INFECTION AND IMMUNITY, Mar. 1982, p. 1032–1040
0019-9567/82/031032-09$02.00/0

Vol. 35, No. 3

Purification and Characterization of Toxins A and B of *Clostridium difficile*

N. M. SULLIVAN, S. PELLETT, AND T. D. WILKINS*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 17 August 1981/Accepted 30 October 1981

Toxin preparations were obtained by growing *Clostridium difficile* VPI strain 10463 in 2-liter brain heart infusion dialysis flasks at 37°C for 3 days. The initial step of the purification scheme involved ultrafiltration through an XM-100 membrane filter. Two toxic activities, designated toxins A and B, were separated by ion-exchange chromatography on DEAE-NaCl gradients. Toxin A was purified to homogeneity by an acetic acid precipitation at pH 5.5. Other separation techniques, including CM Sepharose CL-6B, $(NH_4)_2SO_4$ and acetic acid precipitations, and hydrophobic interaction chromatography, were examined in attempts to further purify toxin B. Although these methods failed to increase the specific activity of toxin B, they provided additional evidence that the two toxins are distinct molecules. The toxins are acid and heat labile and are inactivated by trypsin and chymotrypsin, but not by amylase. The molecular weight of toxin A, as estimated by gel filtration and gradient polyacrylamide electrophoresis, ranged from 440,000 to 500,000. The estimated molecular weight of toxin B was 360,000 to 470,000.

Intestinal disturbances are common side effects of antimicrobial therapy. Pseudomembranous colitis in humans and antibiotic-associated colitis in hamsters are diseases associated with the administration of clindamycin or other antibiotics (6–8, 17, 20). The causative agent of most of these diseases appears to be *Clostridium difficile* and its toxin (7, 10, 17, 30). Fecal filtrates from over 90% of patients with documented pseudomembranous colitis (6, 8) and cecal contents of hamsters injected with clindamycin are cytotoxic in tissue cultures and cause death when injected intracecally into normal hamsters (7, 8). The effects of the toxin are neutralized by antisera against partially purified *C. difficile* culture supernatants (16) and by a fortuitous cross-reaction with *C. sordellii* antisera obtained from the U.S. Bureau of Biologics (8, 10).

Methods for the partial purification of *C. difficile* toxin (3, 20, 26, 28, 28a, 30) have been described by research groups, and there are discrepancies in the characterization of the toxin. Recently, Bartlett et al. reported that two toxins are produced by *C. difficile*: one (toxin A) causes fluid accumulation in rabbit ileal loops and has been called an "enterotoxin," and the other (toxin B) has been described as primarily a cytotoxin (8, 28a; N. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res., **28**:285, 1980). We have found that both toxins are cytotoxic and animal lethal, although toxin B is at least 1,000-fold more cytotoxic than toxin A. Initial data indicate that each toxin is produced in sufficient concentrations in vitro and in vivo to cause cytopathic effects in tissue culture and to kill animals. At present, it is not known which toxin(s) is primarily responsible for the pathology seen in pseudomembranous colitis and antibiotic-associated colitis.

In this paper, we describe the purification and characterization of toxin A and the partial purification and characterization of toxin B.

MATERIALS AND METHODS

Bacterial strain. *C. difficile* VPI strain 10463 was grown in 2-liter brain heart infusion (dialysis flasks for 72 h at 37°C (3).

Chemicals and reagents. Column supports for ion-exchange chromatography (DEAE-Sepharose CL-6B and CM Sepharose CL-6B), hydrophobic interaction chromatography, concanavalin A 4B affinity chromatography, and gel filtration (Sepharose 6B) were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Reagents for the inactivation studies were obtained from Sigma Chemical Co., St. Louis, Mo., and included trypsin (bovine pancreatic, type III), chymotrypsin (bovine pancreatic), bacterial amylase (type IIa), and soybean inhibitor (type I-S).

Polyacrylamide gels were prepared from the following chemicals: electrophoresis pure acrylamide (Bio-Rad Laboratories, Richmond, Calif.), electrophoresis pure sodium dodecyl sulfate (Bio-Rad), electrophoresis-pure *N,N'*-methylenebisacrylamide (Eastman Organic Chemicals, Rochester, N.Y.), *N,N,N',N'*-tetramethyl-

ethylenediamine (Bio-Rad), and electrophoresis-pure ammonium persulfate (Isolab, Inc., Akron, Ohio).

Protein and chloride determination. Protein was quantitated by the Bio-Rad method (Bio-Rad) (9). Chloride concentrations were determined by titration, using Sigma chloride kit no. 830 (Sigma).

Cytotoxicity and animal lethality assays. The cytotoxicity assay was performed in a 92-well flat-bottom microtiter plate (Costar, Data Packaging, Cambridge, Mass.). Chinese hamster ovary cells (CHO-K1) (Flow Laboratories, Inc., McLean, Va.) were grown to confluency in F12 medium (Flow Laboratories) containing 10% fetal calf serum. The cells were trypsinized (0.025% trypsin), incubated for 5 min in 5% CO_2 at 37°C, and washed with two 5-ml volumes of F12 medium containing 2% fetal calf serum. Approximately 1,000 cells were dispensed per well. Samples of toxin preparations were titered in 2- or 10-fold dilutions within the wells after the cells had become fixed to the bottom of the well (3 h). The tissue culture dose was defined as the reciprocal of the highest dilution which rounded 100% of the CHO-K1 cells.

