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Interaction of Monoclonal Antibodies with Pertussis Toxin and Its Subunits

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The binding of two monoclonal antibodies to crude and pure preparations of pertussis toxin was examined. Antibody P11B10 reacted with an epitope present on the S2 subunit of pertussis toxin by immunoblot techniques. Radioimmunoprecipitation analysis and immunoaffinity chromatography with P11B10 indicated that subunits S2 and S3 were closely associated. Antibody P7B10 was unreactive in immunoblots and radioimmunoprecipitation but was able to bind and retain toxin subunits during affinity chromatography. The P7B10 epitope may thus be labile to detergent treatment or radioiodination; or the epitope may form as a result of subunit association. Neither antibody alone nor both in combination neutralized the histamine-sensitizing activity of pertussis toxin in passive-transfer experiments. However, toxin isolated by immunoaffinity chromatography with either monoclonal antibody was able to sensitize mice to histamine challenge.

Bordetella pertussis causes a localized infection of the ciliated epithelium of human bronchi and trachea, resulting in the disease whooping cough. A variety of systemic disturbances in man and experimental animals has been attributed to *B. pertussis* products, although not all of the systemic effects are recognized to occur in whooping cough. Systemic effects include sensitization to nonspecific stress (such as cold or endotoxin shock), enhancement of anaphylaxis, immunopotentiation, hypoglycemia, lymphocytosis, and sensitization to histamine (13, 18, 21, 23, 34, 35). A single moiety, pertussis toxin (PT; lymphocytosis-promoting factor, histamine-sensitizing factor, or islet cell-activating protein), is responsible for the induction of hypoglycemia, lymphocytosis, and histamine sensitization. Purification of active pertussis toxin in reasonable amounts has been difficult, although several successful purification schemes have been reported (1, 9, 17, 19, 20, 26, 33).

Based on the studies of Tamura et al. (29), PT appears to consist of five subunits designated S1 (molecular weight, 28,000), S2 (23,000), S3 (22,000), S4 (11,700), and S5 (9,300). Subunit S1 (A protomer) is responsible for the enzymic activity of PT. It catalyzes the transfer of ADP-ribose from NAD to one of the guanine nucleotide regulatory components in the receptor-adenylate cyclase system of C6 glioma cells (2, 10). The B oligomer (a pentamer formed from the association of dimer S2-S4, dimer S3-S4, and subunit S5) is believed to be involved in the binding of PT to target cells (10, 29, 30). The action of PT results in the enhancement of receptor-linked and GTP-dependent adenylate cyclase activity (29). This mode of action shows similarity to the mechanism of other bacterial toxins which ADP-ribosylate cellular proteins (29).

We have isolated hybridomas which produce monoclonal antibody to various *B. pertussis* antigens (D. W. Frank and C. D. Parker, J. Biol. Stand., in press). In this paper we report the interactions of two stable hybridoma antibodies, P11B10 and P7B10, with pertussis toxin.

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MATERIALS AND METHODS

Sources of reagents. Reagent sources were as follows: Goat anti-mouse immunoglobulin G (IgG) conjugated to peroxidase and purified mouse IgG, Cappel Laboratories, West Chester, Pa.; nitrocellulose paper BA85, Schleicher and Schuell, Keene, N.H.; Amicon ultrafiltration cell and Diaflo PM-10 ultrafiltration membrane, Amicon Corporation, Danvers, Mass.; BALB/c and CFW mice, Charles River Breeding Laboratories, Inc., Wilmington, Mass.; 2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, Wis.; RPMI-1640 tissue culture medium, Kansas City Biologicals, Kansas City, Mo.; Iodobeads, Pierce Chemical Co., Rockford, Ill.; $Na^{125}I$, New England Nuclear, Boston, Mass.; X-Omat XAR-2 film, Kodak, Rochester, N.Y.; bovine serum albumin (BSA) fraction V, U.S. Biochemicals, Cleveland, Ohio; DEAE Affi-gel blue, Affi-gel 10, low-molecular-weight markers, and gel electrophoresis materials, BioRad, Richmond, Calif.; 4-chloro-1-naphthol, morpholinepropanesulfonic acid, phenylmethylsulfonyl fluoride, and all other reagents were from Sigma Chemical Co., St. Louis, Mo.

Toxin preparations. Purified PT preparations were generously provided by Erik Hewlett, Departments of Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville, Va., and James L. Cowell, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Md. James Cowell also contributed purified filamentous hemagglutinin and goat antisera to lymphocytosis-promoting factor (LPF) used in this study. A fraction of *B. pertussis* enriched for toxin was provided by John J. Munoz, Rocky Mountain Laboratories, Hamilton, Mont. Crude toxin was prepared in our own laboratory from *B. pertussis* 165 culture supernatants by the method of Munoz et al., (20) but not including the chromatography or succeeding steps.

