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Purification and Characterization of *Clostridium difficile* Toxin

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Recent evidence indicates that toxigenic *Clostridium difficile* strains are a major cause of antimicrobial-associated ileocecitis in laboratory animals and pseudomembranous colitis in humans. *C. difficile* ATCC 9689 was cultivated in a synthetic medium to which 3% ultrafiltrated proteose peptone was added. Purification of the toxin from broth filtrate was accomplished through ultrafiltration (100,000 nominal-molecular-weight-limit membrane), precipitation with 75% $(NH_4)_2SO_4$, and chromatographic separation using Bio-Gel A 5m followed by ion-exchange chromatography on a diethylaminoethyl-Sephadex A-25 column. The purified toxin displayed only one band on polyacrylamide gel electrophoresis, and approximately 170 pg was cytopathic for human amnion cells. The isolated toxin was neutralized by *Clostridium sordellii* antitoxin, heat labile (56°C for 30 min), and inactivated at pH 4 and 9; it had an isoelectric point of 5.0, increased vascular permeability in rabbits, and caused ileocecitis in hamsters when injected intracecally. Treatment of the toxin with trypsin, chymotrypsin, pronase, amylase, or ethylmercurithiosalicylate caused inactivation, whereas lipase had no effect. By gel filtration, its molecular weight was estimated as 530,000. Upon reduction and denaturation, the toxin dissociated into 185,000- and 50,000-molecular-weight components, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Extensive dissociation yielded only the 50,000-molecular-weight component. The toxin appears to be protoplasmic and is released into the surrounding environment upon autolysis of the cells. Attempts to correlate specific enzymatic activity with the toxin have been unsuccessful. These studies will help delineate the role of *C. difficile* toxin in antimicrobial-associated colitis and diarrhea.

The occurrence of diarrhea in patients being administered certain antimicrobial agents is a well-recognized problem in clinical medicine. The severity of this diarrhea ranges from a mild, self-limiting process to a fulminating colitis. Chemotherapeutic agents often associated with diarrhea or colitis or both include penicillin (31), ampicillin (20, 26), cephalexin (33), lincomycin (41, 50), clindamycin (20, 28, 34, 39, 50), tetracycline (19), chloramphenicol (19), and co-trimoxazole (7). In recent years, clindamycin, lincomycin, and ampicillin have been the agents most frequently associated with this disease and, thus, the antibiotics which have been examined the most extensively (3, 19, 36).

There have been many theories to account for antimicrobial-associated colitis (17). Recent evidence has indicated that a toxin-producing bacterium located in the intestinal tract may be the cause of antimicrobial-associated colitis. This has been shown through two divergent lines of research. Stools obtained from patients with antimicrobial-associated colitis are cytopathic in tissue culture, and this toxicity is neutralized by polyvalent gas gangrene and *Clostridium sordellii* antitoxins (2, 4, 32, 42). Toxigenic strains of *Clostridium difficile* have been isolated from the stools of many patients with antimicrobial-associated colitis, and the toxin produced by this organism was similarly neutralized by polyvalent gas gangrene and *C. sordellii* antitoxins (4, 15, 18). The other line of evidence for a bacterial etiological agent came from work with hamsters (5, 8). Administration of clindamycin to hamsters causes the proliferation of a toxigenic strain of *C. difficile* in the intestinal tract of the animals, leading to acute ileocecitis. Ileocecitis was consistently produced in hamsters by intracecal injection of whole cells or cell-free broth filtrate of *C. difficile* (5). *C. difficile* toxin obtained from both humans and hamsters displayed identical characteristics: acid and alkaline lability, heat

lability (56°C for 30 min), non-dialyzability, neutralization by *C. sordellii* antitoxin, and trypsin sensitivity.

In this investigation we report a method for purifying the cytotoxin produced by *C. difficile* and define parameters which are important for its production. The isolated toxin was characterized chemically and biologically.

MATERIALS AND METHODS

Effect of culture conditions on growth and toxin production. Different media were examined for their ability to support growth and toxin production by *C. difficile* ATCC 9689. The following broth media were examined: chopped-meat-glucose (23), peptone-yeast extract (23), peptone-yeast extract-glucose (23), brain heart infusion (BBL Microbiology Systems, Cockeysville, Md.), thioglycolate (47) (Difco Laboratories, Detroit, Mich.), and thioglycolate with 0.6% glucose (47) (BBL Microbiology Systems). Also examined was a synthetic medium devised by Varel and Bryant (49) for the growth of *Bacteroides fragilis*. The synthetic basal medium consisted of the following components per liter of distilled water: glucose, 5 g; KH_2PO_4, 0.9 g; NaCl, 0.9 g; $CaCl_2 \cdot 2H_2O$, 0.026 g; $MgCl_2 \cdot 6H_2O$, 0.02 g; $MnCl_2 \cdot 4H_2O$, 0.01 g; $CoCl_2 \cdot 6H_2O$, 0.001 g; hemin, 0.001 g; $FeSO_4 \cdot 7H_2O$, 0.004 g; $(NH_4)_2SO_4$, 0.04 g; cysteine·HCl, 0.5 g; sodium carbonate, 4.0 g; thiamine hydrochloride, 1 mg; calcium-D-pantothenate, 1 mg; nicotinamide, 1 mg; riboflavin, 1 mg; pyridoxine hydrochloride, 1 mg; *p*-aminobenzoic acid, 50 μg; biotin, 12.5 μg; folic acid, 12.5 μg; and vitamin B_{12}, 5 μg. This medium was prepared as described previously (49). To examine the effects of amino acids on growth and toxin production, the following were added to 1 liter of basal medium: 0.1 g each of L-histidine hydrochloride, L-tryptophan, glycine, L-tyrosine; 0.2 g each of L-arginine hydrochloride, L-phenylalanine, L-methionine, L-threonine, and L-alanine; 0.3 g each of L-lysine, L-serine, L-valine, L-isoleucine, L-proline, and L-aspartic acid; 0.4 g of L-leucine; and 0.9 g of L-glutamic acid. Commercially available nutrients were individually added to the basal medium with subsequent analysis for growth and toxin production. These included 1% solutions (wt/vol) of proteose peptone no. 3 (Difco Laboratories), peptone (Difco Laboratories), yeast extract (BBL Microbiology Systems), proteose peptone no. 3 with yeast extract, tryptone (Difco Laboratories), casein hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio), phytone (BBL Microbiology Systems) and Casamino Acids (Difco Laboratories). In addition to a 1% concentration, proteose peptone no. 3 was examined at concentrations (wt/vol) of 0.5, 2.0, 3.0, and 5.0%. Brain heart infusion (43 g/liter) and proteose peptone no. 3 (30 g/liter) broths were prepared in distilled water and subjected to positive-pressure ultrafiltration using a 90-mm Hi-Flux cell with a PTHK (100,000 nominal molecular weight limit [NMWL]) membrane (Millipore Corp., Bedford, Mass.). The resulting filtrates were used in place of distilled water for preparation of the basal medium.