For the mouse lethality experiments, 6-week-old ICR male mice were used (Flow Laboratories). The toxin preparations were diluted in twofold dilutions in 50 mM Tris-hydrochloride buffer (pH 7.5), and 0.5 ml of each dilution was injected intraperitoneally. Deaths were recorded at 18 h. The LD_{100} was defined as the reciprocal of the highest dilution required to kill 100% of the mice injected.

Toxin purification. *C. difficile* VPI strain 10463 was grown in 12 2-liter dialysis culture flasks as previously described. After centrifugation at 8,000 × *g* for 10 min and filtration through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.), the culture supernatant (~750 ml) was concentrated to 50 ml by ultrafiltration at 4°C, using an XM-100 membrane filter (Amicon Corp., Lexington, Mass.) with a thin-channel type concentrator. The retentate was washed with 1,500 ml of 50 mM Tris-hydrochloride buffer (pH 7.5) at 4°C and concentrated to a final volume of 40 to 50 ml.

The concentrated supernatant was loaded onto a DEAE-Sepharose CL-6B column (2.5 by 10 cm) which had been equilibrated with 50 mM Tris-hydrochloride (pH 7.5). After the sample was loaded, the column was washed with 200 ml of 50 mM Tris-hydrochloride (pH 7.5) containing 0.05 M NaCl. The sample was eluted first with a 300-ml linear NaCl gradient in 50 mM Tris-hydrochloride buffer (0.05 to 0.25 M NaCl), followed by 150 ml of 50 mM Tris-hydrochloride (pH 7.5) containing 0.3 M NaCl. A second 300-ml linear gradient (0.3 to 0.6 M NaCl) in the same buffer followed the 0.3 M NaCl wash. The flow rate of the columns was 55 to 60 ml/h (gravity) at 4°C. Fractions (4.2 ml) were collected and assayed for cytotoxicity by using CHO-K1 cells.

The fractions containing the highest cytotoxic titers were pooled, filter sterilized, and stored at 4°C. The toxins that eluted in the first and second NaCl gradients were designated toxins A and B, respectively.

From 5 to 10 ml of the toxic fractions from the first DEAE gradient (toxin A) was dialyzed against 1 liter of 0.01 M sodium acetate buffer (pH 5.5) at 4°C for 18 to 24 h. The dialysate was centrifuged to recover the precipitate at 169 × *g* for 10 min, washed with 5 ml of the same acetate buffer, and recentrifuged. The precipitate was solubilized in 5 to 10 ml of 50 mM Tris-hydrochloride, (pH 7.5) containing 0.05 M NaCl, and the solution (toxin A) was filter sterilized and stored at 4°C.

PAGE. Davis gels with a 5% lower gel and an upper stacking gel (12) and gradient gels (2.5 to 27%) (Isolab) were used to demonstrate the removal of contaminating proteins during the purification procedure. Slab gels (7.5 by 7.5 by 0.3 cm) were used for both procedures. Davis gel samples contained approximately 100 μg of protein, 10% glycerol, and 1 to 2 μl of 0.05% bromphenol blue. The buffer system for the Davis gels was Tris-glycine (pH 8.4), whereas polyacrylamide gel electrophoresis (PAGE) with gradient gels was performed with Tris-borate (pH 8.4). For Davis gels, electrophoresis was performed at 60 V until the marker dye was at the interface between the stacker and the lower gel, then increased to 125 V until the dye had migrated to 0.5 cm from the bottom of the gel. The gradient PAGE gels were first preelectrophoresed for 30 min at 75 V before sample loading, then the voltage was increased to 125 V for 18 to 24 hours.

After electrophoresis, both types of PAGE gels were fixed with 10% trichloroacetic acid for 1 h and stained with 0.025% Coomassie brilliant blue R-250 in 10% acetic acid. The gels were destained with a 10% trichloroacetic acid solution.

Crossed IEP. Crossed immunoelectrophoresis (IEP) was also used as a method to demonstrated the removal of contaminating proteins during the purification procedure. Crossed IEP was performed with the LKB 2117 Multiphor apparatus and IEP kit (LKB Instruments, Inc., Rockville, Md.). The general methodology presented in the LKB instruction manuals and application notes 85 and 249 and in the quantitative IEP manual of Axelsen et al. (4) was followed. Gels were pressed, washed, stained, and destained. The antisera used for the second dimension was goat antisera produced against crude culture supernatants of *C. difficile* strain 10463 (16).

Elution of the toxins from PAGE gels. The two toxins were eluted from PAGE gels to localize the toxic activity for toxin B and to confirm that the purified toxin A was the major protein band. Samples of toxin A (600 μg) and toxin B (3.0 mg) were loaded onto an entire slab gel. When electrophoresis was completed, a longitudinal slice of the gel was cut, fixed, and stained with Coomassie blue as previously described. The remainder of the gels were cut horizontally in 3-mm slices, homogenized in 50 mM Tris-hydrochloride (pH 7.5) containing 0.05 M NaCl, and stored 18 to 24 h at 4°C. The fractions were then assayed for cytotoxic activity.