Production of monoclonal antibodies. Monoclonal antibody-producing cell lines were obtained by slight modifications of techniques developed by Kohler and Milstein (11) and described in detail elsewhere (Frank and Parker, in press). Briefly, BALB/c mice were immunized with a toxin-enriched fraction of *B. pertussis* from J. J. Munoz, hybrid colonies were screened in enzyme immunoassay with our

crude toxin as antigen, hybrid wells which gave a positive reaction were cloned at least twice by limiting dilution, and the monoclonal nature of each cell line was confirmed by metabolic labeling of antibodies and isoelectric focusing analysis (25). P11B10 and P7B10 monoclonal antibodies were of the IgG1 isotype.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with *B. pertussis* antigen preparations on a 7.5 to 20% polyacrylamide gradient gel by the Laemmli system (12) modified (27) as follows: (i) inclusion of 0.5 M urea in the solubilization buffer, stacking gel, and resolving gel; and (ii) reduction of bis-acrylamide in the resolving gel stock from 0.8 to 0.54%. Electrophoresis was performed at 0.5 W constant power for 15 h for gels that were 12 cm by 14 cm by 0.75 mm. Protein concentrations were determined by the method of Lowry et al. with BSA as a standard (14). Gels were usually loaded with 5 to 15 μg of protein per lane. Gels were stained with Coomassie blue by the method of Fairbanks et al. (6) or with silver by the method of Morrisey (16).

Proteins separated by electrophoresis were electrophoretically transferred to nitrocellulose paper as described by Towbin et al. (32), and excess binding sites on the nitrocellulose were blocked by incubation at 35°C in a solution containing 0.9% NaCl, 10 mM Tris, and 3% BSA (pH 7.4) for 1 h. The nitrocellulose was washed briefly in 0.9% NaCl–10 mM Tris buffer (pH 7.4) (wash buffer) and incubated in undiluted hybridoma culture supernatant. Test sheets were allowed to incubate overnight at 4°C, washed 10 times by gentle agitation for 5 min in wash buffer, and incubated for 2 h at room temperature in an optimal dilution (1:2,000) of goat anti-mouse IgG conjugated to peroxidase. The nitrocellulose was washed 10 times as before and developed with 4-chloro-1-naphthol as the chromagen (8). For calculation of molecular weights in immunoblots, molecular weight markers from each gel were transferred to nitrocellulose and stained with amido black.

Ascites and antisera production. For ascites fluid production, retired BALB/c breeder females were pretreated with an injection of 0.5 ml of 2,6,10,14-tetramethyl pentadecane, and 1 to 4 weeks later each mouse was injected intraperitoneally with 10^7 hybridoma cells which had been washed three times in serum-free RPMI-1640. Ten to 14 days later, ascitic fluids from each mouse were collected, cells were removed by centrifugation, and the supernatant fluids were stored frozen in small samples until use. For antiserum production, three injections of 2 μg of our crude PT (heated at 56°C for 0.5 h just before use) were given intraperitoneally into adult CFW female mice at two week intervals. One week after the last injection, the mice were exsanguinated, the blood was pooled, and the serum was collected. Serum was stored frozen in small samples.

Radiolabeling of crude PT. Crude PT was radioiodinated by the method of Markwell with Iodobeads (15). Five beads were washed three times in 0.1 M phosphate-buffered saline (pH 7.2). Crude PT (100 μg) and 1-mCi carrier-free $Na^{125}I$ in 1 ml of phosphate-buffered saline containing 2 M urea were added to the washed beads. Labeling mixtures were gently swirled for 15 min at room temperature. The reaction was stopped by removing the beads from the reactants. Free $Na^{125}I$ was removed by dialysis of labeled toxin against five changes of 200 ml of phosphate-buffered saline containing 2 M urea.

RIP procedure. A variety of methods were examined before radioimmunoprecipitation (RIP) was achieved. Monoclonal antibodies were conjugated to agarose beads, or used in solution; antibody was precipitated with an antiimmunoglobulin or with *Staphylococcus aureus* cells positive for protein A. These procedures all immunoprecipitated toxin when P11B10 was used, but not when P7B10 was used. High backgrounds resulted with each method, but the background was lower in the technique with soluble antibody and *S. aureus* cells. Titrations revealed that the optimal concentration of *S. aureus* cells was 100 μl of a 5% (vol/vol) cell suspension. The final reaction mixture consisted of iodinated toxin in an amount to yield about 10^6 cpm, 5 μl of monoclonal ascitic fluid or immune serum, 5 μl of fetal calf serum, and 30 μl of 0.5 M NaCl–25 mM Tris (pH 7.4), in a final volume of 45 μl. Reaction mixtures were held at room temperature for 2 h, and 100 μl of fixed and washed *S. aureus* Cowan strain were added to each mixture (5). This step was followed by another 2-h incubation at room temperature after which the staphylococci were washed five times by centrifugation with 1.5 ml of 0.5 M NaCl–25 mM Tris and solubilized for SDS-PAGE. Insoluble debris was removed by centrifugation, and total counts in each supernatant were measured in a Beckman 8000 gamma counter (Irvine, Calif.). To compare gamma counts in individual bands of RIP reaction mixtures, reactions containing P11B10 ascites, goat anti-LPF antisera, and P3x63Ag8.653 ascites were prepared containing 10-fold more reagents than described above. The reaction mixtures were incubated, washed, and solubilized for electrophoresis. Lanes, 3.5 cm in width, were loaded with 300,000 cpm, and the labeled peptides were separated by SDS-PAGE on an 11 to 20% acrylamide gradient gel. After electrophoresis, each lane was cut into 2.5-mm slices, and the radioactivity in each slice was measured in the gamma counter. Slices showing peaks of radioactivity were eluted with 200 μl of 0.125 M Tris–0.1% SDS–0.1 mM EDTA and reelectrophoresed on gradient gels to determine the polypeptide composition of each fraction. Bands on dried gels were visualized by autoradiography.