Preparation of broth filtrates and cell extracts. Colonies of *C. difficile* ATCC 9689 from a blood agar plate were inoculated into 10.0 ml of each medium described above and incubated overnight (18 h). One milliliter of each culture was then used to inoculate 100.0 ml of the same medium with subsequent incubation for 72 h. All incubations were at 37°C under anaerobic conditions (80% nitrogen, 10% carbon dioxide, and 10% hydrogen). Broth filtrates were prepared by centrifuging each culture at 12,000 × *g* for 30 min. The supernatant was passed through a 0.45-μm membrane filter, divided into aliquots, and frozen at −70°C. The cellular pellet was washed several times in cold 50 mM potassium phosphate buffer at pH 7.0. The packed cells were resuspended in buffer, placed in a 25-ml rosette cell (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), and sonically treated in an ice bath with a Branson Sonifier (Branson Ultrasonics Corp., Danbury, Conn.), equipped with a microtip adaptor, at a 100-W power setting. The sonic disruption was performed in 2-min bursts, each followed by a 3-min cooling period. Cell breakage was continued until 90% or more of the cells were ruptured. The temperature of the suspension did not exceed 11°C during this procedure. Cellular debris was removed by centrifugation at 20,000 × *g* for 20 min. The supernatant was divided into aliquots and stored at −70°C.

Growth curve and toxin production. *C. difficile* ATCC 9689 was inoculated into 10.0 ml of basal medium supplemented with ultrafiltered (100,000-NMWL membrane) proteose peptone no. 3 (BMP broth). After anaerobic incubation at 37°C for 12 h, 0.1 ml was inoculated into 100.0 ml of freshly prepared, sterile BMP broth. During subsequent incubation, aliquots were removed for analysis of broth filtrate and cell extract cytotoxic titers, viable cell count, absorbance at 500 nm, and heat-resistant spore population. Dilutions for viable cell count were prepared in 0.05% yeast extract solution with subsequent plating on brucella blood agar plates. The total viable heat-resistant spore population was determined by heating aliquots at 80°C for 10 min and plating on blood agar.

Tissue culture. Cytotoxicity was determined in human amnion cell cultures (Microbiological Associates, Bethesda, Md.). Cells were received as monolayers in 150-cm^2 flasks. The monolayer was removed by trypsinization (0.25% trypsin in calcium- and magnesium-free Hanks balanced salt solution), counted in a Neubauer chamber, and suspended in medium 199 containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. One milliliter of the cell suspension, diluted to a final concentration of 75,000 cells per ml, was dispensed into each well of a plate containing 24 16-mm-diameter wells (Microbiological Associates). After 24 h of incubation at 37°C in a 5% CO_2 atmosphere, each well had a confluent monolayer of cells. The medium was replaced with medium 199 containing 2% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Samples analyzed for toxicity were introduced directly into each well (50 μl/well). At 24 and 48 h after exposure to toxin, each well was examined for evidence of cytotoxicity (increased refractility, change in morphology, or partial loss of adherence). The reciprocal of the highest dilution resulting in complete rounding of the cells was defined as the number of cytotoxic units (CU) per 50-μl sample. Specific cytotoxic activity

was expressed as CU per milligram of protein. Antitoxin neutralization was performed by mixing equal volumes of antitoxin and toxin and incubating at room temperature for 60 min, followed by cytotoxicity determination. Antitoxins examined included polyvalent gas gangrene antitoxin (Lederle Laboratories, Pearl River, N.Y.), as well as the individual U.S. standard clostridial antitoxins present in the polyvalent antitoxin (*C. perfringens*, *C. septicum*, *C. histolyticum*, *C. oedematiens*, and *C. sordellii*) (kindly provided by E. Seligmann, Food and Drug Administration, Bureau of Biologics, Rockville, Md.).

Toxin purification. Broth filtrate from a 24-h culture of *C. difficile* ATCC 9689, grown in BMP broth, was prepared and subjected to pressure ultrafiltration at 4°C using a 90 mm Hi-Flux cell with a PTHK (100,000 NMWL) membrane (Millipore Corp.). The retentate was brought to 75% $(NH_4)_2SO_4$ saturation by the slow addition of solid $(NH_4)_2SO_4$. During addition of $(NH_4)_2SO_4$, the pH was maintained at 7.0 through the addition of 1 N NaOH. The precipitate resulting after standing overnight was harvested by centrifugation at 15,000 × *g* for 30 min, resuspended in 50 mM potassium phosphate buffer at pH 7.0, and extensively dialyzed against the same buffer.

A 5.0-ml portion of the above solution was applied to a Bio-Gel A 5m column and equilibrated with 50 mM potassium phosphate buffer at pH 7.0. The sample was eluted under constant pressure at a flow rate of 20 ml/h. The effluent was monitored for optical absorbance at 280 nm, with 5.0-ml fractions collected. Each fraction was titrated for cytotoxicity, and those fractions displaying toxicity were pooled and concentrated to 5.0 ml under positive pressure with an Amicon ultrafiltration cell with a UM 10 membrane (Amicon Corp., Lexington, Mass.). This concentrating procedure resulted in no loss of toxin or change in specific activity. The concentrated, partially purified toxin was stored at −70°C.

