Construction of a Diphtheria Toxin A Fragment-C180 Peptide Fusion Protein Which Elicits a Neutralizing Antibody Response against Diphtheria Toxin and Pertussis Toxin

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A genetically engineered gene fusion was constructed which encoded a nontoxic derivative of the A fragment of diphtheria toxin joined to the C180 peptide of the S1 subunit of pertussis toxin. The product of this gene fusion, termed the DTA-C180 protein, was purified from the periplasm of Escherichia coli to approximately 80% purity. The DTA-C180 protein possessed an apparent molecular weight of 43,000 by reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The DTA-C180 protein was cleaved into two tryptic peptides, which migrated with apparent molecular weights of approximately 22,000. One tryptic peptide reacted with diphtheria antitoxin, while the other tryptic peptide reacted with anti-C180 peptide immunoglobulin G. The DTA-C180 protein did not inhibit protein synthesis or stimulate clustering morphology in Chinese hamster ovary cells. The DTA-C180 protein elicited an immune response, in guinea pigs, against both the DTA and C180 peptide components of the fusion protein, with alum being a more efficient adjuvant than Freund's adjuvant for eliciting neutralization titers. Neutralization titers elicited by DTA-C180 protein were weaker than those elicited by diphtheria toxoid and pertussis toxin 9K/129G, a genetically engineered double mutant of pertussis toxin. Three doses of DTA-C180 protein yielded a neutralization titer of 1/750 against pertussis toxin in Chinese hamster ovary cells and a neutralization titer of 1/50 against diphtheria toxin in Vero cells. This is the first report of a protein derived from a recombinant S1 subunit that elicits a neutralizing titer against pertussis toxin.

Bordetella pertussis produces extracellular and cell-associated factors which contribute to its pathogenesis (28). One of these virulence factors, pertussis toxin (PT), has also been shown to elicit a protective immune response, in animals, against both intracerebral and aerosol *B. pertussis* challenge (25). PT is a hexameric protein composed of six subunits, designated S1, S2, S3, S4, and S5, which are organized noncovalently with 1:1:1:2:1 stoichiometry, respectively (27). S1 catalyzes the ADP-ribosylation of the α subunit of selected G proteins, while the remaining five subunits (termed the B oligomer) are responsible in vivo for the delivery of S1 to the G protein.

The whole-cell pertussis vaccine, while effective, has been implicated as the source of acute reactions after immunization; this has been outlined in a recent document from the Centers for Disease Control (6). In an attempt to generate an effective but less-reactive pertussis vaccine, several acellular pertussis vaccines have been produced (for a summary, see reference 23). In Japan, an acellular pertussis vaccine, composed of a partially purified and detoxified culture supernatant fluid of *B. pertussis*, is used as an immunogen (26). The Centers for Disease Control has recommended that an acellular pertussis vaccine be used interchangeably with the whole-cell vaccine for the fourth or fifth dose of the routine series of vaccinations against pertussis (6).

Recently, more defined acellular pertussis vaccine candidates have been engineered which possess double mutations within the S1 subunit of PT (16, 23). For example, PT9K/

129G does not express detectable ADP-ribosyltransferase or cytotoxic activities but retains the ability to elicit a protective immune response, in animals, against challenge by *B. pertussis* (23). These genetically engineered pertussis toxoids, alone or with other immunogens of *Bordetella*, may prove to be effective acellular pertussis vaccines.

Future acellular pertussis vaccines may be composed of immunogens which consist of defined pertussis epitopes. Sato and coworkers (24) showed that the monoclonal antibody 1B7, which recognized an epitope on the S1 subunit of PT, conferred passive protection to mice against challenge by *B. pertussis*. Subsequent studies localized the 1B7 epitope to the amino-terminal region of S1 (4, 7, 22). These results indicated that the S1 subunit might be a candidate as a defined pertussis immunogen. However, the S1 subunit, as either a native protein or fusion protein, failed to elicit neutralizing antibody titers against PT in mice, rabbits, or guinea pigs regardless of the dose of antigen and adjuvant used (20). Unsuccessful attempts to elicit a protective immune response to S1 in guinea pigs followed immunization schedules which included intradermal administrations of between 20 and 300 μg of immunogen alone or with alum or Freund's adjuvant (23a).