Affinity chromatography. Monoclonal antibodies were purified from ascitic fluids by DEAE Affi-gel blue chromatography (3). Twenty milligrams of purified IgG was then attached to a 1-ml slurry of Affi-gel 10 by the method of Staehelin et al. (28) in a coupling buffer of 0.1 M morpholinepropanesulfonic acid (pH 7.5). Unreacted sites on the affinity gels were blocked with an equal volume of 0.1 M ethanolamine HCl (pH 8) for 1 h at room temperature. Gel slurries were packed into 3-ml plastic syringes, washed with 10 bed volumes of elution buffer (4 M sodium thiocyanate, 2 M urea), and equilibrated with 0.25 M NaCl–12.5 mM Tris (pH 7.6) (column buffer). Crude PT (1 mg in column buffer with 10 μM phenylmethylsulfonyl fluoride) was allowed to adsorb slowly to each column and was incubated at room temperature for 2 h. After being washed with 30 ml of column buffer, toxin subunits were eluted with 4 M sodium thiocyanate–2 M urea. Fractions containing protein were pooled, dialyzed against 0.05 M Tris (pH 7.6), and examined by SDS-PAGE.

Concentrated culture supernatants were also used as starting material for immunoaffinity columns. Briefly, 1 liter of *B. pertussis* 165 was cultivated for 48 h at 35°C with shaking in the liquid minimal medium described previously (27). Cells were removed from spent medium by centrifugation at 3,000 × *g* and fluids were passed through a 0.45-μm membrane filter. Filtered supernatants were concentrated ca. 350 times by Amicon PM-10 membrane filtration. Material adhering to the membrane was removed by gentle washing with 1 M NaCl–50 mM Tris (pH 7.6). The membrane wash