Diethylaminoethyl-Sephadex A-25 was suspended in 5 volumes of 0.1 M tris(hydroxymethyl)aminomethane buffer at pH 7.5 containing 0.1 N NaCl. This was equilibrated for 24 h, poured into a column (1.6 by 20 cm), and equilibrated with the same buffer at a flow rate of 30 ml/h. Approximately 5.0 ml of the pooled and concentrated fractions obtained after gel filtration was applied to this column. The column was washed with buffer until the absorbance of the eluate at 280 nm was zero. The toxin was eluted with a 0.1 to 1.0 N linear gradient of NaCl. The effluent was monitored for optical absorbance at 280 nm, with 2.5-ml fractions collected. Each fraction was titrated for cytotoxicity, and those fractions displaying toxicity were pooled and concentrated under positive pressure with an Amicon ultrafiltration cell with a UM 10 membrane. The purified toxin was extensively dialyzed against 50 mM potassium phosphate buffer at pH 7.0 and stored at −70°C.

Proteins were quantitated by the method of Lowry et al. (35) with crystallized bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Analytical PAGE. Determination of protein homogeneity was performed by polyacrylamide gel electrophoresis (PAGE) by the method of Davis (11), utilizing a 7.5% lower gel and an upper stacking gel. Protein samples containing approximately 150 μg of protein, 15 mg of sucrose, and bromophenol blue marker dye were added to the top of the gel. Different concentrations of protein were examined to achieve optimum visualization and band separation. Electrophoresis was performed at 2 mA/tube until the tracking dye was about 1 cm from the end of the gel. Gels were stained for protein with 0.25% Coomassie brilliant blue R-250 in a mixture of acetic acid-methanol-water (10:45:45, vol/vol/vol). Removal of excess dye was accomplished with a solution of 5% methanol and 7.5% acetic acid in a diffusion destainer (Bio-Rad Laboratories, Richmond, Calif.).

Molecular weight estimation. Ascending chromatography, performed in a column (2.6 by 70 cm) of Bio-Gel A 5m, was used for estimating the molecular weight of the toxin (1). The K_{av} values of standard proteins (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were related to that of the toxin (1). Both the purified toxin and ultrafiltered BMP broth supernatant were examined. The cytotoxic titer of the effluent was used to obtain a K_{av} value of the toxin.

Sodium dodecyl sulfate (SDS)-PAGE was performed by the method described by Weber and Osborn (51). The acrylamide gel contained 0.1 M sodium phosphate (pH 7.0), 5.0% polyacrylamide, and 0.1% SDS. For complete dissociation, standard protein samples (Pharmacia Fine Chemicals, Inc.) were dissolved in 0.01 M sodium phosphate buffer (pH 7.2) containing 2.5% SDS and 5% β-mercaptoethanol, and the solution was heated at 100°C for 5 min. Partial dissociation of the proteins was performed at 60°C for 15 min in buffer containing 1% SDS and 1% β-mercaptoethanol. Purified toxin, suspended in 50 mM potassium phosphate buffer at pH 7.0, was dialyzed against 0.01 M sodium phosphate buffer, pH 7.2, and dissociated by both methods. Conditions for electrophoresis, staining, and destaining were the same as described for analytical PAGE.

Isoelectric focusing. Gel isoelectric focusing was performed by the procedure of Righetti and Drysdale (43) in a gel with an acrylamide concentration of T = 4% and C = 5% (22). Bio-lyte 3/10 (Bio-Rad Laboratories) was used as the source of ampholytes. A constant current of 0.5 mA/tube was applied until the voltage reached 300 V, after which the voltage was held at this level for the remaining 20 h of the run. Focused gels were stained and destained by methods described for analytical PAGE. The pH gradient was determined using standard proteins (Sigma Chemical Co.).

Effect of different temperatures on toxin. To examine the effect of different temperatures on the toxin, *C. difficile* ATCC 9689 was inoculated into BMP broth and incubated anaerobically for 24 h. Broth filtrate was then prepared, divided into aliquots, and stored under the following conditions: −70, −20, 4, 20 to 25, and 37°C. One sample was immediately examined for cytotoxic titer. At regular time intervals, a vial at each temperature was removed and stored at −70°C until the cytotoxicity titer could be determined. To examine the effect of repeated freezing and thawing on the toxin, a sample of the broth filtrate was frozen at −70°C; on successive days it was removed and thawed, a sample was taken for cytotoxicity determi-

nation, and the sample was refrozen. The sample removed was similarly frozen at −70°C for later toxicity analysis.

Effect of pH on toxin. Purified toxin was diluted to 1.5 μg in 1.0 ml of distilled water. By using concentrated HCl and 4 N NaOH, the pH was adjusted to 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0, 10.0, and 11.0, with distilled water added to give a final volume of 2.0 ml in each sample. The adjusted toxin samples were kept at 4°C for 24 h, after which the pH was adjusted to 7.0, and the final volume was brought to 3.0 ml with distilled water. The cytotoxicity titer of each sample was determined.

Enzymatic inactivation of the toxin. Purified toxin was diluted to 1.5 μg in 1.0 ml of 50 mM potassium phosphate buffer at pH 7.0. Bacterial amylase, bacterial lipase, trypsin, chymotrypsin, and pronase were examined for their effect on the toxin. All enzymes were tested at a final concentration of 2.5 mg/ml except pronase, which was used at 0.05 mg/ml. Bacterial lipase was prepared in 0.05 M citrate-phosphate buffer at pH 6.0, and bacterial amylase was suspended in 0.05 M phosphate buffer at pH 7.0. Trypsin, chymotrypsin, and pronase were prepared in 0.05 M tris(hydroxymethyl)aminomethane buffer at pH 7.4. Equal volumes of enzyme and toxin were mixed, incubated for 24 h at 37°C, and examined for cytoxicity titers.

Thimerosal inactivation of toxin. Equal volumes of thimerosal (ethylmercurithiosalicylate) at different concentrations (see below) and 3.0 μg of toxin per ml were mixed and incubated at 37°C for 24 h. After incubation, serial twofold dilutions of each mixture were prepared in medium 199, with subsequent analysis for cytotoxicity. The ability of cysteine to protect the toxin against thimerosal inactivation was examined by preparing a solution of cysteine (1 mg/ml of 50 mM phosphate buffer at pH 7.0) and boiling it to drive off residual oxygen. Equal volumes of cysteine, thimerosal (10 μg/ml of 50 mM phosphate buffer at pH 7.0), and toxin were mixed, incubated at 37°C for 24 h, and tested for cytotoxicity.