In this study, a genetically engineered gene fusion has been constructed which encodes a nontoxic derivative of the A chain of diphtheria toxin (DT) joined to the C180 peptide of the S1 subunit of PT. The C180 peptide is a genetically engineered deletion peptide composed of the first 180 amino-terminal amino acids of the S1 subunit which includes the 1B7 epitope (2). This gene fusion, termed the DTA-C180 protein, is stable when expressed in *Escherichia coli* and immunogenic in guinea pigs, and anti-DTA-C180 protein sera neutralize the cytotoxic activities of both DT and PT.

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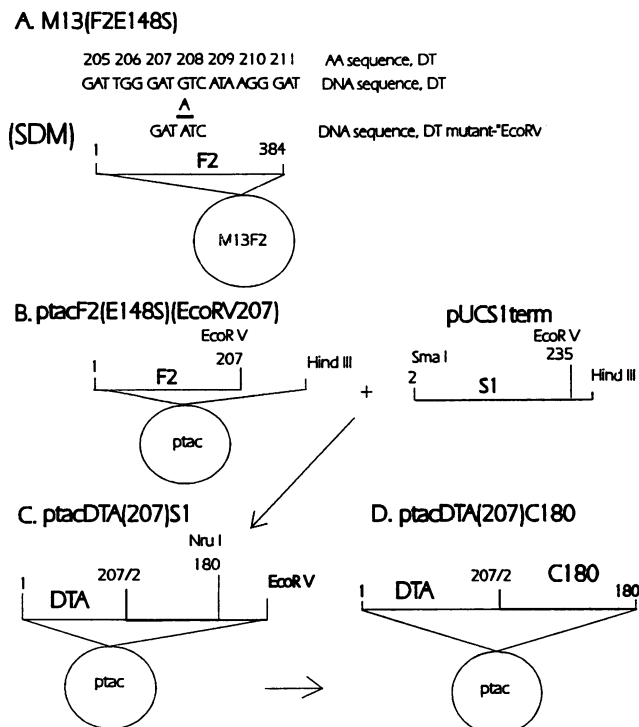† Present address: Department of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226.

FIG. 1. Construction of ptacDTA(207)C180. ptacDTA(207)C180 was constructed in four steps, A through D. Details of this construction are described in Materials and Methods.

MATERIALS AND METHODS

Materials. [Adenylate ^{32}P-phosphate]NAD was purchased from Dupont. Trypsin, trypsin inhibitor, and rabbit anti-horse immunoglobulin G (IgG) were from Sigma.

Construction of the ptacDTA(207)C180 vector. ptacDTA (207)C180 was engineered in four steps (Fig. 1).

(i) **Site-directed mutagenesis of M13F2(E148S).** The *Eco*RI-*Hin*dIII fragment of ptacF2(E148S) (1a) was subcloned into the respective restriction sites of M13mp18, yielding M13F2 (E148S). F2 refers to a 5' fragment of the DT structural gene which has been described previously (1a). Single-stranded DNA of M13F2(E148S) was subjected to site-directed mutagenesis (1a) to introduce a single base substitution (G to A) at the first nucleotide of codon 208 of the F2 gene, yielding M13F2(E148S)(*Eco*RV207). An antisense oligonucleotide, 5' ATC CCT TAT GAT ATC CCA ATC 3' (the mutated base is in boldface), was used to generate the G-to-A substitution. This mutation introduced a unique *Eco*RV restriction site within the DNA-encoding residues 207 and 208 of the F2 gene.

(ii) **Construction of ptacF2(E148S)(*Eco*RV207).** The *Bam*HI-*Hin*dIII fragment from the replicative form of M13F2(E148S)(*Eco*RV207) was subcloned into the respective sites of ptac18 (12), yielding ptacF2(E148S)(*Eco*RV207).

(iii) **Construction of ptacDTA(207)S1.** A *Sma*I-*Hin*dIII fragment from pUCS1term (2), which encoded DNA for a Gly residue followed in frame by residues 2 through 235 of the S1 subunit of PT, was subcloned into the *Eco*RV-*Hin*dIII restriction sites of ptacF2(E148S)(*Eco*RV), yielding ptacDTA(207)S1.

(iv) **Construction of ptacDTA(207)C180.** ptacDTA(207)S1 was digested with *Nru*I and *Eco*RV, ligated, and transformed into *E. coli* TG1. Several transformants were identi-

fied which had *Sal*I-*Hin*dIII fragments approximately 165 bases smaller than the *Sal*I-*Hin*dIII fragment of ptacDTA (207)S1; one of these transformants was termed ptacDTA (207)C180. The junction between the DTA-C180 components of the gene fusion was sequenced to confirm the correct nucleotide sequence. The gene product of ptacDTA(207) C180 was termed the DTA-C180 protein.