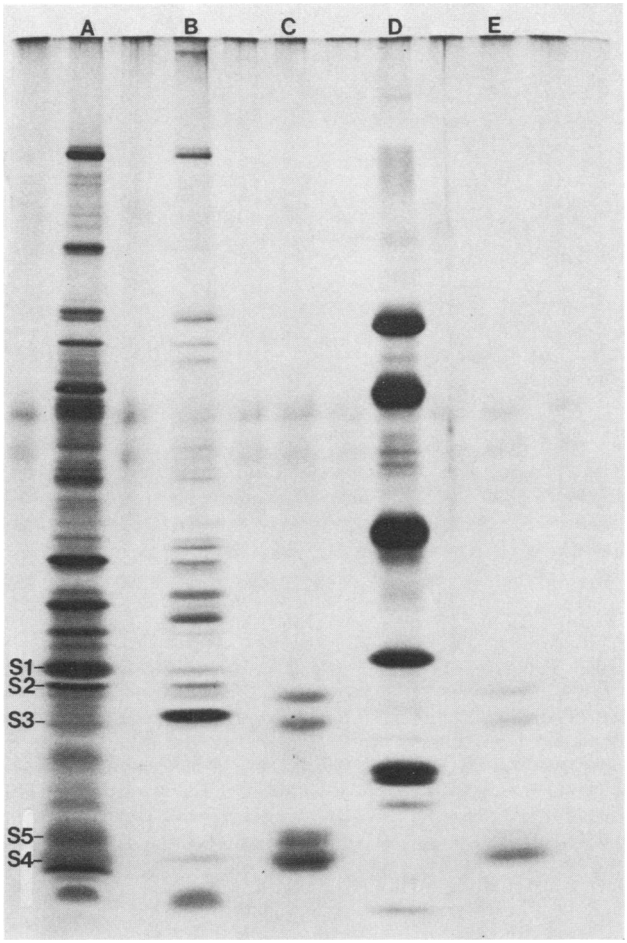

FIG. 1. SDS-PAGE of PT preparations. Various toxin preparations were electrophoresed in a 7.5 to 20% gradient acrylamide gel and stained with silver stain. Lanes: A, crude PT from Munoz; B, crude PT prepared in our laboratory; C, purified PT from Cowell; D, molecular weight markers phosphorylase *b* (92,500), BSA, (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500); and E, purified PT from Hewlett. Horizontal dashes indicate the locations of the presumed PT subunits as determined by molecular mass in multiple electrophoresis experiments. The specific PT subunits are designated at the left. Our gel system contained 0.5 M urea, which caused S4 and S5 to reverse their apparent molecular weights. Visual inspection of the stained gel showed a faint band at the position of S1 in lanes C and E. Note that the relative proportions of the five peptides appear to vary among the four preparations. The lowest band seen in lanes A and B is LPS as determined by immunoblots of similar gels with serum or monoclonal antibody specific for Bordetella LPS (data not shown).

fluid and culture medium concentrate were pooled and dialyzed against column buffer. This material (1.3 mg of protein) was applied, washed, and eluted from immunoaffinity columns as described above for crude toxin fractions.

In vivo toxin activity and toxin neutralization tests. Histamine-sensitizing activity was used as an in vivo assay for pertussis toxin activity (22). Dilutions of eluates from immunoaffinity columns were injected intravenously into male CFW mice (14 to 16 g). Three days later, the animals were challenged intraperitoneally with 0.36 mg of histamine base per 10 g of body weight (24). For toxin neutralization tests, animals were pretreated on day −1 by an intraperitoneal injection of 0.2 ml of antiserum, monoclonal ascitic fluid, or saline. On day 0, all mice were given an intravenous injection of 1 μg of crude toxin (heated at 56°C for 0.5 h just before injection to inactivate dermonecrotic toxin. Histamine challenge was given on day 3 as above. Death within 3 h of histamine challenge was attributed to the histamine-sensitizing activity of PT in both assays.

RESULTS

Immunoblot analysis. Preliminary studies had indicated that monoclonal antibodies from hybrids P11B10 and P7B10 reacted in enzyme immunoassay to crude PT fractions (Frank and Parker, in press). Crude PT preparations contained PT and several other polypeptides, as well as lipopolysaccharide (LPS), as shown in the SDS-PAGE profile of this material (Fig. 1, lanes A and B). Purified toxin preparations showed five polypeptide bands with molecular weights of 29,000 to 30,000, 25,000 to 26,000, 23,000, 14,000, and 12,000 in our gel system, apparently corresponded to PT

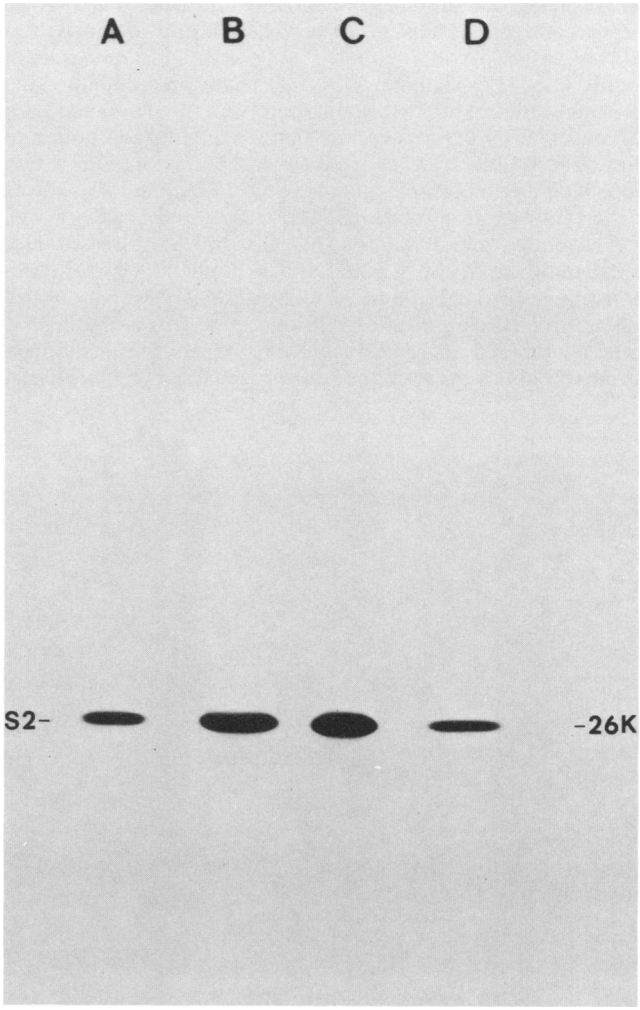

FIG. 2. Immunoblot analysis of PT preparations. After electrophoresis of 5 μg of protein per lane in a 7.5 to 20% gradient acrylamide gel, proteins were electrophoretically transferred to nitrocellulose paper and reacted with P11B10 culture supernatant. Molecular weight markers, run on the same gel, were transferred to nitrocellulose and stained with amido black. Lanes: A, purified PT from Hewlett; B, crude PT from Munoz; C, purified PT from Cowell; and D, crude PT from our laboratory.