Ileocecitis in hamsters. Intracecal injection of the toxin was made into male Syrian hamsters (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), weight 90 to 100 g, by the procedure of Bartlett et al. (5), with the exception that ether was used for anesthesia instead of sodium pentobarbital. Animals were observed daily for signs of diarrhea or moribund condition or both and were sacrificed 3 days after the injection. Hamsters were judged as having ileocecitis if their cecum was enlarged and hemorrhagic.

Vascular permeability in rabbits. Test and control materials were assayed for their ability to increase vascular permeability in rabbits by the procedure of Evans et al. (14). Samples were injected 48 h, 24 h, 12 h, 6 h, 4 h, 3 h, 2.5 h, 2 h, 1.5 h, 1 h, 45 min, 30 min, and 15 min before intravenous injection of 2.0% Evans blue dye in 0.15 M NaCl. Phosphate buffer controls were injected 48 h, 24 h, 12 h, 6 h, 3 h, 1.5 h, 45 min, and 15 min before introduction of the dye. Phospholipase C (100 μg/ml of normal saline), injected 2 h before Evans blue, served as the positive control (46). Samples were tested in two rabbits, and the mean diameter was determined. Those preparations causing a bluing greater than 5 mm in diameter were considered positive for vascular permeability factor.

Enzyme assays. Cell-free filtrates and extracts prepared from 24-h brain heart infusion broth cultures of 13 strains of *C. difficile* were examined for enzymatic activity. These strains were isolated from a variety of sources and included both toxigenic and nontoxigenic organisms as determined in human amnion tissue culture cells. In addition, the purified toxin from *C. difficile* ATCC 9689, used at a concentration of approximately 50 μg/ml, was examined for these activities. The method of Kunitz (30), based on the increased ultraviolet absorption during the course of deoxyribonucleic acid depolymerization, was used to assay deoxyribonuclease activity. The deoxyribonucleic acid agar plate methods of Smith et al. (45) and Jeffries et al. (24) were also used to measure deoxyribonuclease activity. Phospholipases A_2 and C, with soybean lecithin as the substrate, were assayed by methods described in the *Worthington Enzymes* manual (Worthington Biochemicals Corp., Freehold, N.J.). Gelatinase activity was assayed on plates containing 0.4% gelatin and agar (12). After 18 h of incubation at 37°C, the plates were flooded with acid and observed for clearing. Caseinase activity was measured by procedures described in the manual. Acetylcholinesterase activity was assayed by titrimetric determination of acid produced by acetylcholine hydrolysis (21). The amount of acid-soluble oligonucleotides liberated from the hydrolysis of ribonucleic acid by ribonuclease was determined by the procedure of Kalnitsky et al. (25). Neuraminidase hydrolysis of mucin was assayed by the procedure described in the *Worthington Enzymes* manual. Hyaluronic acid, when mixed with an acid-albumin solution, becomes turbid. Hyaluronidase activity was measured by its ability to decrease the turbidity (48). Collagenase activity, using bovine Achilles' tendon collagen as the substrate, was determined using the procedure described in the *Worthington Enzymes* manual.

RESULTS

Effect of medium composition on growth and toxin production. The quantity of toxin present in broth filtrates of *C. difficile* after 72 h of growth in different media is shown in Table 1. Toxin yield is expressed as the reciprocal of the toxin titer. All media, except the synthetic basal medium by itself and with the addition of amino acids, displayed growth after 24 h of incubation. These two media required 48 h for visible growth. The omission of B vitamins or glucose from the basal medium decreased the toxin yield two- to fourfold. Yeast extract could be substituted for B vitamins with no loss of toxin yield. Peptides appear to be important for toxin production, as the addition of amino acids to the basal medium in the absence of peptides permitted growth of *C. difficile* but did not permit toxin production. Toxin yields were highest when *C. difficile* was grown in chopped-meat-glucose broth, brain heart infusion broth,

TABLE 1. *C. difficile ATCC 9689 toxin production in different media*

MEDIUM		Reciprocal of toxin titer[b]
Chopped meat glucose		8192
Proteose peptone #3 + yeast extract		1024
Proteose peptone #3 + glucose + yeast extract		4096
Brain heart infusion		8192
Thioglycollate		2048
Thioglycollate + glucose		4096
Basal[a] Medium	Amino acid mixture	ND[c]
	1% phytone	1024
	1% yeast extract	2048
	1% casein hydrolysate	1024
	1% bacto-tryptone	1024
	1% bacto-peptone	1024
	0.5% proteose peptone #3	512
	1.0% proteose peptone #3	2048
	2.0% proteose peptone #3	4096
	3.0% proteose peptone #3	8192
	5.0% proteose peptone #3	4096
	Brain heart infusion (<100,000mw components)	512
	3% proteose peptone #3 (<100,000mw components)	2048

[a] Each nutrient listed was added to a synthetic basal medium as described in the text.

[b] Titer expressed as reciprocal of highest filtrate dilution causing complete rounding of human amnion tissue culture cells.

[c] No detectable cytotoxic activity.

and basal medium containing 3% proteose peptone no. 3. When ultrafiltered brain heart infusion or 3% proteose peptone no. 3 was added to the basal medium, the toxin yield decreased when compared with the addition of the nonfiltered nutrients. The synthetic basal medium containing ultrafiltered 3% proteose peptone no. 3 was chosen as the medium for subsequent toxin purification. Toxin yield was sacrificed to permit easier isolation.