Expression and purification of the DTA-C180 protein. Three liters of L broth was inoculated with a 1/100 dilution of an overnight culture of *E. coli* TG1/ptacDTA(207)C180. After 2 h of incubation with shaking at 37°C, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture (A_{595}, 0.1) and the incubation was continued for an additional 4 h (A_{595}, 0.5). Cells were concentrated by centrifugation at 6,000 × g for 10 min. Cell pellets were resuspended in 25 mM Tris-HCl (pH 8.0) containing 30% sucrose (100 ml) and incubated with 8 ml of 8.0 mg of lysozyme per ml in 0.1 M EDTA on ice for 40 min. At this time, the periplasm was separated from cellular material by centrifugation at 8,000 × g for 50 min at 4°C. The periplasm was precipitated with ammonium sulfate (final concentration, 50%). The 50% ammonium sulfate precipitate was resuspended in 25 ml of 25 mM Tris-HCl (pH 7.6) and chromatographed on Sephacryl S-200HR resin (550 ml of resin equilibrated in 25 mM Tris-HCl [pH 7.6]). Fractions from the S-200HR chromatography, which contained the DTA-C180 protein, were pooled and chromatographed on DEAE-Sephacel resin (6 ml of resin equilibrated in 25 mM Tris-HCl [pH 7.6]). The column was washed sequentially with 15-ml volumes of 25 mM Tris-HCl (pH 7.6) containing 50, 100, 150, and 200 mM NaCl. Fractions from the 150 mM NaCl wash, which contained the majority of the DTA-C180 protein, were pooled and precipitated with ammonium sulfate (final concentration, 50%). Under these conditions, about 1 mg of DTA-C180 protein was purified per 1 liter of cells.

Trypsin digestion of the DTA-C180 protein. Reaction mixtures (20 μl) contained 0.1 M Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 0.5 μg of DTA-C180 protein, and 0.5 ng of trypsin. At the indicated times, digestions were terminated by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, with or without β-mercaptoethanol, and boiling for 5 min. The tryptic digests were subjected to SDS–13.5% PAGE. Gels were analyzed by Coomassie blue staining or subjected to Western blotting (immunoblotting). Western blots were probed with either rabbit anti-C180 peptide IgG (3) or horse diphtheria antitoxin (Connaught Laboratories). When probed with diphtheria antitoxin, the blots were incubated with a secondary antibody, rabbit anti-horse IgG. Blots were then probed with ^{125}I-labeled protein A and subjected to autoradiography. When appropriate, authentic C180 peptide or DT was included in the Western blot analysis.

DEAE chromatography of trypsin-digested DTA-C180 protein. Reaction mixtures (2 ml) contained 0.1 M Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 100 μg of DTA-C180 protein, and 100 ng of trypsin. After 45 min, 200 ng of trypsin inhibitor and 20 mM dithiothreitol (DTT) were added. This tryptic digest was chromatographed on DEAE-Sephacel resin (1 ml of resin equilibrated in 25 mM Tris-HCl [pH 7.6]–20 mM DTT). The resin was washed with a stepwise gradient of NaCl (2-ml aliquots of 25 mM Tris-HCl [pH 7.6]–20 mM DTT containing 25 through 150 mM NaCl, in 25 mM increments).

Cytotoxic activity of DTA-C180 protein. Chinese hamster ovary (CHO) cells were cultured in HAM's F12 medium supplemented with 10% newborn calf serum. DT, PT, and

DTA-C180 protein were serially diluted in 25 mM Tris-HCl (pH 7.6) containing 1.0 mg of egg albumin per ml and assayed for biological activity.

(i) **Inhibition of protein synthesis (1a).** Confluent lawns of CHO cells were incubated in 1.0 ml of fresh culture media containing the indicated concentrations of DT or DTA-C180 protein. After 3 h, media were removed and cells were incubated in 0.5 ml of fresh media containing 2 μCi of [35S]methionine per ml. After 5 h, media were removed and cells were washed twice with cold 7.5% trichloroacetic acid (TCA) and then once with phosphate-buffered saline. Cells were dissolved in 0.4 ml of 0.1 N NaOH. Incorporation of [35S]methionine into TCA-precipitable material was determined by liquid scintillation counting.