subunits S1 to S5 (Fig. 1, lanes C and E). However, in gel systems which contain urea, S4 and S5 have been observed to exchange places (M. Tamura, personal communication), so that we believe that S5 moves with the higher apparent molecular weight in our studies. Toxin preparations were subjected to SDS-PAGE, transferred to nitrocellulose, and examined for specific immunoreactivity. Culture supernatant fluid from monoclonal P11B10 reacted as shown in Fig. 2. Note that P11B10 reacted with a single polypeptide in each toxin preparation. The molecular weight of this peptide corresponded to the S2 subunit (26,000). In similar analyses, monoclonal antibody P7B10 did not react with any toxin preparation. Neither P11B10 nor P7B10 reacted in immunoblots with purified LPS, purified filamentous hemagglutinin, or Triton-insoluble envelope preparations from *B. pertussis* used as antigen (data not shown).

RIP analysis. RIP was used to assess the binding activity of P11B10 and P7B10 to undenatured, ^{125}I-labeled crude toxin preparations. Figure 3 shows the results of such an experiment in which labeled toxin was incubated with antibody, precipitated, denatured and examined by SDS-PAGE. Three control reactions were performed, and included ^{125}I-labeled crude toxin incubated with anti-LPS monoclonal ascitic fluid (Fig. 3, lane A), crude toxin plus diluent (lane B), and crude toxin plus ascitic fluid from mice injected with the parental myeloma cell line (lane C). Autoradiograms of these control reactions showed several bands, including two nonspecific bands (molecular weight, 35,000 and 45,000) as well as four bands (30,000, 26,000, 23,000, and 14,000) which corresponded to subunits S1, S2, S3, and S5, respectively. Total radioactivity in washed and solubilized control reactions ranged from 1,178 to 1,261 cpm/μl. A positive control reaction of labeled crude toxin and goat anti-LPF (Fig. 3, lane D) yielded increased amounts of all 5 PT subunits (radioactivity of the precipitate averaged 6,198 cpm/μl; four-

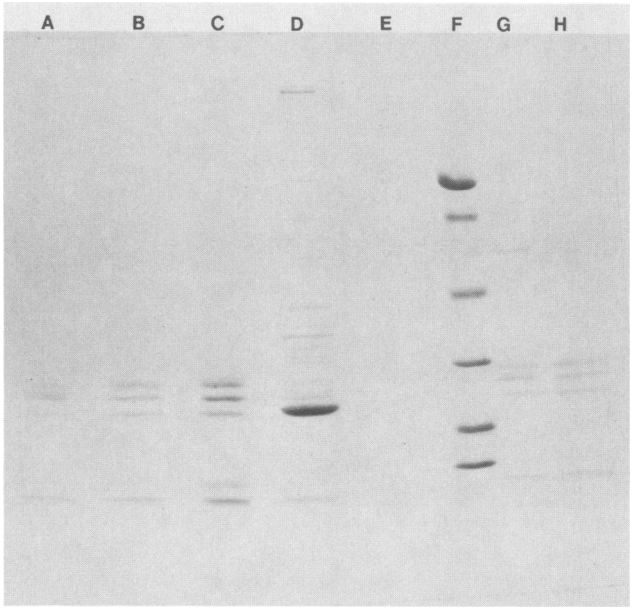

FIG. 4. Affinity purification of PT subunits. Crude PT was adsorbed to Affi-gel beads conjugated to P7B10, P11B10, or purified normal mouse IgG. The affinity gels were washed (0.25 M NaCl, 12.5 mM Tris, [pH 7.6]) and eluted. Eluates were analyzed by electrophoresis through a 7.5 to 20% gradient gel and stained with Coomassie blue. Lanes: A, P11B10 eluate; B, 7B10 eluate; C, Cowell purified PT; D, crude PT; E, normal mouse IgG eluate; F, molecular weight markers phosphorylase *b* (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000) soybean trypsin inhibitor (21,500), and lysozyme (14,400); G, P11B10 eluate (with Amicon PM-10 concentrated *B. pertussis* culture supernatant as starting material); and H, P7B10 eluate (with Amicon PM-10 concentrated *B. pertussis* culture supernatant as starting material).