Growth and toxin production. Toxin production was compared with various growth parameters of *C. difficile*. After inoculation into BMP broth, samples were removed at various time intervals to determine viable cell counts, absorbance at 500 nm, heat-resistant spore population, and the quantity of toxin present in the broth filtrate and cell extract (Fig. 1). There was little change in any of the parameters examined from the time of inoculation to 4 h post-inoculation. During logarithmic growth, occurring 4 to 12 h post-inoculation, there was a slight increase in the toxin titer of the broth filtrate. The quantity of toxin present in the filtrate rapidly increased 12 to 24 h post-inoculation, during which time the viable cell count and absorbance decreased. For the remainder of the experiment (24 to 96 h post-inoculation), the viable cell count, absorbance, and filtrate toxin titer remained approximately the same. Although not shown in Fig. 1, the amount of toxin present in the cell extract, when expressed in relation to milligrams of protein, remained at a steady level from 0 to 12 h, after which it steadily decreased to a level which stayed approximately constant from 24 to 96 h post-inoculation. A heat-resistant spore population became evident at 48 h (5 spores per ml) and slowly increased to 150 spores per ml at 96 h. The pH of BMP broth immediately after inoculation was 7.3. The pH decreased to 6.0 at 12 h, after which it slowly increased to 6.4 by the end of the experiment.

Purification of the toxin. The amounts of toxin and protein obtained after each purification step are shown in Table 2. A 24-h broth filtrate of *C. difficile* was first concentrated by using positive-pressure ultrafiltration through a 100,000-NMWL membrane. Approximately 5% of the toxin was lost during ultrafiltration. Changing the exclusion limit of the membrane had no effect on toxin retention as 10,000- and 50,000-NMWL membranes also resulted in a 5% loss of toxic activity. A 1,000,000-NMWL membrane did not retain the toxin. The second purification step was precipitation with 75% $(NH_4)_2SO_4$. A majority of the toxin precipitated between 50 and 75% $(NH_4)_2SO_4$ saturation, and increasing the concentration above 75% did not result in additional precipitated toxin. The toxin was further purified by gel filtration on a column of Bio-Gel A 5m. Preliminary experiments revealed incomplete resolution of toxin and void volume when Sephadex G-200, Bio-Gel A 0.5m, or Bio-Gel A 1.5m were used as the separating matrix. As shown in Fig. 2, Bio-Gel A 5m allowed complete separation of the void volume and toxin. When the toxic fractions were pooled, concentrated, and analyzed by PAGE, two major and some minor bands were seen (Fig. 3). The concentrated toxic fractions obtained after gel filtration were applied to a column of diethylaminoethyl-Sephadex A-25 and eluted with a linear NaCl gradient. The toxin eluted at an NaCl concentration of 0.4 N and corresponded to a clearly resolved protein peak (Fig. 4). Analysis of the concentrated toxic fractions by PAGE and isoelectric focusing revealed only one protein band. The resulting toxin was purified 2,000-fold from the broth filtrate with a 58% recovery. Approximately 170 pg of purified toxin was sufficient to cause complete rounding of the human

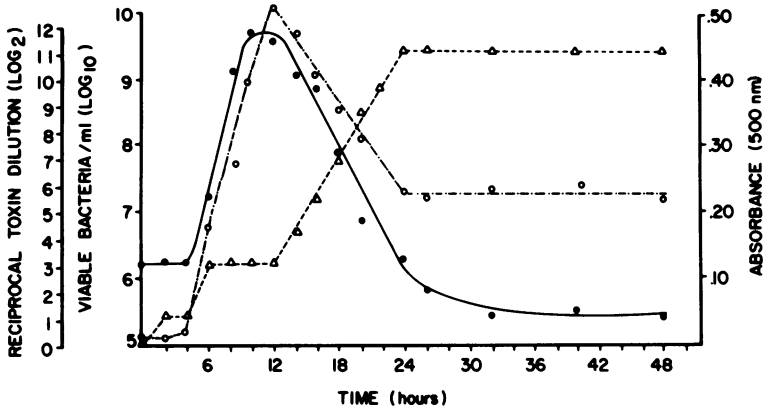

FIG. 1. *Growth of C. difficile ATCC 9689 in basal medium containing ultrafiltered peptones. Symbols: ●, viable cell count; △, reciprocal of toxin dilution (log_2); ○, absorbance at 500 nm.*

TABLE 2. *Purification of C. difficile toxin*

Stage	Purification Step	Volume (ml)	Protein		Cytotoxin		Specific activity	Percent[b] starting material	Purification[c]
			mg/ml	mg	C.U./ml[a]	total C.U.	C.U./mg		
0	Cell-free filtrate	4000	14.4	57684	4.1×10^4	1.6×10^8	2.8×10^3	100	1
1	Ultrafiltration	270	5.5	1485	5.9×10^5	1.6×10^8	1.1×10^5	95	36
2	75% $(NH_4)_2SO_4$	80	4.9	389	1.4×10^6	1.1×10^8	2.8×10^5	67	93
3	Bio-Gel A 5m	35	1.6	56	3.1×10^6	1.1×10^8	2.0×10^6	65	653
4	DEAE-Sephadex A-25	20	0.8	16	4.8×10^6	9.5×10^7	5.9×10^6	58	2067

[a] CU per milliliter was defined as the highest dilution of the sample resulting in complete rounding of human amnion cells × 20 (correction factor for final dilution made in tissue culture well).

[b] Percent starting material based on total CU at step in purification ÷ total CU in starting material × 100.

[c] Purification is based on specific activity at step in purification ÷ specific activity of starting material.

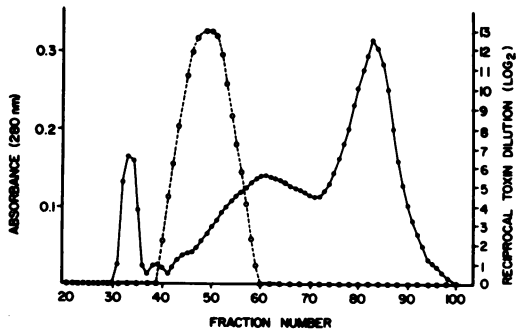

FIG. 2. *Gel filtration of $(NH_4)_2SO_4$-precipitated protein on a Bio-Gel A 5m column (2.6 by 70 cm) with 50 mM potassium phosphate buffer, pH 7.0, as the eluant. The 5.0-ml sample, applied to the column, contained 25.0 mg of protein. Symbols: ●, absorbance at 280 nm; ○, reciprocal of toxin dilution (log_2).*

amnion cell line. Higher concentrations of purified toxin (10 μg) were required to cause gross ileocecitis in hamsters. Heating (56°C for 30 min) of the purified toxin and preincubation with polyvalent gas gangrene or *C. sordellii* antitoxins caused inactivation. Antitoxins directed against *C. perfringens*, *C. oedematiens*, *C. septicum*, and *C. histolyticum* had no effect on the toxin.