(ii) **Stimulation of CHO cell clustering (14).** One milliliter of 10^5 CHO cells was incubated with the indicated concentrations of PT or DTA-C180 protein. After 18 to 24 h, cell morphology was examined by using a light microscope. The minimum concentration required to elicit a clustering response was defined as the protein concentration required to cluster approximately 50% of the cells. Cell clustering was scored in 33% increments of the number of cells exhibiting a clustered morphology.

ADP-ribosylation of transducin (9). Reaction mixtures (125 μl) contained 0.1 M Tris-HCl (pH 7.6), 20 mM DTT, 1 μM [adenylate ^{32}P-phosphate]NAD (specific activity, 2.5 × 10^3 cpm per 1 pmol of NAD), 0.1 μM transducin, and either C180 peptide or DTA-C180 protein. At timed intervals, 20 μl of the reaction mixture was added to 6 μl of SDS-PAGE sample buffer containing β-mercaptoethanol and boiled for 5 min. Samples were subjected to SDS-10% PAGE. Incorporation of radiolabel was determined by scintillation counting of the band corresponding to the α subunit of transducin. Linear velocity for the ADP-ribosylation of transducin was defined as the millimoles of ADP ribose incorporated into the α subunit of transducin per minute per mole of enzyme.

Guinea pig immunization and antiserum testing. The immunogenicity of the DTA-C180 protein was tested in guinea pigs, an animal model used to test the immunogenicity of diphtheria toxoids (5) and pertussis vaccines (17). Two immunization schemes were used. In one case, 0.5 ml of phosphate-buffered saline containing 50 μg of the DTA-C180 protein was mixed with 0.5 ml of complete Freund's adjuvant for the first dose and incomplete Freund's adjuvant for subsequent doses. In the second immunization scheme, 50 μg of the DTA-C180 protein was mixed with 1 mg of aluminum hydroxide in 1 ml of phosphate-buffered saline. Three guinea pigs were immunized for each scheme. Immunizations were given at 0, 28, and 93 days. Serum samples were taken at days 28, 42, and 109. The antisera obtained were tested for their ability to (i) recognize PT and DT in Western blotting, by using 1 μg of target protein per lane, and (ii) neutralize the toxic effect of PT and DT in vitro by the CHO cell (14) and Vero cell (18) assays, respectively, as described previously (17). Serial dilutions of the antisera were tested for the ability to neutralize the toxic effects of 25 pg of PT per ml and 45 pg of DT per ml in microtiter plates.

RESULTS

Construction of ptacDTA(207)C180. The construction of ptacDTA(207)C180 is outlined in Fig. 1 and described in Materials and Methods. The ptacDTA(207)C180 vector produces a fusion protein, termed the DTA-C180 protein, composed of amino acids 1 through 207 of the A chain of DT (E148S), glycine, amino acids 2 through 180 of the C180
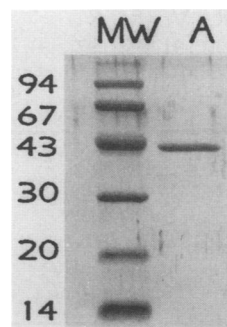

FIG. 2. SDS-PAGE of the DTA-C180 protein. Purified DTA-C180 protein (lane A) and protein standards (lane MW) (molecular masses, in kilodaltons, are shown to the left of the panel) were subjected to reduced SDS–13.5% PAGE. The Coomassie blue-stained gel is shown.

peptide of the S1 subunit of PT, and isoleucine (in that order). The glycine and isoleucine residues, at the junctions between the DTA and C180 peptide sequences and at the carboxyl terminus of the fusion protein, respectively, are products of the engineering protocol. The engineered *Eco*RV restriction site, within the DNA-encoding residues 207 and 208 of the A fragment of DT of ptacF2(E148S)(*Eco*RV207), allows convenient insertion of heterologous DNA downstream of the DTA domain.

Purification of the DTA-C180 protein. The DTA-C180 protein was purified from the periplasm of *E. coli* TG1/ptacDTA(207)C180, by using a three-step procedure: ammonium sulfate precipitation, gel exclusion chromatography, and, finally, DEAE chromatography. The purity of the DTA-C180 protein was estimated by densitometry to be approximately 80% (Fig. 2). The DTA-C180 protein migrated by reduced SDS-PAGE with an apparent molecular mass of 43 kDa, similar to its predicted molecular mass, which indicated that the trypsin-sensitive region (R190, V191, R192, and R193) within the DTA portion of the DTA-C180 protein was intact (Fig. 3A, oxidized and reduced).