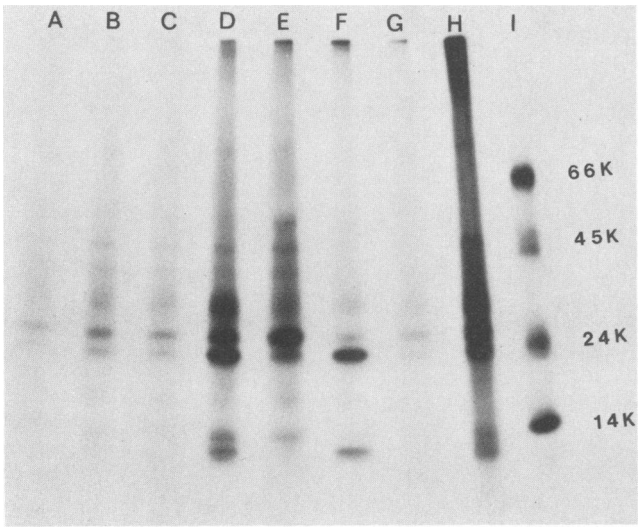

FIG. 3. Autoradiogram of an RIP reaction with labeled PT. Crude PT prepared in our laboratory was radioiodinated and reacted with various antisera or buffers. Toxin-antibody complexes were precipitated by a protein A staphylococcus technique, and the precipitates were solubilized and subjected to SDS-PAGE. Lanes: A, WC8H4 ascites fluid (an LPS-specific monoclonal antibody); B, diluent only; C, P3x63Ag8.653 ascites fluid (parental myeloma tumor line); D, goat antibody to LPF; E, P11B10 ascites fluid; F, mouse antibody to crude PT; G, P7B10 ascites fluid; H, ^{125}I-labeled crude PT; I, ^{125}I-labeled molecular weight markers BSA (66,200) ovalbumin (45,000), trypsinogen (24,000), and lysozyme (14,400).

to fivefold higher than negative controls), but also showed nonspecific bands at molecular weights of 35,000 and 45,000. Mouse polyclonal antiserum to PT specifically precipitated only S3 and S4 subunits (Fig. 3, lane F). The reaction mixture containing P11B10 ascites (Fig. 3, lane E) showed a band at 56,000, as well as nonspecific bands at 45,000 and 35,000 and small amounts of S1 and S5. Bands corresponding to S2 and S3 appeared to be specifically immunoprecipitated. After gel slicing, the P11B10 S2 and S3 bands were found to contain threefold and twofold higher radioactivity, respectively, than control bands. Radioactivity recovered from P11B10 RIP reactions averaged 2,682 cpm/μl. P7B10 ascites (Fig. 3, lane G) did not appear to immunoprecipitate toxin, and radioactivity from these solubilized reaction mixtures averaged only 1,470 cpm/μl.

Immunoaffinity chromatography. Three immunoaffinity chromatography columns were prepared by coupling mouse IgG isolated from P11B10 ascites fluids, P7B10 ascites fluids, and normal mouse serum to Affi-gel 10 beads. Crude PT preparations were placed on each column. Unbound material was removed by washing, and bound material was eluted with urea-sodium thiocyanate solution. Eluates were analyzed by SDS-PAGE (Fig. 4). Both P11B10 (Fig. 4, lane A) and P7B10 (lane B) bound intact PT, whereas normal mouse IgG (lane E) did not. In further experiments, in which we simulated the ionic conditions of the RIP assay by doubling the ionic strength of incubation and wash buffers, P11B10 bound the S2 and S3 subunits of PT (Fig. 5). Dark staining material in the region of 55,000 and 68,000 molecular weight was also present but seemed to be artifactual silver

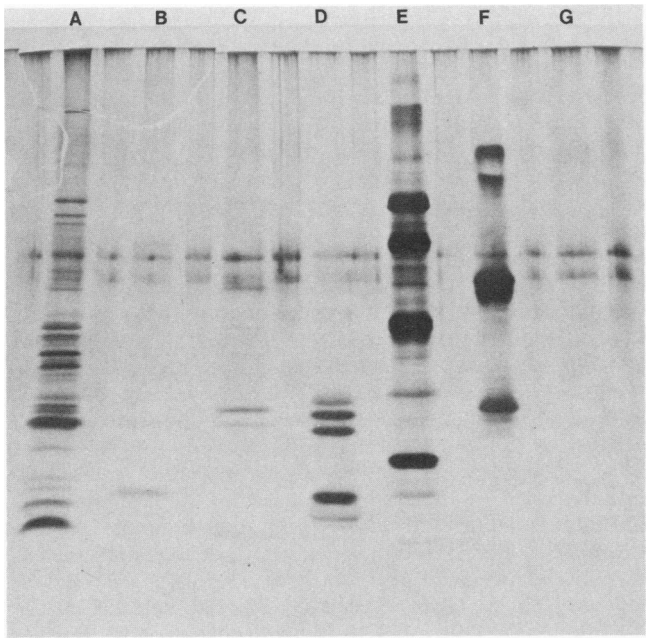

FIG. 5. Affinity chromatography with higher ionic strength buffers. Crude PT was adsorbed to Affi-gel beads conjugated to P11B10 or BSA. Incubation and wash buffers consisted of 0.5 M NaCl–25 mM Tris (pH 7.6). Each column was eluted, and the eluates were analyzed on a silver-stained 7.5 to 20% gradient acrylamide gel. Lanes: A, crude PT starting material, B, material eluted from the BSA column; C, P11B10 eluate; D, purified PT from Cowell; E, molecular weight markers phosphorylase B (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500); F, monoclonal antibody P11B10 used for the affinity column; and G, solubilizing buffer only.

staining due to the presence of 2-mercaptoethanol in the solubilizing buffer. A control lane containing only solubilizing buffer is shown in Fig. 5, lane G. Note that the same artifactual bands in the buffer control also appear in lanes containing protein (7, 31).

