
<th>Peptide-BSA conjugate</th>
<th>CRM‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1–17</td>
<td>Mean</td>
<td>1,602</td>
<td>10,120</td>
<td>16,340</td>
<td></td>
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<tr>
<td>Range</td>
<td>440–3,300</td>
<td>5,100–24,000</td>
<td>4,200–31,500</td>
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<td></td>
</tr>
<tr>
<td>Peptide 169–186</td>
<td>Mean</td>
<td>4,042</td>
<td>ND§</td>
<td>4,400</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>510–14,000</td>
<td>2,800–7,200</td>
<td></td>
<td></td>
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<tr>
<td>Rabbit serum</td>
<td>Peptide 1–17</td>
<td>42,496</td>
<td>94,000</td>
<td>&gt;1.4 IU/ml</td>
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</tr>
<tr>
<td>Peptide 169–186</td>
<td>10,138</td>
<td>ND§</td>
<td>&gt;1.4 IU/ml</td>
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<tr>
<td>Toxoid‡</td>
<td>122,400</td>
<td>2,452</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All samples were analyzed in triplicates. ND, not determined.

*Values are expressed as titers. Data are from five mice and one rabbit for each of the peptide-CRM conjugates.

†Immunizing antigen.

‡Values for rabbit serum are expressed as international units (IU)/ml and were obtained by comparing the diphtheria toxin neutralizing capability of the serum sample to that of an international standard for diphtheria vaccines (World Health Organization) by the rabbit skin test as prescribed in the European Pharmacopoeia.

§Antibodies to peptide 169–186-CRM or -BSA conjugates could not be evaluated due to the strong binding of immunoglobulins to these peptide conjugates.

†This serum was obtained by hyperimmunization with the one-component vaccine used in the Swedish field trial (4). The CHO-cell titer of the serum is 8000.

**DISCUSSION**

Monoclonal antibodies with neutralizing activity and site-directed mutagenesis were utilized by several groups to identify the amino acid residues involved in the ADP-ribosylating activity of PT and to produce immunogenic molecules devoid of enzymatic activity (17–21). It is clear however that the functional groups of the toxin being altered by these procedures frequently are involved in immunogenicity and enzyme activity (17).

We present here a different approach based on mapping relevant epitopes of PT by use of synthetic peptide antigens. Two peptide epitopes from the enzymatic subunit S1 of PT were conjugated to CRM 197 and tested for immunogenicity and efficacy by in vivo challenge with PT. Peptide 1–17 represents the N terminus of the S1, and this domain of the toxin molecule has been implicated in the catalytic activity of PT by monoclonal antibody mapping, which showed that amino acid residues 7–15 are essential for the binding of neutralizing antibodies (22–24). The other peptide epitope, peptide 169–186, is located close to the C terminus of S1, the region in cholera toxin that is thought to be involved in NAD+ binding (25). We have selected these peptides and four additional peptides as functional epitopes recognized by infant convalescent serum and by animal hyperimmune serum raised against PT (10).

Conjugation of the peptides by way of the thiol group of a cysteine residue added to the C terminus of the peptides (and not belonging to the PT sequence) to NH2 groups of the CRM molecule provided uniquely attached peptides to the antigenic carrier protein, since the only thiol groups present on peptides were that of the C terminal cysteine. By the present procedure crosslinking of CRM molecules could be avoided as well as crosslinking of the peptides.

The two peptide-CRM conjugates were antigenic in mice and rabbits (Table 1), as shown in ELISA with native toxin and the BSA conjugates of the peptides as coating antigens. The strong antibody binding properties of 169–186-BSA or 169–186-CRM conjugate exclude the possibility of using
these as coating antigens even at dilute solution, such as that that is possible for PT itself. The avidity of the binding of these peptide conjugates to antibodies is in the same range as that noted for biotin-avidin interactions (unpublished results).

Rabbit antiserum against 1–17-CRM conjugate inhibited the ADP-ribosylation by PT in the presence of saturating concentrations of the substrates NAD\(^+\) and transducin (Fig. 2). This result is in agreement with studies showing that mutations in this N-terminal region inhibit the enzymatic activity as well as binding of neutralizing antibodies (22-24). All neutralizing antibodies directed against S1 and thus far published show a requirement for an intact N-terminal region of S1 for their binding.

In accordance with the suggestion that pertussis is a toxin-mediated disease (5) where protection could be achieved by neutralization of the enzymic activity of PT, the peptide 1–17-CRM conjugate proved to act as protective antigen in mice against PT challenge (Fig. 3). It is noteworthy that mice even with low titers (e.g., 440) against PT were fully protected in the leukocytosis test.

The 169–186-CRM conjugate was also strongly immunogenic in mice and rabbits. The rabbit antiserum inhibited the CHO-cell aggregation caused by PT (50 ng/ml). The same CRM conjugate proved as protective antigen in mice when challenged with 0.5 \(\mu\)g of PT (Fig. 3), despite its lack of inhibitory effect on the enzymatic activity of PT toward transducin. A possible explanation would be that antibodies to 169–186-CRM conjugate with their high avidity may prevent the attachment of pertussis holotoxin oligomer to cells and the subsequent release of the enzymatic subunit. This early prevention of ADP-ribosylation is not observed, however, in the in vitro assay used here, where a soluble substrate transducin, is present (Fig. 2).

The in vivo challenge with PT applied here uses a dose that gives a 2- to 5-fold increase in leukocyte counts and the measured response, leukocytosis, is a prominent feature of the clinical disease. Protection against leukocytosis thus represents a valuable indicator of the protective efficacy of the peptide-CRM conjugates. The applied carrier CRM is not only a well-defined, pure, immunogenic carrier protein but in itself is also a useful immunogen against diphtheria in a future diphtheria/tetanus/pertussis vaccine. To the best of our knowledge, in vivo protection against PT by a vaccine based on the immunodominant enzymatic subunit S1 of the toxin alone has not been reported previously. Experiments using recombinant S1 have not shown any protective immunogenicity thus far (26, 27).

In summary, we have identified two peptide sequences from the immunodominant enzymic subunit of S1 of PT that can elicit protective antibody response by different mechanisms. Vaccines based on synthetic peptides have been developed for influenza virus (28), group A streptococci (29), and foot-and-mouth disease virus (30, 31). The identification of peptide conjugates as described here may represent a first step in the development of a chemically well-defined, safe, and efficacious pertussis vaccine.

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