Susceptibility of the DTA-C180 protein to trypsin. Reduced SDS-PAGE of tryptic digests of the DTA-C180 protein yielded two stable peptides (Fig. 3B, reduced). Both tryptic peptides migrated with apparent molecular masses of approximately 22 kDa. In contrast, nonreduced SDS-PAGE of tryptic digests of the DTA-C180 protein yielded a single protein with an apparent molecular weight identical to the unnicked DTA-C180 protein (Fig. 3B, oxidized). Thus, it appeared that the trypsin-sensitive region within the DTA portion of the DTA-C180 protein, i.e., R190, V191, R192, and R193, was preferentially cleaved by trypsin and that the disulfide bond of C186 and C201 had formed within the DTA portion of the DTA-C180 protein. We cannot rule out the possibility that some DTA-C180 protein has formed a disulfide bond between C41 of the C180 peptide and C186 of DTA.

The identities of the two tryptic peptides of the DTA-C180 protein were determined by measuring their antigenicity to either anti-C180 peptide IgG or diphtheria antitoxin. A tryptic digest of the DTA-C180 protein was subjected to reduced SDS-PAGE, and the gels were stained with Coomassie blue (Fig. 4, protein) or transferred to nitrocellulose and probed with either anti-C180 peptide IgG (Fig. 4, anti-C180) or diphtheria antitoxin (Fig. 4, anti-DT). Each tryptic peptide reacted with only one antiserum, with the
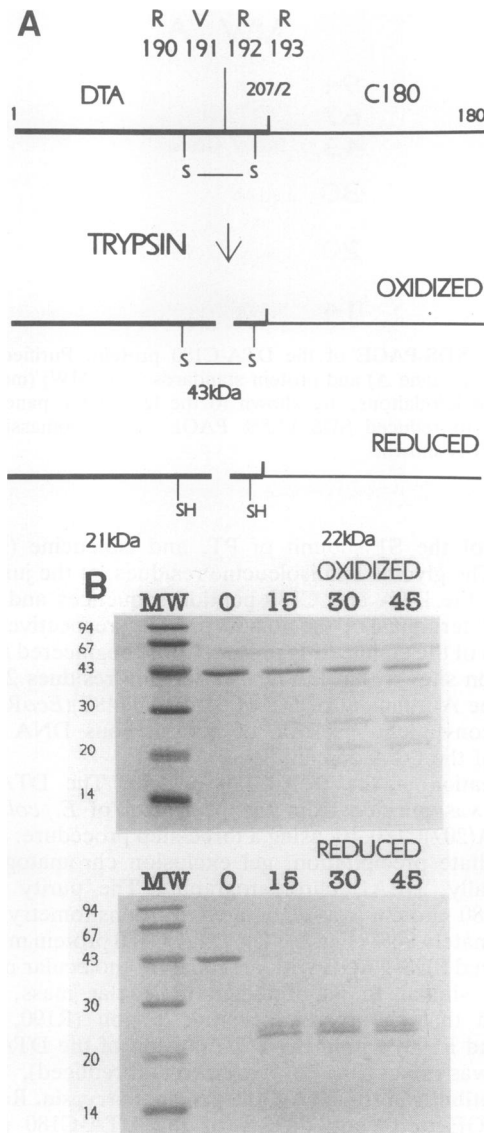
FIG. 3. Trypsin digestion of DTA-C180 protein. (A) Preferential cleavage of the trypsin-sensitive region of the DTA-C180 protein (R190, V191, R192, and R193) would yield a 43-kDa protein by nonreduced SDS-PAGE (oxidized) and two peptides of 21 and 22 kDa by reduced SDS-PAGE (reduced). (B) Twenty-five micrograms of DTA-C180 protein per ml was incubated with 25 ng of trypsin per ml in 0.1 M Tris-HCl (pH 8.0) containing 2 mM CaCl₂. At the indicated times (minutes of incubation with trypsin are shown above each lane), 20 μl of the tryptic digest was added to SDS-PAGE sample buffer with (reduced) or without (oxidized) β-mercaptoethanol and samples were boiled for 5 min. Samples were subjected to SDS–13.5% PAGE, and gels were stained with Coomassie blue. Protein standards were coelectrophoresed (lane MW) (molecular masses, in kilodaltons, are shown to the left of the panel).
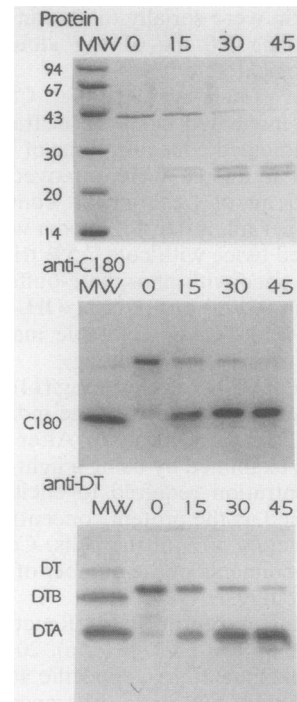

