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Differential Cytotoxic Effects of Toxins A and B Isolated from *Clostridium difficile*

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Toxin A and toxin B preparations of *Clostridium difficile* have been shown to affect metabolic functions of intact HeLa cells with different kinetics. The cytotoxins were purified from dialyzed filtrates of *C. difficile* strain VPI 10463 by hydrophobic interaction chromatography and ion-exchange chromatography and were concentrated by dialysis or by ultrafiltration. The toxins, which are immunologically unrelated, were analyzed by polyacrylamide gel electrophoresis and by immunochemistry with the Western blot technique. Toxin A was resolved into one major cytotoxic protein and a minor, rapidly migrating species that did not comigrate with toxin B. Toxin B was resolved into one major and three minor cytotoxic proteins. One protein comigrating with toxin A had no cytotoxic activity. The highly purified toxin A at 1.0 mg/ml caused loss of intracellular K^+ and inhibition of protein synthesis in HeLa cells within 1 h. These effects correlated with morphological changes indicating cytotoxicity. At lower protein concentrations of toxin A (10- to 100-fold less), however, cytotoxic effects were seen at 120 min, whereas no changes in K^+ levels or protein synthesis were yet evident. The toxin B preparation, 1,000-fold more toxic than toxin A, was diluted to equivalent cytotoxicity as measured in the overnight assay. Toxin B caused loss of K^+ and inhibition of protein synthesis well after cytotoxic morphological changes were complete. In contrast, at higher protein concentrations (2- to 2,000-fold more), intracellular K^+ was lost completely by 120 min. The effects on cell rounding and protein synthesis were incomplete at 120 min, but increased with the toxin B concentration.

Clostridium difficile produces toxins that are considered the pathogenic agents of antibiotic-associated pseudomembranous colitis in humans and animals (3, 12, 16). *C. difficile* culture filtrates are cytotoxic, as are both toxin A and toxin B, the two toxic activities that have been separated from these filtrates (2, 18, 19). Cytopathic changes are expressed morphologically in various cultured mammalian cells as cell rounding with increased refractility (7, 9, 15). In association with such morphological changes, culture filtrates cause a decrease in mitotic figures and a reduction of tissue culture cloning efficiency in HeLa cells (11). Effects noted with partially purified toxin mixtures include elevation of guanylate cyclase activity in gut homogenates and inhibition of adenylate cyclase (21). Na^+-K^+ ATPase is not affected. Studies with toxin mixtures partially purified from cell extracts or from culture filtrates show irreversible loss of fibronectin from fibroblast surfaces (1) and decreased uptake and incorporation of precursors into DNA, RNA, and protein (10). Dinitrophenol eliminates all morphological effects, suggesting that toxin uptake is an energy-dependent process. Indirect immunofluorescence with anti-actin antiserum shows disruption of straight microfilament bundles without leakage of [^{3}H]uridine from prelabeled lung fibroblasts (MRC-5) (20). A range of structural alterations of myofilament bundles and dense bodies was confirmed by transmission electron microscopy in smooth muscle cells intoxicated with partially purified toxin B (22). The changes correlate with cell rounding. Other cell organelles show no morphological alterations. These results suggest that one or both toxins may have an effect on actin, but no biochemical evidence is available to confirm this.

To understand the events underlying the morphological changes, purified toxins are needed. Attempts to purify *C. difficile* cytotoxic filtrates led to the finding that two toxic proteins can be separated by ion-exchange chromatography (2, 18, 19): toxin A and toxin B. Toxin A was purified to homogeneity by ultrafiltration, ion-exchange chromatography, and acetic acid precipitation (17) and shown to be immunologically distinct from toxin B (18, 19). Toxin B was partially purified, but could not be distinguished from several contaminating proteins when analyzed by polyacrylamide gel electrophoresis (PAGE). Toxin B is at least 1,000-fold more cytotoxic than toxin A per milligram of protein. In this investigation, we have modified earlier procedures (18); cytotoxins were purified from dialyzed filtrates of *C. difficile* by hydrophobic interaction chromatography and ion-exchange chromatography and were concentrated by dialysis or by ultrafiltration. A preliminary report of this purification procedure has been made (S. W. Rothman, J. E. Brown, and D. A. Foret, Abstr. XII Int. Congr. Microbiol. p. 12, 1982).