Molecular weight. The K_{av} value obtained from three individual experiments corresponded to a molecular weight of 530,000 (Fig. 5). The toxin eluted as one symmetrical peak.

The molecular weight of the dissociated toxin was determined by PAGE in SDS. When the toxin was heated at 60°C for 15 min in the presence of 1% SDS and 1% β-mercaptoethanol, two protein bands were detected, corresponding to molecular weights of 185,000 and 50,000, re-

spectively. More extensive dissociation and reduction resulted in only the 50,000-molecular-weight peptide. These data suggest that the toxin is composed of numerous polypeptide chains.

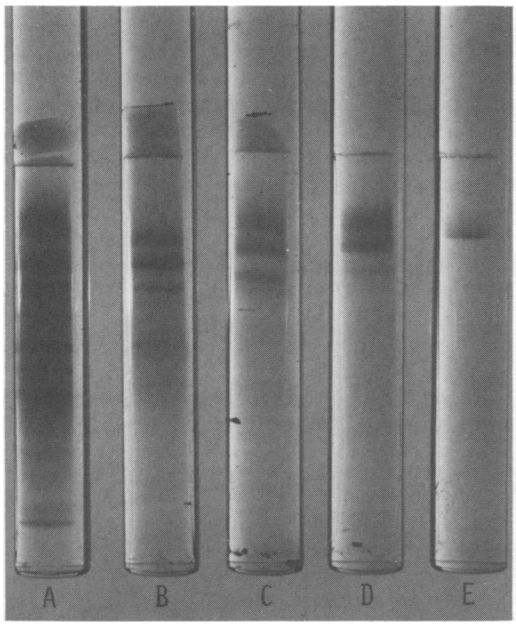

FIG. 3. *Protein heterogeneity at different stages in the purification of C. difficile ATCC 9689 toxin as monitored by PAGE. (A) Broth filtrate; (B) concentrated broth filtrate (100,000-NMWL membrane); (C) $(NH_4)_2SO_4$ precipitation of concentrated broth filtrate; (D) toxic material resulting from gel filtration (Bio-Gel A 5m) of $(NH_4)_2SO_4$-precipitated protein; (E) toxic fraction resulting after ion-exchange (diethylaminoethyl-Sephadex A-25) separation of pooled and concentrated cytotoxic fractions obtained from gel filtration.*

Isoelectric focusing. The purified toxin possessed an isoelectric point of approximately 5.0 as shown in Fig. 6. Proteins of known isoelectric points were used to construct a standard curve. Three individual experiments were performed with the isoelectric point of the toxin ranging

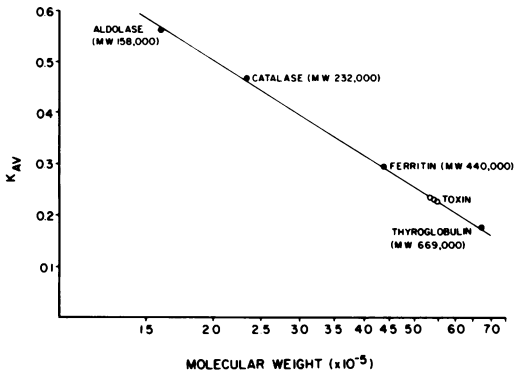

FIG. 5. *Molecular weight (M.W.) determination of purified C. difficile cytotoxin on a column (2.5 by 70 cm) of Bio-Gel A 5m.*

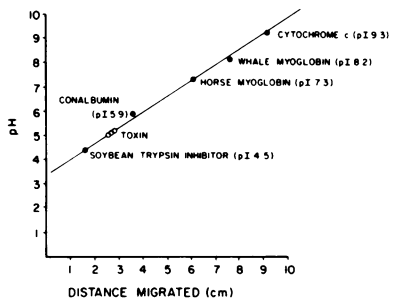

FIG. 6. *Isoelectric focusing of C. difficile toxin. Comparison of the electrophoretic mobilities of standard proteins with that of C. difficile toxin.*

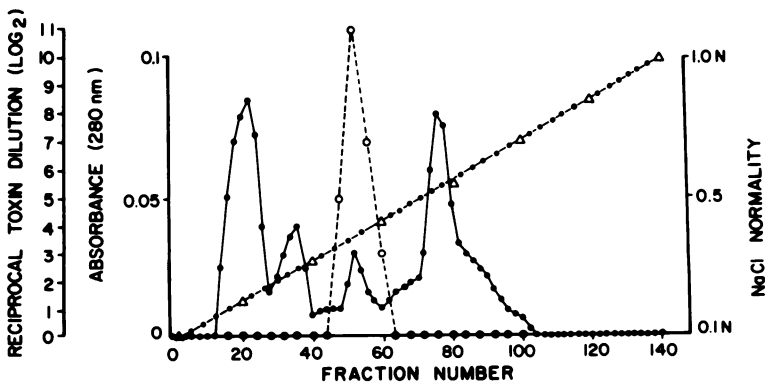

FIG. 4. *Chromatography of partially purified C. difficile cytotoxin from a Bio-Gel A 5m column (Fig. 2) on a column (1.6 by 20 cm) of diethylaminoethyl-Sephadex A-25. A linear gradient of NaCl was used for elution. Symbols: ●——●, absorbance at 280 nm; ○- - -○, reciprocal of toxin dilution (log_2); △-●-△, NaCl normality.*

from 4.9 to 5.1. Similar results were obtained when the toxin was incorporated into the polyacrylamide gel before polymerization rather than layered on top of the gel.