Gel filtration chromatography. Gel filtration chromatography was used as a possible method of purification. Samples (5 ml) of crude culture supernatant were put onto a column (2.5 by 7.0 cm) of Sepharose CL-6B and eluted at 4°C with 50 mM Tris-hydrochloride buffer (pH 7.5) at a flow rate of 25 to 30 ml/h.

Determination of native molecular weight. Descending chromatography, performed with a Sepharose CL-6B column (1.5 by 77 cm), was used for estimating the native molecular weights of the toxins (1). The K_{av} values of high molecular weight standard proteins (Pharmacia Fine Chemicals) aldolase, catalase, ferritin, and thyroglobulin, were used to obtain a standard curve. The cytotoxic titers of the fractions were used to locate the toxins for the calculation of their K_{av}

values, and the molecular weights of the two toxins were estimated from the standard curve.

Gradient polyacrylamide gels were also used to estimate the molecular weights of the toxins. Relative mobilities were calculated for each toxin. The toxins were detected by eluting the activity from the gels.

Ammonium sulfate precipitations. Ammonium sulfate precipitations were performed on crude culture supernatants to determine the precipitation properties of the two toxins. Finely ground ammonium sulfate was added slowly in 5% increments to crude culture supernatant (4°C) with gentle mixing. After each addition, the sample was mixed for 15 to 20 min, adjusted to pH 7.0 with 2 N NaOH, and then filtered through a 0.45-μm membrane filter. The samples were analyzed for cytotoxicity and animal toxicity.

Acetic acid precipitations. Samples (1.0 ml) of toxin A or B were dialyzed for 18 h at 4°C against 100-ml volumes of 0.01 M acetate buffer (pH 3.0 to 6.0) in 0.3-pH-unit increments. The dialysates were filtered with a 0.45-μm membrane filter, and the filter, which retained the precipitated material, was placed in 50 mM Tris-hydrochloride (pH 7.5). The precipitates and supernatant were assayed for cytotoxic activity and protein concentrations.

Hydrophobic interaction chromatography. Toxin A (5 ml; 0.6 mg of protein per ml) and toxin B (5 ml; 0.7 mg of protein per ml) were adsorbed separately onto phenyl-Sepharose 4B. In a batch procedure, the toxins were diluted 1:10 (vol/vol) in 0.01 M phosphate buffer (pH 6.8) containing 0, 5, 10, 15, or 30% ammonium sulfate and added to the gel. Each toxin was then eluted with increasing concentrations of ethylene glycol (0 to 50%). In addition, toxin samples were diluted 1:10 in 5% $(NH_4)_2SO_4$–0.01 M PO_4 buffer (pH 6.8) and put onto phenyl-Sepharose 4B columns (2.5 by 5 cm). The columns were washed with 100 ml of the same buffer, and a linear gradient was then applied consisting of 90 ml of 5% $(NH_4)_2SO_4$ and 90 ml of 50% ethylene glycol in 0.01 M PO_4 buffer (25). Fractions (2 ml) were collected and assayed for cytotoxicity.

Carboxymethyl-Sepharose 6B chromatography. Ion-exchange chromatography on carboxymethyl-Sepharose 6B was examined as a possible method of purification for both toxins. Batch techniques were used with two different buffer systems: 0.003 M phosphate buffer (pH 5.8) and 0.05 M acetate buffer (pH 3.8). Toxin A (2 ml) and toxin B (5 ml) were concentrated to 0.5 ml in a B15 minicon concentrator (Amicon) and diluted 1:10 with starting buffer. The columns (2.5 by 10.0 cm) were washed with 2 volumes of buffer, and samples were eluted by stepwise increases of sodium, and phosphate or acetate concentrations (18, 27).

Effects of storage at different temperatures. Acetic acid-precipitated toxin A (0.6 mg/ml), partially purified toxin B (DEAE; 0.6 mg/ml), and crude culture supernatant (1.4 mg/ml) were stored at −20, 4, 20 to 25, and 37°C. Samples were examined for loss of cytotoxicity daily for 1 week and then once per week for 4 weeks. The toxins were also incubated at 56°C, and samples were taken at time intervals (0, 6, 10, 15, 30, 60, and 120 min) and examined for loss of cytotoxic activity.

Effects of pH. Purified toxin A and partially purified toxin B (both 0.6 mg/ml) were diluted 1:10 in the following pH buffer systems and incubated for 2 h at room temperature: 0.2 M glycine-HCl (pH 2.0), 0.05 M acetate (pH 4.0), 0.05 M Tris-hydrochloride (pH 7.5), and 0.2 M glycine-NaOH (pH 10.0). The samples then were diluted 1:10 in 0.05 M Tris-hydrochloride buffer (pH 7.5) and examined for loss of activity by the cytotoxicity and animal lethality assays.

Enzymatic inactivation of the toxins. Bacterial amylase, trypsin, and chymotrypsin were examined for their effect on the toxins. To demonstrate that the enzymes were active before the toxin inactivation experiments, standard enzyme assays were performed by the methods described in the *Worthington Enzymes Manual* (Worthington Biochemicals Corp., Freehold, N.J.). Trypsin and chymotrypsin were mixed in approximately equimolar amounts with the toxins and incubated at 37°C for 24 h. Samples were taken for cytotoxicity assays at different time intervals during the incubation period. The final concentrations of the toxins were 0.3 mg/ml. Soybean trypsin inhibitor was added to each sample before determination of cytotoxicity.