The average PT yield from columns in which our crude toxin extract (concentrated by differential salt solubility) was used as starting material was 0.2 μg/ml of original culture supernatant. Use of Amicon-concentrated culture supernatant as starting material doubled the average PT yield to 0.4 μg/ml of original culture supernatant.

In vivo toxin neutralization and toxin activity tests. An initial titration demonstrated that a dose of 100 ng of our crude toxin mixture was sufficient to sensitize five of five mice to a histamine challenge, whereas at a 50-ng dose, two of five animals were sensitized. When mice were pretreated with either goat anti-LPF or mouse anti-crude PT antisera, 1 μg of crude toxin did not sensitize to the lethal effects of a histamine challenge. P11B10 ascitic fluid, P7B10 ascitic fluid, P11B10 and P7B10 ascites fluid mixed together, or an anti-LPS monoclonal antibody did not neutralize the ability of crude PT to sensitize mice to histamine.

Materials eluted from P11B10, P7B10, and normal mouse IgG immunoaffinity columns were tested for histamine-sensitizing activity in vivo. The median histamine-sensitizing dose for both P11B10 and P7B10 eluates was 40 to 50 ng (Table 1). Eluates from normal mouse IgG columns failed to sensitize mice to histamine at the highest dose tested (2 μg per mouse).

DISCUSSION

Four independent PT preparations were used in this study. Our crude PT (from *B. pertussis* 165) and Munoz toxin (from *B. pertussis* $3779BL_2S_4$) were isolated by using techniques developed by Munoz et al. (20). Purified PT supplied by Cowell and Hewlett was isolated with *B. pertussis* Tohama phase I and *B. pertussis* 114 respectively, as producer strains (4, 9, 33). It should be noted that *B. pertussis* 114 was derived from strain $3779BL_2KS_3$ and is essentially the same strain used by Munoz ($3779BL_2S_4$). Regardless of source or purity of PT, these preparations generally contained five polypeptide bands at molecular weights of 29,000 to 30,000, 25,000 to 26,000, 23,000, 14,000, and 12,000 in SDS-PAGE analysis. These molecular weight assignments differ slightly from those published by Tamura et al. (29) in which PT subunits migrated at molecular weights of 28,000, 23,000, 22,000, 11,700, and 9,300. The molecular weight disparity could be due to differences in bacterial strains used for toxin production, but is most likely to be due to differences in the gel systems used to resolve toxin subunits. For the estimation of toxin subunit molecular weight, Tamura et al. (29) used 12.5% acrylamide tube gels, whereas our separation gel system was a 7.5 to 20% acrylamide gradient slab gel containing 0.5 M urea. Differences in solubilizing buffers do not account for the molecular weight disparity, since we solubilized our samples in the 4 M urea–1% SDS buffer of Tamura et al. (29) and found no change in subunit molecular weight assignment. The most likely explanation is that urea in our gel affected the relative mobilities of the peptides, with S4 and S5 being most affected; it should be noted that all our subunit assignments are presumed ones.

Immunoblot analyses of the four toxin samples revealed that monoclonal P11B10 reacted specifically to subunit S2

TABLE 1. Mouse toxicity of PT isolated by immunoaffinity chromatography

Material	Treatment	Dose (μg of protein)[a]	Mice, dead/total[b]
Saline	None		0/5
Crude PT[c]	Exposed to elution buffer and heated, 56°C, 0.5 h	1.0	4/5
Crude PT	Heated, 56°C, 0.5 h	1.0	5/5
Crude PT	Heated, 80°C, 0.5 h	1.0	0/5
Crude PT	Eluted from normal mouse IgG column	2.0	0/5
Crude PT	Eluted from P11B10 column	2.0	5/5
		1.0	5/5
		0.2	5/5
		0.05	2/5
Amicon PT[d]	Eluted from P11B10 column	0.05	3/5
Crude PT	Eluted from P7B10 column	2.0	5/5
		1.0	5/5
		0.2	5/5
		0.05	3/5
Amicon PT	Eluted from P7B10 column	0.05	4/5

[a] Injected intravenously into male CFW mice (14 to 16 g) on day 0 in a volume of 0.1 ml.

[b] The number of dead mice per total number of mice tested after challenge with 0.36 mg of histamine per 10 g of body weight. Histamine challenge was done 3 days after toxin injection.

[c] Crude PT was culture supernatant concentrated by differential salt extraction.

[d] Amicon PT was culture supernatant concentrated by Amicon PM-10 ultrafiltration.