Effect of temperature on toxin. The cell-free filtrate from a 24-h BMP broth culture of *C. difficile* ATCC 9689 was divided into aliquots and placed at different temperatures. When stored at 37°C or at room temperature (20 to 25°C), the toxin lost activity, with no cytotoxicity being demonstrated after 10 days or 4 weeks, respectively (Fig. 7). Filtrates stored at 4 or −20°C displayed an initial loss of activity at 2 weeks, after which they remained stable for the duration of the experiment. There was no loss of activity when the toxin was stored at −70°C. The toxin was stable to repeated freezing and thawing.

Inactivation of toxin. Trypsin, chymotrypsin, pronase, and amylase inactivated the toxin, whereas bacterial lipase had no discernible effect. Figure 8 shows the effect of different amylase concentrations on the toxin. The toxin was completely inactivated at an amylase concentration of 10 mg/ml, with 1 mg/ml displaying no effect. When assay procedures described previously (13; *Worthington Enzymes* manual) were used, no proteolytic activity (gelatinase and caseinase) was found in the amylase preparation. These data suggest that the toxin produced by *C. difficile* is a glycoprotein.

Thimerosal (ethylmercurithiosalicylate) was also examined for its effect on the purified toxin. Concentrations of thimerosal as low as 5 ng/ml partially inactivated the toxin, with 500 μg/ml required for complete inactivation. Cysteine protected the toxin against thimerosal inactivation, and this protective ability was greatly enhanced if the cysteine was first boiled before it was added to the toxin and thimerosal.

The toxin was most stable at pH 7.0, whereas at pH 4.0 and 9.0 it was completely inactivated.

Vascular permeability. All toxin injections given before Evans blue caused an increased vascular permeability in rabbits. Negative controls injected less than 1 h before Evans blue displayed a weak positive reaction, but this was minimal (2 to 4 mm in diameter) and was easily distinguished from the test injections. The maximum area and intensity of bluing, with a mean diameter of 15 mm, occurred with injections given 2 h before introduction of dye. The center of the bluing displayed small, indurated white areas having a diameter of approximately 4 mm. Heating (56°C for 30 min) destroyed the vascular permeability factor, as did preincubation of the toxin with polyvalent gas gangrene or *C. sordellii* antitoxins.

Enzymatic activity. The cell-free filtrates and extracts prepared from broth cultures of 13

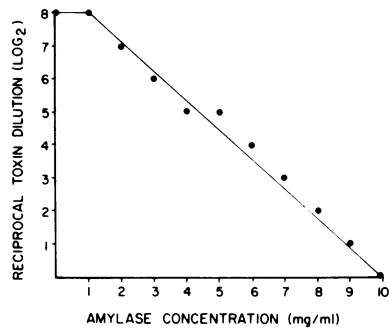

FIG. 8. *Amylase inactivation of C. difficile cytotoxin.*

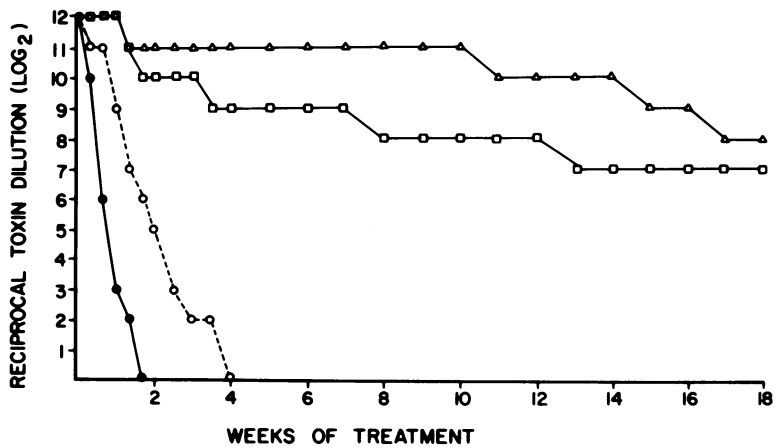

FIG. 7. *Effect of different temperatures on C. difficile cytotoxin. Symbols:* □, *4°C;* △, *−20°C;* ○, *20 to 25°C;* ●, *37°C.*

C. difficile strains (nine toxigenic and four nontoxigenic), as well as the purified toxin, were examined for various enzymatic activities. These enzymes included deoxyribonuclease, ribonuclease, phospholipases A_2 and C, gelatinase, caseinase, acetylcholinesterase, neuraminidase, hyaluronidase, and collagenase. No enzymatic activity could be associated with the purified toxin. Low levels of ribonuclease and deoxyribonuclease activities were demonstrated in the cellular extracts of *C. difficile*. Ribonuclease activity was also demonstrated in 6 of 13 *C. difficile* filtrates. No relationship existed between ribonuclease activity and toxigenicity (four toxigenic and two nontoxigenic). *C. difficile* was devoid of all other enzymatic activities.

DISCUSSION

Recent evidence has shown toxigenic *C. difficile* to be a major cause of antimicrobial-associated colitis in humans (2, 4, 17, 32, 42). The *C. difficile* toxin purified and characterized in this investigation is similar to, if not identical with, the toxin found in stools of patients with antimicrobial-associated colitis and laboratory animals with ileocecitis (2, 4, 5, 17, 32). Previous investigators have shown this toxin to be heat labile (56°C for 30 min), acid and alkaline sensitive, neutralized by polyvalent gas gangrene or *C. sordellii* antitoxins, causative of increased vascular permeability in rabbits, lethal to hamsters if injected intracecally, and inactivated by trypsin and pronase.

A synthetic medium containing low-molecular-weight peptides was used for isolation of the toxin. These peptides were obtained by filtering a peptone solution through a 100,000-NMWL membrane. Subsequent passage of broth filtrate through an identical membrane permitted removal of contaminating medium proteins from the toxin. The toxin's large molecular weight (>500,000) made this step possible with only minimal loss of toxin. As previously described for *C. perfringens* alpha toxin (44), the addition of peptides to the synthetic basal medium was essential for *C. difficile* toxin production, although other investigators have successfully obtained toxin production in a protein-free medium (N. Harvie, S. Ellis, D. Mason, A. Allo, G. Rifkin, J. Silva, and R. Fekety, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., Abstr. no. 130, 1978). Bartlett et al. (4) found that glucose increases toxin production by *C. difficile*. In this investigation, glucose and B vitamins were found to increase toxin production.