FIG. 4. Identification of the tryptic peptides of DTA-C180 protein. Twenty-five micrograms of DTA-C180 protein per ml was incubated with 25 ng of trypsin per ml in 0.1 M Tris-HCl (pH 8.0) containing 2 mM CaCl₂. At the indicated times (minutes of incubation with trypsin are shown above each lane), 20 μl of the tryptic digest was added to SDS-PAGE sample buffer containing β-mercaptoethanol and samples were boiled for 5 min. Samples were subjected to SDS–13.5% PAGE. Proteins in gels were identified by Coomassie blue staining (Protein) or transferred to nitrocellulose and incubated with rabbit anti-C180 IgG (anti-C180) or horse DT antisera (anti-DT). Blots incubated with horse DT antisera were also treated with the secondary antibody rabbit anti-horse IgG. Blots were then incubated with ^{125}I-labeled protein A. Protein standards were coelectrophoresed in the appropriate analysis: protein panel, MW lane shows the migration of protein standards (in kilodaltons); anti-C180 panel, MW lane shows the migration of C180 peptide; anti-DT panel, MW lane shows the migration of DT, DTB, and DTA components of a nicked preparation of DT. A Coomassie blue-stained gel and autoradiograms are shown.

peptide reactive to anti-C180 possessing a lower apparent molecular weight than the peptide reactive to diphtheria antitoxin. The relative migrations of the tryptic peptides were determined with respect to the migration of the intact DTA-C180 protein (Fig. 4, lane 0).

DEAE chromatography of trypsin-digested DTA-C180 protein. DTA-C180 protein was subjected to limited trypsin digestion and then chromatographed onto DEAE-Sephacel

in the presence of 20 mM DTT. The two tryptic peptides of the DTA-C180 protein eluted from the column at different NaCl concentrations, with the peptide reactive to diphtheria antitoxin eluting between 50 and 75 mM NaCl and the peptide reactive to anti-C180 eluting between 100 and 125 mM NaCl. Western blots were performed to confirm the identity of the peptides. These data indicated that the DTA and C180 peptide components of the DTA-C180 protein dissociated upon reduction of the disulfide bond of the DTA-C180 protein (C186—C201).

Cytotoxic activity of the DTA-C180 protein. The DTA-C180 protein was not cytotoxic to CHO cells. Measurement of the incorporation of [^{35}S]methionine into TCA-precipitable material by CHO cells showed that while 7×10^{-10} M DT inhibited protein synthesis by 50%, 2×10^{-7} M DTA-C180 protein, the highest concentration tested, did not inhibit protein synthesis. Microscopic examination showed that while 8×10^{-12} M PT stimulated 50% of the CHO cells to show a clustering morphology, 2×10^{-7} M DTA-C180
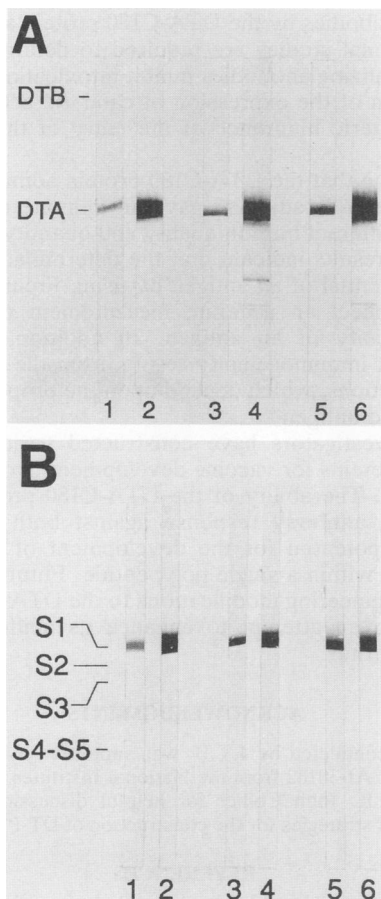
FIG. 5. Western blotting DT and PT with antisera against DTA-C180 protein. DT (A) and PT (B) were subjected to Western blotting by using antisera against DTA-C180 protein. Lanes: 1, 3, and 5, antisera obtained by immunizing with alum after one, two, and three doses, respectively; 2, 4, and 6, antisera obtained by immunizing with Freund's adjuvant after one, two, and three doses, respectively. The lines after DTA and DTB indicate the electrophoretic migration of the A and B subunits of DT. The lines after S1, S2, S3, S4, and S5 indicate the electrophoretic migration of the corresponding PT subunit.