The concentrations used for bacterial amylase were 10.0, 1.0, and 0.1 mg/ml. Equal volumes of the amylase concentrations and toxins (0.6 mg/ml) were mixed and incubated for 2 and 24 h at 25°C. A tube containing toxin (0.6 mg/ml) and buffer was used as a control. Samples were examined for loss of cytotoxicity.

TABLE 1. Purification of toxins A and B of *C. difficile*

Stage	Purification step	Vol (ml)	Protein		Cytotoxicity		Sp act (CU/mg)	Starting material (%)	Fold purification
			mg/ml	Total mg	CU/ml[a]	Total CU			
1	Crude culture supernatant	750	1.4	1,090	5.8×10^{8}[b]	4.4×10^{11}	4.0×10^{8}	100	
2	Ultrafiltration, XM-100	42	12.4	525	7.2×10^{9}[b]	3.0×10^{11}	5.7×10^{8}	70	1.4
3	CL-6B DEAE								
	Toxin A	48	2.0	96	3.0×10^{6}	1.5×10^{8}	1.5×10^{6}		
	Toxin B	24	0.6	14.4	3.2×10^{9}	7.7×10^{10}	5.3×10^{9}	18	13
4	Acid precipitation, toxin A	48	0.6	28.8	2.3×10^{6}	1.1×10^{8}	3.8×10^{6}		2.6[c]

[a] CU/ml, 100% tissue culture dose.
[b] Values for toxin B only.
[c] Fold purification (toxin A only) after stage 3.

Compositional analyses of the toxins. Samples of toxin A (0.5 ml) and toxin B (0.6 mg/ml) were examined for the presence of hexose (15), pentose (14), and phosphorus (13).

Because the cytotoxin (toxin B) was previously reported to be a glycoprotein (26), a concanavalin A column (0.5 by 3.0 cm) was used in an attempt to adsorb toxin B (2, 24). A 0.5-ml sample (0.2 mg of protein per ml) in 50 mM acetate buffer (pH 5.8) containing 0.9% NaCl was loaded onto the column and washed with 20 ml of the same buffer. Fractions (0.5 ml) were collected and assayed for cytotoxicity.

Protease assays. Samples (0.1 ml) of crude culture filtrate (1 mg/ml) and toxins A and B were examined for protease activity, with azocasein (Sigma) as the substrate (20).

Production of purified toxin A antiserum. Antiserum was raised in a goat against a Formalin-treated preparation of acetic acid-precipitated toxin A by the methods previously described (16). The goat received a subcutaneous injection of 1.5 ml of the toxoid once per week over a period of 8 weeks. The antiserum was examined by (i) neutralization tests in CHO-K1 cells (16) and (ii) by crossed IEP by using crude culture supernatants in the first dimension.

RESULTS

Toxin purification. The cytotoxic activity of the cell-free culture supernatant (crude culture supernatant) was 5.8×10^8 cytotoxic units (CU) per ml. Toxin B was at least 1,000-fold more cytotoxic than toxin A; therefore, the cytotoxicity owing to toxin A in crude culture supernatants could not be determined (Table 1).

The specific activities of the toxins at each step in the purification scheme are given in Tables 1 and 2. There was an initial loss of about 30% of the cytotoxicity (toxin B) when the crude culture filtrate was concentrated with the thin-channel ultrafiltration device. Active cytotoxin did not go through the ultrafilter, so we assume the loss was owing to either binding to the filter or inactivation.

The DEAE-Sepharose CL-6B column separated two toxin activities in *C. difficile* culture supernatant which we have designated toxin A and toxin B (Fig. 1). Most of the protein eluted in the first gradient immediately after toxin A, so we sacrificed some of the toxin A peak to achieve greater purification. When this was done carefully, both PAGE and crossed IEP showed that many of the contaminating proteins were eliminated (Fig. 2 and 3). Approximately six protein bands were observed in the toxin A preparation after ion-exchange chromatography when fractions were pooled carefully; at least six additional bands were observed when the entire cytotoxic peak was included. Toxin A was further purified by precipitating the toxin in acetate buffer at pH 5.5. Under these conditions, the remaining contaminating proteins remained in solution and the precipitated toxin A could be obtained by centrifugation. Analysis of the acid-precipitated toxin A by PAGE and crossed IEP (Fig. 2 and 3) revealed that the preparation was homogeneous and that the toxic activity was associated with the protein band (Fig. 4).

Toxin B presented a greater problem. We had a much higher specific activity in the toxin B fraction after the DEAE-Sepharose CL-6B column than had been reported previously for purified cytotoxin, yet our preparation obviously contained several contaminants (Fig. 2). We are not sure which, if any, of the protein bands represents the toxin, since the toxicity was eluted from an area with at least three protein bands (Fig. 4). Crossed IEP also showed several contaminants (Fig. 3). We have tried many different methods to further purify toxin B, but in all cases the specific activity did not increase and often decreased because of the instability of the toxin.

Biological activity. Both purified toxin A and the toxin B preparations were cytotoxic and caused the same morphological alterations in CHO-K1 cells. Toxin B was at least 1,000-fold more cytotoxic than toxin A; the cytotoxicity of toxin A was not because of slight contamination with toxin B, since specific antiserum to toxin B (21a) did not neutralize this cytotoxicity. The specific activities of toxins A and B were 3.8×10^6 CU/mg and 5.3×10^9 CU/mg, respectively (Table 1). The specific activities of toxins in the mouse lethality assay were 6.4×10^3 LD_{100}/mg for toxin A and 2.1×10^4 LD_{100}/mg for toxin B (Table 2).