(molecular weight 25,000 to 26,000). P11B10 was unreactive with the other polypeptides in crude PT as well as other subunits in pure PT. Although not rigorous proof, this observation suggested that S2 is not a precursor of the lower-molecular-weight subunits of the toxin. Monoclonal P7B10 showed no immunoblot activity against crude PT preparations to which it bound in enzyme immunoassays. Denaturation of the epitope recognized by P7B10 may account for this observation.

RIP and immunoaffinity chromatography procedures were developed to examine reactivity of these antibodies to nondenatured toxin preparations. Our crude PT represented a subset of proteins that were enriched by virtue of solubility in high ionic strength solutions (1 M NaCl, 50 mM Tris [pH 7.6]) and insolubility under low ionic strength conditions (5 mM Tris [pH 8]). When such a fraction was radiolabeled with ^{125}I and used in RIP analysis, we consistently observed faint bands in negative control reactions. These background bands occurred in negative control reactions with and without serum and could not be eliminated by the use of detergents (Nonidet P-40, Tween 20), different incubation buffers, or different techniques to precipitate toxin-antibody complexes. The presence of background bands in negative control RIP reactions may be due to insolubility of components in crude PT, since purified fractions of PT have been observed to form insoluble aggregates when stored in 2 M urea (29). The RIP incubation conditions which allowed antibody binding (i.e., not containing urea) could have resulted in partial insolubility of PT and the moieties of molecular weight 35,000 and 45,000. Comparison of counts per minute per band present in control reactions and P11B10 reactions demonstrated that this monoclonal antibody immunoprecipitated subunits S2 and S3. Under the conditions of the RIP assay, our data suggest that S2 and S3 were closely associated. These data, however, do not eliminate the possibility that P11B10 recognized an epitope on subunit S2 that also exists on nondenatured subunit S3.

The RIP results were confirmed and extended by immunoaffinity chromatography of PT. When RIP buffer (0.5 M NaCl, 25 mM Tris) was used to equilibrate and load a column containing P11B10-conjugated agarose beads, the column retained the S2 and S3 subunits of PT. Use of one-half-strength RIP buffer (0.25 M NaCl, 12.5 mM Tris) resulted in retention of all five subunits on the beads. In 5 M urea, PT forms both S2-S4 and S3-S4 dimers (29). Our unexpected finding of S2-S3 association in both RIP and immunoaffinity chromatography assays with P11B10 suggests that these subunits may associate under our less stringent solvent conditions. Alternatively, antibody binding to subunit S2 may stabilize S2-S3 association and destabilize S2-S4 association.

The specificity of antibody P7B10 for PT was demonstrated only by immunoaffinity chromatography. The binding of untreated toxin to P7B10 affinity columns showed that P7B10 recognized an antigenic site of PT. The PT epitope recognized by P7B10 appears to be labile to denaturation, since P7B10 is unreactive in immunoblots. The failure of P7B10 to bind PT in RIP experiments is more difficult to explain, since we used a variety of RIP techniques and buffers. These techniques included double antibody immunoprecipitation and the use of agarose beads conjugated with purified P7B10 (data not shown). We can only speculate that the epitope recognized by P7B10 was altered by radioiodination. Radioiodination is known to impair the subunit association of the toxin (30). Taken together, these results suggest that the antigenic site recognized by P7B10 may result from PT tertiary or quaternary structure.

Immunoaffinity chromatography of both crude toxin extracts and Amicon-concentrated culture supernatants resulted in the isolation of biologically active PT. The estimated mean median histamine-sensitizing dose of 40 to 50 ng per mouse falls within the range of some investigators (9, 17, 34) but is somewhat higher than the dose reported by recent studies (4, 19). The possible presence of protein contaminants in our preparation or differences in the sensitivity of mouse strains to PT may explain these observations (17, 21). It is possible that less harsh elution conditions may increase PT toxic activity. We have already found that the histamine-sensitizing activity was 20% higher and the PT protein yield from immunoaffinity columns was doubled when the starting material was Amicon-concentrated culture supernatant rather than crude toxin extract. This increase in yield and activity probably reflects the great reduction in time and handling of crude material.

In conclusion, the binding activity of two monoclonal antibodies to PT subunits was assessed by immunoblot, RIP, and immunoaffinity chromatography techniques. Monoclonal antibody P11B10 bound specifically to subunit S2 of PT. In experiments with this antibody, subunits S2 and S3 appeared to be closely associated under certain solvent conditions. Antibody P7B10 recognized native PT but not radioiodinated or denatured PT. Thus, P7B10 may bind an epitope resulting from the secondary structure of a subunit or from the association of several subunits. These monoclonal antibodies were shown to be useful in purification of biologically active PT and may be useful in assay of PT. P11B10 recognizes even denatured PT subunit S2 and may thus be a sensitive detection device for PT. P7B10 appears to recognize only intact, unaltered PT. It may thus serve to assess PT denaturation or subunit dissociation.

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