TABLE 1. PT and DT neutralization by antisera against DTA-C180 peptide

Neutralization of:	Addition to DTA-C180	Serum dilution[a] after after immunization		
		1	2	3
PT	Alum	0	1/40	1/750
	Freund's adjuvant	0	0	1/20
DT	Alum	0	0	1/50
	Freund's adjuvant	0	0	1/2

[a] The highest dilution of serum capable of full toxin neutralization is reported. 0, absence of neutralization.

blotting (Fig. 5) showed that the antisera recognized specifically only the S1 subunit of PT and the DTA fragment of DT. Specific reactivity was observed after the first dose and increased after the second and third immunizations. Animals immunized with alum gave a weaker response than those immunized with Freund's adjuvant. Toxin neutralization activity did not directly correlate with the results obtained in Western blotting (Table 1), as neutralizing antibodies were observed in only one case after the second dose and in other cases after the third dose. In addition, the neutralization titers obtained with alum were higher than those obtained with Freund's adjuvant.

In comparison, two doses of 10 µg of PT9K/129G and 37 µg of diphtheria toxoid, in guinea pigs, elicited a neutralization titer against PT of between 1/1,280 and 1/2,560 in CHO cells and a neutralization titer against DT of between 1/320 and 1/640 in Vero cells. After this immunization, guinea pigs were resistant to challenge with DT, while mice immunized with one dose of the same vaccine were resistant to aerosol and intracerebral challenge with virulent B. pertussis (23a). The immunization protocols used to evaluate the protective properties of this vaccine were previously described (17).

DISCUSSION

The DTA-C180 protein was expressed as a soluble and stable protein within the periplasm of E. coli. Biochemical analysis indicated that both the DTA and C180 peptide portions of the DTA-C180 protein retained native functional properties: the tryptic site (R190, V191, R192, and R193) within the DTA portion of the DTA-C180 protein was intact and preferentially cleaved with trypsin; the C186—C201 disulfide bond, which formed within the DTA-C180 protein, was reduced by DTT in the absence of denaturant; and the DTA-C180 protein catalyzed the ADP-ribosylation of transducin at a rate similar to that of authentic C180 peptide. In addition, our studies indicated that there was little chemical interaction between the DTA and C180 peptide portions of the DTA-C180 protein, since the tryptic peptides of DTA and C180 dissociated upon disulfide bond reduction of trypsin-digested DTA-C180 protein.

A DTA derivative of DT was chosen as the diphtheria component of the DTA-C180 protein because of its inherent lack of cytotoxicity. DT follows the A-B model for structure-function (8), in which ADP-ribosyltransferase activity resides within the DTA portion of DT, and receptor binding and membrane translocation functions reside within the DTB portion of DT. Consistent with the A-B model, earlier studies showed that DTA was less cytotoxic by several orders of magnitude to Vero cells than DT (8). To further reduce the cytotoxic potential of the DTA-C180 protein, a

protein, the highest concentration tested, did not elicit a clustering CHO cell response.

ADP-ribosylation of transducin by C180 peptide and DTA-C180 protein. The linear velocity of C180-peptide-mediated ADP-ribosylation of transducin is about 1% of that catalyzed by the S1 subunit of PT (9). DTA-C180 protein was observed to retain this limited capacity to catalyze the ADP-ribosylation of transducin. Specific activities were determined to be 15 mmol of ADP ribose incorporated into the α subunit of transducin per min per mol of DTA-C180 protein and 30 mmol of ADP ribose incorporated into the α subunit of transducin per min per mol of C180 peptide (see Materials and Methods for reaction conditions).