Molecular weight determinations. The K_{av} val-

TABLE 2. Mouse lethality of toxins A and B during purification

Stage	Purification step	Animal toxicity (LD_{100}/ml)	Sp act (LD_{100}/mg)
1	Crude culture supernatant	3.2×10^3	1.6×10^3
2	Ultrafiltration, XM-100[a]		
3	CL 6B DEAE		
	Toxin A	1.3×10^4	6.4×10^3
	Toxin B	1.3×10^4	2.1×10^4
4	Acid precipitation, toxin A	6.4×10^3	1.1×10^4

[a] Animal toxicities were not done on this stage.

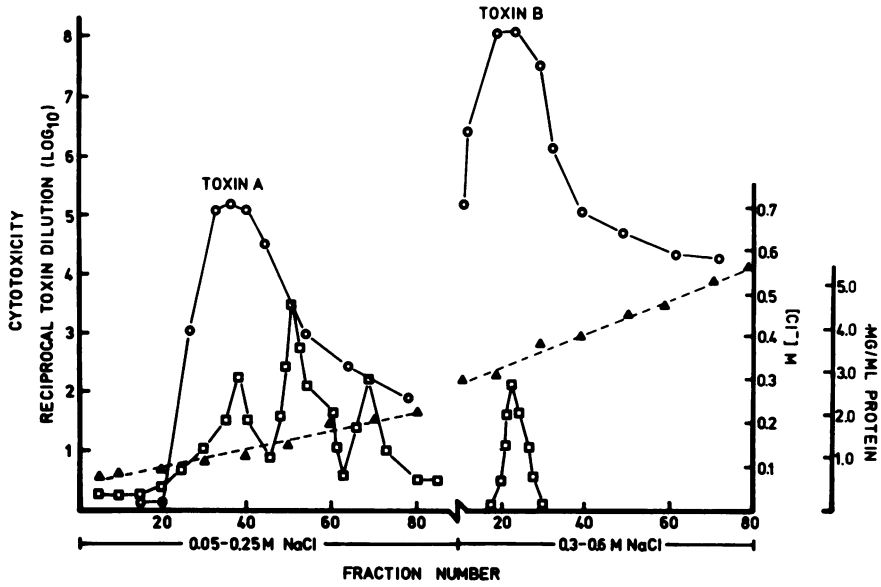

FIG. 1. Separation of toxins A and B by ion-exchange chromatography. A sample (42 ml) of concentrated culture supernatant was loaded onto a column (2.5 by 10.0 cm) of DEAE-Sepharose CL-6B, and toxins A and B were eluted with two linear NaCl gradients (0.05 to 0.25 M NaCl and 0.3 to 0.6 M NaCl). A 150-ml wash with 50 mM Tris-hydrochloride (pH 7.5) containing 0.3 M NaCl followed the first gradient. Symbols: ○, cytotoxicity (100% tissue culture dose); □, protein concentration; and △, chloride molarity.

ues of both toxins, as determined by gel filtration, corresponded to a molecular weight of approximately 440,000 to 500,000.

The relative mobility, as determined by gradient PAGE, indicated that toxin A had a molecular weight of 550,000, whereas the molecular weight of toxin B was 360,000.

Difference in the properties of toxins A and B. Various methods including $(NH_4)_2SO_4$ precipitation, acetic acid precipitation, hydrophobic interaction chromatography, CM-Sepharose 6B chromatography, and agarose electrophoresis were examined to determine whether they increased the specific activity of toxin B. Although these methods did not result in increased purification of toxin B, they did demonstrate distinct differences between toxins A and B (Table 3).

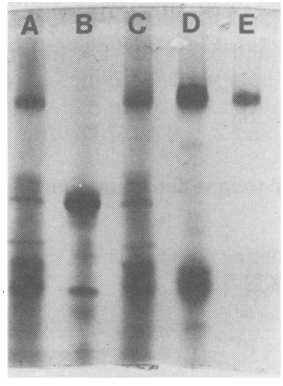

FIG. 2. Analysis of toxin preparations by electrophoresis in 5% Davis gels. Samples (100 μg of protein) include: (A) crude culture supernatant, (B) toxin B (DEAE), (C) crude culture supernatant, (D) toxin A (DEAE), and (E) toxin A (acetic acid precipitation).

Stability of toxins A and B at different temperatures and pH. Both toxins were stable from −20 to 37°C for up to 4 weeks. There was a 99% loss of activity within 6 min at 56°C for both toxins.

Toxin A was stable at pH 4 and 10, whereas toxin B was not stable at either pH. Both toxins were inactivated at pH 2.0.

Trypsin and chymotrypsin inactivation. Trypsin and chymotrypsin inactivated both toxins. There was no activation nor interconversion of one toxin to the other. *C. difficile* has been reported to be weakly proteolytic (19); however, we could not detect proteolytic activity in the culture supernatant or in the toxin preparations with the azocasein method.