C. difficile multiplied rapidly in the peptone-enriched basal medium, reaching maximum viable cell count approximately 12 h post-inoculation. After a short stationary phase, the viable cell count and absorbance dropped rapidly. It was during this decrease of viable cells that the quantity of toxin in the filtrate increased. The specific activity of cytoplasmic toxin (CU per milligram of protein) decreased when the toxin titer present in the filtrate increased. This suggests that the toxin is protoplasmic, being released into the medium when the cells undergo autolysis. A protoplasmic toxin of *C. difficile* was reported earlier to cause a fatal infection in laboratory animals (44). Physical and chemical agents which cause cell lysis or a weakening of the cell wall may increase broth filtrate toxin yield. This may explain the results of Onderdonk and Bartlett (Program Abstr. Intersci. Conf. Antimicrob. Agents. Chemother. 18th, Atlanta, Ga., Abstr. no. 128, 1978), who increased filtrate toxin yields by increasing the oxidation reduction potential or the temperature at which the medium was incubated. Both of these factors could cause increased cell lysis of this anaerobic organism. Antibiotics which act on the cell wall may increase toxin release from *C. difficile*; the importance of this phenomenon in antimicrobial-associated colitis should be examined.

Ultrafiltration, ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography were used to purify the toxin present in broth filtrate. This procedure resulted in a 2,000-fold purification of the toxin and a final toxin specific activity of 5.9×10^6 CU/mg of protein. Approximately 170 pg of toxin was necessary to cause complete rounding of human amnion cells. The purified toxin displayed only one band on disc gel electrophoresis and isoelectric focusing. Attempts to extract the toxin by diffusion from the polyacrylamide gel have been unsuccessful. The explanation for this is unclear.

The molecular weight of the toxin, as determined by gel filtration, was approximately 530,000. Taylor et al. (N. S. Taylor, A. Onderdonk, and J. Bartlett, Program Abstr. Intersci. Conf. Antimicrob. Agents. Chemother. 18th, Atlanta, Ga., Abstr. no 127, 1978) reported the toxin as having a molecular weight of between 300,000 and 500,000. Pronase, trypsin, chymotrypsin, and amylase inactivated the toxin, indicating that it is a glycoprotein. The amylase preparation used in these experiments contained no detectable proteolytic activity. The high molecular weight of the toxin may be one reason for its apparent slow release from the cytoplasm of *C. difficile*. When reduced and dissociated in SDS and β-mercaptoethanol, the toxin displayed 185,000-molecular-weight and 50,000-molecular-weight peptides. Extensive reduction and dissociation of the toxin yielded only the 50,000-

molecular-weight fraction. These data suggest that the native toxin is composed of several polypeptide chains.

Cholestyramine and colistipol hydrochloride bind to *C. difficile* toxin, preventing its activity in tissue culture (9, 16, 29). This evidence suggested that the toxin was acidic or neutral. In this investigation, the isoelectric point was found to be 5.0. This is in contrast to many other enterotoxins, which are usually basic or neutral (9).

Thimerosal (ethylmercurithiosalicylate) inactivated *C. difficile* toxin at low concentrations, and this inactivation could be prevented by the simultaneous addition of reduced cysteine. A similar situation of thimerosal inactivation and cysteine protection has been described for urease (6). With urease, we feel that organomercurial compounds attach to essential sulfhydryl groups, preventing its enzymatic action. Cysteine, with its readily available sulfhydryl group, binds to organomercurial compounds, thereby preventing their action. We have found that other heavy-metal compounds, which are less toxic to the host than is thimerosal, may also inactivate *C. difficile* toxin. Examples of such compounds include ferrous sulfate, ferrous gluconate, magnesium hydroxide, and aluminum hydroxide. It has been demonstrated recently that bismuth subsalicylate, indomethacin, and phenylbutazone inhibit the secretory activity of *Escherichia coli* and *Vibrio cholerae* (13, 37, 40). These substances may exhibit a similar inhibitory action against *C. difficile* enterotoxin. One approach to the treatment of antimicrobial-associated colitis has been the administration of an antibiotic, such as vancomycin, to which *C. difficile* is susceptible (27). This type of treatment may rid the intestinal tract of *C. difficile* but probably has little or no impact on toxin already released. Indeed, a cell wall-active agent such as vancomycin might induce the release of additional toxin from cells. The concomitant administration of a bactericidal antibiotic and a compound which inactivates *C. difficile* toxin may prove to be a more effective means of treatment.

Clostridial species produce a wide variety of toxins, many of which possess known enzymatic activity (44). In this investigation, 13 strains of *C. difficile*, as well as the purified toxin, were examined for enzymatic activity. Of the 10 enzymes assayed, only low levels of deoxyribonuclease and ribonuclease were detected, and these were primarily present in the cell extracts. Phospholipases A_2 and C, gelatinase, caseinase, acetylcholinesterase, neuraminidase, hyaluronidase, and collagenase were not demonstrated. The only virulence factor of *C. difficile* described to date has been the enterotoxin.

The availability of a purified toxin from *C. difficile* will aid in delineating the exact role of this organism in antimicrobial-associated colitis and other diseases. Marrie et al. (38) have recently reported the isolation of a toxigenic *Clostridium sporogenes*, as well as *C. difficile*, from stools of a patient with lincomycin-associated pseudomembranous colitis.

Several theories have been proposed to explain the ability of *C. sordellii* antitoxin to neutralize *C. difficile* toxin (18). These theories include cross-reactivity and a contamination or misidentification of the original *C. sordellii* culture used to prepare the antitoxin (4). Our study of the *C. sordellii* strain originally used to produce the antitoxin (kindly supplied by E. Seligmann) indicates that it is a pure culture and is correctly identified. We have, as yet, been unable to demonstrate in vitro cytotoxin production by *C. sordellii* (unpublished observation). Of particular interest is our finding that *C. sordellii* toxin (Food and Drug Administration Biologic Standard) is cytotoxic to human amnion cells (unpublished data). Immunological and biochemical studies of purified *C. sordellii* and *C. difficile* toxins are needed to resolve this phenomenon of cross-neutralization.

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