Immunogenic properties of the DTA-C180 protein. The sera obtained from guinea pigs immunized with the DTA-C180 protein mixed with either aluminum hydroxide (alum) or Freund's adjuvant were initially tested individually by Western blotting and toxin neutralization assays. Since the titers of the individual sera in each group were consistent, we report here the results obtained with pooled sera. Western

nonrevertible mutation, E148S, was included within the DTA component. The E148S mutation has been shown to reduce by about 800-fold the cytotoxic potential of DT to cultured cells (1, 29). The C180 peptide derivative of S1 of PT was chosen as the pertussis component of the DTA-C180 protein, also because of its inherent lack of cytotoxicity. The C180 peptide is a recombinant derivative of S1, which possesses about 1% of the capacity of S1 to ADP-ribosylate transducin (9, 15) and lacks the capacity to elicit cytotoxic activity against CHO cells (1). Consistent with these defined properties of the two components of the DTA-C180 protein were our observations that the DTA-C180 protein did not possess detectable cytotoxic activity on CHO cells. Together, these data are consistent with the construction of the DTA-C180 protein as a biologically null protein, an ideal property for an immunogen. If necessary, additional mutations may be engineered into either the DTA or C180 peptide component of the DTA-C180 protein to further reduce biological activity.

The DTA-C180 protein elicited a neutralizing antibody response against both DT and PT.

(i) Neutralization titer of the diphtheria component of the DTA-C180 protein. Only after the third dose of DTA-C180 protein was a neutralization titer against DT detected, and, relative to a neutralization titer elicited by two doses of diphtheria toxoid, this titer was weak.

Earlier studies showed that polyclonal sera against DTA did not neutralize DT (10, 21), but monoclonal antibodies, with epitopes within DTA, neutralized DT (30). Together with our results, these studies suggest that while DTA possesses an epitope capable of eliciting a neutralizing antibody response, the epitope is not immunodominant within native DTA. We did not determine the neutralization potential of DTA in guinea pigs, which prevented direct comparison between the relative immunogenicity of the DTA-C180 protein and DTA; however, from our data and previous data (10, 21), it appears that both molecules elicit either no or only a weak neutralizing response. Our current research is directed toward modifying the diphtheria component of the DTA-C180 protein to attempt to elicit a neutralizing titer against DT which approaches diphtheria toxoid.

(ii) Neutralization titer of the pertussis component of the DTA-C180 protein. By using alum as an adjuvant, two doses of the DTA-C180 protein elicited a detectable neutralizing titer against PT. By the third dose, the DTA-C180 protein elicited a neutralization titer against PT which approached the titer of two doses of PT9K/129G. This is the first report that a recombinant derivative of the S1 subunit can elicit a neutralizing titer against PT and shows the potential feasibility for the development of an epitope-defined pertussis vaccine. For comparison, after one dose of PT9K/129G, mice are resistant to challenge by *B. pertussis* (17).

Studies on antibody-mediated neutralization of pertussis toxin showed that a neutralizing epitope was localized within the S1 subunit (24). However, attempts to elicit a neutralizing antibody response, by using recombinant S1 (rS1) as the immunogen, to date, have been unsuccessful (20). In retrospect, the inability of rS1 to elicit a neutralizing antibody response may be due to the urea extraction required to solubilize rS1 in *E. coli* extracts (20). Urea-extracted rS1 may not refold into a structure amenable to elicit a neutralizing antibody response. The DTA-C180 protein is expressed in a soluble form in *E. coli*, which eliminates the use of denaturants, such as urea, in the purification protocol. The mechanisms for the observed elicitation of DT or PT neu-

tralizing antibodies by the DTA-C180 protein are not apparent. Additional studies are required to determine whether these neutralizing antibodies inhibit intoxication directly via an inhibition of the expression of catalytic activity or indirectly via steric hindrance of the entry of the toxins into cells.

The finding that the DTA-C180 protein administered with alum or Freund's adjuvant gave an immune response that was quite different both in quality and quantity was surprising. These results indicate that the determination of immunogenic potential of an antigen by using Freund's adjuvant may not reflect an accurate measurement of the actual immunogenicity of an antigen. In addition, our studies suggest that immunogenicity results from the antigen-adjuvant interactions, which depend upon the properties of both adjuvant and antigen.

Other investigators have constructed various forms of chimeric proteins for vaccine development in other systems (11, 13, 19). The ability of the DTA-C180 protein to elicit neutralizing antibody response against both DT and PT shows the potential for the development of a DT or PT immunogen within a single polypeptide. Future studies will focus on engineering modifications to the DTA-C180 protein construct in an attempt to enhance its ability to elicit a neutralizing titer.

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