Tests for carbohydrates and phosphorus. Rolfe and Finegold (26) has reported that the cytotoxin (toxin B) appeared to be a glycoprotein based on the inactivation of the toxin by large amounts of bacterial amylase (10 mg/ml). We were unable to demonstrate any inactivation of our toxin A or B preparations even at the unusually high-test concentration of 5 mg of the enzyme per ml. A small amount of hexose (24 μg/mg of protein) and pentose (60 μg/mg of protein) was found in the

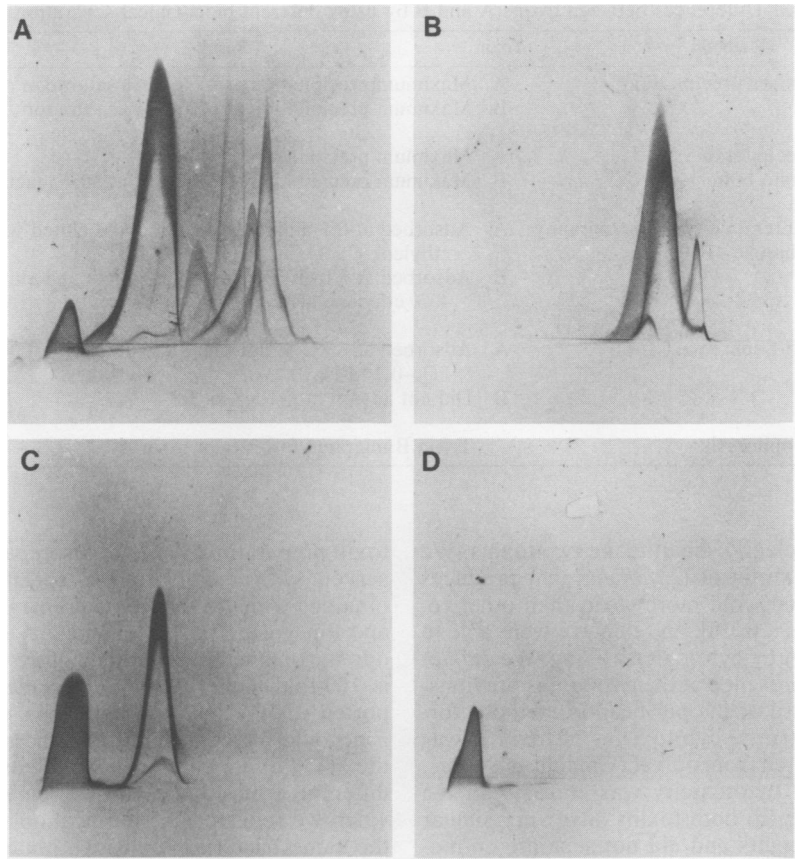

FIG. 3. Analysis of toxin preparations by crossed IEP. All of the wells in the first dimension initially contained 50 μg of protein. The upper part of each gel contained 125 μl of antiserum prepared against *C. difficile* strains 10463 culture supernatant. (A) Crude culture supernatant, (B) toxin B (DEAE), (C) toxin A (DEAE), and (D) toxin A (acetic acid precipitation).

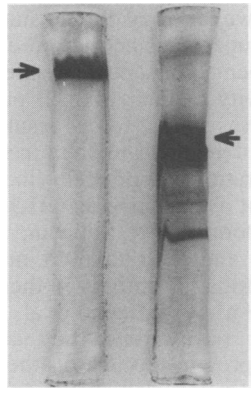

FIG. 4. Localization of cytotoxic activity of toxins A and B on 5% Davis slab gels. Samples (600 μl) of toxin A (600 μg) and toxin B (3.0 mg) were layered across the surface of the gels. One vertical slice was fixed and stained. The other gel was sliced horizontally into 3-mm sections, and the sections were assayed for cytotoxic activity. Cytotoxic activities are indicated by arrows.

toxin B preparation (0.6 mg/ml), but toxin B was not adsorbed when passed through a concanavalin A column. Toxin A did not have detectable amounts of hexose or pentose. Neither toxin preparation contained detectable phosphorus.

Specificity of toxin A antiserum. Neutralization tests were done with the purified toxin A antiserum. Toxin A activity was neutralized with the antiserum to a titer of 520 to 1,040. There was no detectable neutralization of toxin B activity.

Analysis of the antiserum by crossed IEP showed the presence of only one precipitin band which corresponded to the toxin A band.

DISCUSSION

Our results confirm the observation of Bartlett et al. that *C. difficile* produces at least two toxins (8). Other research groups had not separated these two toxins previously, both because the strains of *C. difficile* that were used produced amounts of toxin A which were not detectable by cytotoxicity assay and because the methods

TABLE 3. Differences between toxins A and B by using different biochemical separation methods

Method	Toxin	Result
Ammonium sulfate precipitation	A	Maximum precipitation at 45 to 50% saturation
	B	Maximum precipitation at 60 to 65% saturation
Acetic acid precipitation (0.01 M acetate buffer)	A	Maximum precipitation at pH 5.5
	B	Maximum precipitation at pH 3.8 (but 90% inactivated)
Hydrophobic interaction chromatography (phenyl-Sepharose 4B)	A	Adsorbed at 0% ammonium sulfate and eluted with 20 to 25% ethylene glycol
	B	Adsorbed at 5 to 10% ammonium sulfate and eluted with 35 to 40% ethylene glycol
Carboxymethyl-Sepharose CL-6B	A	Adsorbed at pH 5.8, not pH 3.8; eluted with 0.04 M PO_4–0.2 M NaCl
	B	Did not adsorb at pH 5.8 or 3.8
Agarose electrophoresis		Toxin B migrated faster than toxin A

used did not clearly separate the two toxins. We have used a strain of *C. difficile* that produces 1,000- to 10,000-fold more toxin than other reported strains (26, 28), and thus we were able to detect toxin A by cytotoxicity assay. We did not observe the presence of the two toxins previously (29) because of the purification methods formerly used: (i) the cytotoxicity of toxin B was not easily eluted from polyacrylamide gels; thus, the observed cytotoxicity was eluted with the toxin A band; (ii) both toxins have very similar molecular weights and did not separate on molecular sieve columns; and (iii) the brand of DEAE ion-exchanger used (Biogel A) did not clearly separate the two toxins.

The toxin that our research group had earlier obtained in almost pure form (29), as we now know, was toxin A containing as a contaminant about 1% toxin B. The contaminating toxin B accounted for the greater cytotoxic specific activity of that preparation compared to the present toxin A preparation. The degree of purity of a protein is based on the ability to detect contaminants. Therefore, because we could not detect contaminants in the pH 5.5 precipitated toxin A preparation by two types of acrylamide electrophoresis and by the rigorous test of crossed IEP, we have referred to this preparation as purified toxin A.

We have attempted to reproduce results which have been reported by Rolfe and Finegold (26) on the purification of the cytotoxin (toxin B) by using the same strain of *C. difficile* and separation techniques that were described in their paper. As reported, a major protein band was obtained on PAGE, however, analysis by crossed IEP indicated that the major band was not the cytotoxin. Crossed IEP with monospecific toxin B antibodies showed that the cytotoxin was associated with a minor component of the toxin preparation. Another discrepancy was observed with regard to the specific activities obtained with the final toxin preparation of Rolfe and Finegold. The specific activity of toxin B in our starting material (crude culture supernatant) is 100-fold higher than the specific activity reported for the final purified toxin of Rolfe and Finegold. We considered that the sensitivity of the WI38 tissue culture cells they used might be different from our CHO-K1 cells; however, when we repeated the purification scheme with the same bacterial strain, we obtained specific activities similar to those which they reported.

After the completion of this work, we discovered that another group had also reported isolation of two toxins from *C. difficile* (5), and we noted several discrepancies. One discrepancy was that their D-2 (toxin B) preparation did not kill mice; however, they did not state how much toxin B was injected, and therefore their results may have been a concentration effect. The final specific activity of their toxin B preparation in cell culture was similar to our results; however, they did not detect cytotoxicity in their toxin A (D-1) preparations. The cell line they used (HeLa) is not as sensitive as CHO-K1 cells for toxin A (S. Donta and N. Sullivan, submitted for publication), and their toxin A may not have produced cytopathic activity in their assay. Davis gels were used by Banno et al. as their only criterion for purity, and they analyzed low amounts of protein (20 μg) on these gels. Our toxin B preparation also shows only one major protein band when this amount of protein is examined. Thus, we feel that the cytotoxin of *C. difficile* (toxin B) has not been purified to date.

Other fractionation methods for proteins, including ion-exchange chromatography, acetic acid and $(NH_4)_2SO_4$ precipitation, and hydrophobic interaction chromatography were exam-

ined for further purification of toxin B. None of these methods resulted in an increase in specific activity, and in many cases, a decrease was observed. Currently we are attempting to purify toxin B by affinity chromatography on a column of Sepharose 4B with immobilized gamma globulin specific for toxin B.

Three standard sodium dodecyl sulfate-PAGE procedures, using combinations of sodium dodecyl sulfate, urea, and guanidine-hydrochloride (22, 23, 29), were used in an attempt to determine the subunit structure of toxin A. Under these conditions, toxin A was not dissociated into smaller units (J. Libby, unpublished data). These results were confirmed by another research group (J.-S. Chen and K. Blanchard, personal communication). Because of the large mass of toxin A, we believe it is composed of several subunits, and we are currently investigating other procedures for the dissociation of toxin A into subunits.

The difficulty in separating the two toxins, and their similarities, initially led us to believe that they were two interchangeable forms of the same toxin. This appears much less likely because Bartlett et al. obtained antiserum that neutralized only toxin A (8) and we have now produced antisera to toxins A and B separately which neutralized the respective toxin and do not cross-react with each other (21a). There are several procedures (Table 3) that separate the toxins, and we have not observed the conversion of toxin A or B into the other during any of these manipulations. In addition, toxin A also causes fluid influx into the rabbit ileal loop, whereas toxin B does not (7, 21b), and toxin B is a more potent cytotoxin with at least a 1000-fold-greater specific activity than toxin A. This is further evidence which supports the idea that *C. difficile* produces two distinct toxins. It has previously been reported that toxin A is several-fold more lethal for mice than toxin B (8). However, we have found that toxin B is more lethal for mice than toxin A per milligram of protein. With the purified toxin A and the monospecific gamma globulin we now have, we hope to determine the mechanisms of action of these toxins both on tissue culture cells and the colonic mucosa.

ACKNOWLEDGMENTS

We thank David Lyerly for performing the IEP analyses and J. Libby, J.-S. Chen, and K. Blanchard for performing the sodium dodecyl sulfate-PAGE studies.

These studies were supported by Public Health Service grant AI15749-03 from the National Institute of Allergy and Infectious Disease and in part by the State of Virginia.

LITERATURE CITED

1. **Andrews, P.** 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
2. **Aspberg, K., and J. Porath.** 1970. Group-specific adsorption of glycoproteins. Acta Chem. Scand. **24**:1839–1841.
3. **Aswell, J. E., M. Ehrich, R. Van Tassell, G. C. Tsai, L. V. Holdeman, and T. D. Wilkins.** 1979. Characterization and comparison of *C. difficile* and other clostridial toxins, p. 272–275. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
4. **Axelsen, N. H., J. Krøll, and B. Weeke (ed.).** 1973. A manual of quantitative immunoelectrophoresis. Methods and applications. Universitesforlaget, Oslo, Norway.
5. **Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2**:629–635.
6. **Bartlett, J. G., T.-W. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. New Engl. J. Med. **298**:531–534.
7. **Bartlett, J. G., A. B. Onderonk, R. L. Cisneros, and D. L. Kasper.** 1977. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. J. Infect. Dis. **136**:701–705.
8. **Bartlett, J. G., N. S. Taylor, T.-W. Chang, and J. Dzink.** 1980. Clinical and laboratory observations in *Clostridium difficile* colitis. Am. J. Clin. Nutr. **33**:2521–2526.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
10. **Chang, T.-W., J. G. Bartlett, S. L. Gorbach, and A. B. Onderdonk.** 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect. Immun. **20**:526–529.
11. **Chang, T.-W., M. Lauerman, and J. G. Bartlett.** 1979. Cytotoxicity assay in antibiotic-associated colitis. J. Infect. Dis. **140**:765–770.
12. **Davis, B. J.** 1965. Disc electrophoresis. II. Methods and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
13. **DeSiervo, A. J.** 1969. Alterations in the phospholipid composition of *Escherichia coli* B during growth at different temperatures. J. Bacteriol. **100**:1342–1349.
14. **Dische, Z.** 1949. Spectophotometric method for the determination of free pentose and pentose in nucleotides. J. Biol. Chem. **181**:379–392.
15. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colormetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
16. **Ehrich, M., R. L. Van Tassell, J. M. Libby, and T. D. Wilkins.** 1980. Production of *Clostridium difficile* antitoxin. Infect. Immun. **28**:1041–1043.
17. **Fekety, R., J. Silva, R. A. Browne, G. D. Rifkin, and J. R. Ebright.** 1979. Clindamycin-induced colitis. Am. J. Clin. Nutr. **32**:244–250.
18. **Hauschild, A. H. W., and R. Hilsheimer.** 1971. Purification and characteristics of the enterotoxin of *Clostridium perfringens* type A. Can. J. Microbiol. **17**:1425–1432.
19. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
20. **Humphrey, C. D., C. W. Condon, J. R. Cantey, and F. E. Pittman.** 1979. Partial purification of a toxin found in hamsters with antibiotic-associated colitis. Gastroenterology **76**:468–476.
21. **Kreger, A. S., and L. D. Gray.** 1978. Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of pseudomonal protease-induced rabbit cornial damage. Infect. Immun. **19**:630–648.

21a. **Libby, J. M., and T. D. Wilkins.** 1982. Production of antitoxins to two toxins of *Clostridium difficile* and immunological comparison of the toxins by crossed-neutralization studies. Infect. Immun. **35**:374–376.

21b. **Lyerly, D. M., D. E. Lockwood, S. J. Richardson, and**

T. D. Wilkins. 1982. Biological activities of toxins A and B of *Clostridium difficile*. Immun. **35**:1147–1150.

22. **Matheka, H. D., P. J. Enzmann, H. L. Bachrach, and B. Migl.** 1977. The influence of sodium dodecyl sulfate from different sources on the separation of virus proteins in polyacrylamide gel electrophoresis. Anal. Biochem. **81**:9–17.
23. **Moldow, C., J. Robertson, and L. Rothfield.** 1972. Purification of bacterial membrane proteins. J. Membr. Biol. **10**:137–152.
24. **Murthy, R., and A. Herez.** 1973. Purification of human α_1-antitrypsin by affinity chromatography on Sepharose bound Concanavalin A. FEBS Lett. **32**:243–246.
25. **Ochoa, J. L.** 1978. Hydrophobic (interaction) chromatography. Biochemistry **60**:1–45.
26. **Rolfe, R. D., and S. M. Finegold.** 1979. Purification and characterization of *Clostridium difficile* toxin. Infect. Immun. **25**:191–201.
27. **Shannon, L. M., E. Kay, and J. Y. Lew.** 1966. Peroxidase isoenzymes from horseradish roots. I. Isolation and physical properties. J. Biol. Chem. **241**:2166–2172.
28. **Taylor, N. S., and J. G. Bartlett.** 1979. Partial purification and characterization of a cytotoxin from *Clostridium difficile*. Rev. Infect. Dis. 1:379–385.

28a. **Taylor, N. S., G. M. Thorne, and J. G. Barlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.

29. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
30. **Wilkins, T. D., M. Ehrich, R. Van Tassell, and E. Balish.** 1980. Studies on the clostridial toxin associated with antibiotic-induced pseudomembranous colitis, p. 411–417. *In* D. Eaker and T. Wadström (ed.), Natural toxins. Pergamon Press, Inc., Elmsford, N.Y.