We have previously reported that *C. difficile* cytotoxic culture filtrates cause a decrease in intracellular K^+ levels and an inhibition of α-aminoisobutyric acid uptake (17) in intact HeLa cells. This initial inhibition of membrane functions is followed by inhibition of macromolecular synthesis. We have now further investigated the kinetics of the inhibition of cellular membrane functions and protein synthesis using purified toxin A and toxin B. When preparations were adjusted to equivalent cytotoxicity as determined by the overnight assay, the inhibition of measured cellular functions by toxin A occurred simultaneously with cell rounding. Inhibition by toxin B was not observed until cell rounding was complete. At higher protein concentrations, inhibition of cellular functions by toxin B correlated with cell rounding. The results suggest that these toxins have similar modes of action and that the initial effect of both may be on membrane permeability or energy metabolism.

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(A preliminary report of this work has been made [S. W. Rothman, J. E. Brown, A. Diecidue, and D. Foret, Fed. Proc. **42**:1811, 1983].)

MATERIALS AND METHODS

Buffers. Tris buffers were used during purification and are referred to as follows. Buffer 20T8 consisted of 0.020 M Tris-hydrochloride (pH 8.0). With 80 and 100 mM NaCl, this buffer is referred to as 20T8/80 and 20T8/100, respectively.

Preparation and assay of culture filtrates. *C. difficile* strain VPI 10463, kindly supplied by Nadine Sullivan, Virginia Polytechnic Institute and State University, Blacksburg, was grown anaerobically in a dialysis bag suspended in brain heart infusion broth. Growth conditions and preparation of filtrates have been described previously (17). Pooled culture filtrates were dialyzed versus buffer 20T8/100 by ultrafiltration in a hollow fiber dialyzer with an H1P100 cartridge (Amicon Corp., Danvers, Mass.). Sterile filtrates were stored at 4°C until required. Cytotoxicity of samples was measured, after overnight incubation on HeLa cell monolayers, as a change in crystal violet staining of the cell monolayers (16). The endpoint was determined visually or by absorbance (595 nm) measurement of the stained monolayers (Multiskan MC; Flow Laboratories, Inc., McLean, Va.). Absorbance at 405 nm was subtracted from the readings to correct for any anomalies in the plastic. A logarithmic plot of dye absorbance versus the dilution of toxin allowed determination of the highest dilution with detectable cytotoxicity. The minimum cytotoxic dose (MCD) is defined as the reciprocal of the greatest dilution of sterile sample producing detectable cytotoxicity in this assay after incubation overnight. Protein concentrations were determined by the method of Lowry et al. (13). Procedures for the establishment of HeLa cell monolayers have been described previously (17).

Preparation of antitoxin. Rabbit antiserum against crude toxin was prepared as previously described (H. Collins, A. Stone, and S. Rothman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B119, p. 37). Initial subcutaneous inoculations of 1.2 ml containing 6×10^5 MCD of *C. difficile* culture filtrates were given together with excess antitoxin to neutralize lethality. This neutralizing antibody had been prepared in rabbits by subcutaneous inoculation of cytotoxic cecal extracts from guinea pigs (S. Rothman, A. Stone, and H. Collins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B35, p. 20). At the same time, the rabbits were primed by injection of 0.2 ml of complete Freund adjuvant into a hind footpad. Thirteen days later the swollen popliteal lymph node in the thigh above the injection site was inoculated with 0.2 ml containing 10^5 MCD of toxic filtrate plus excess antitoxin. Injection of the same preparation into this node was repeated 20 days later. One week later, neutralizing antibody was detected in serum from these rabbits. Monthly injections of culture filtrate alone (3.5×10^5 MCD) into this node maintained antibody levels.

Partial purification of *C. difficile* toxins. Toxins A and B were purified from dialyzed filtrates of *C. difficile* strain VPI 10463 (S. W. Rothman, J. E. Brown, and D. A. Foret, Abstr. Int. Congr. Microbiol. 1982, p. 112) by hydrophobic interaction chromatography, ion-exchange chromatography, and concentration by dialysis or by ultrafiltration (Table 1). The initial hydrophobic interaction chromatography step was performed at 25°C in an 8- by 7-cm column at a flow rate of 6.3 ml/min. Ammonium sulfate (Ultrapure; Schwartz/Mann, Orangeburg, N.Y.) was added to the filtrate to 15% saturation; no precipitate formed at this salt concentration. The sample (2,600 ml) was then applied to the Phenyl-Sepharose column (Phenyl-Sepharose CL-4B; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with starting buffer (20T8/100 buffer, 15% saturated with ammonium sulfate), followed by elution with starting buffer. No toxin was detected in the initial 1,800-ml eluant of nonbinding protein. Elution with a linear gradient of 0 to 50% ethylene glycol in 20T8/100 buffer (Fig. 1) yielded a single absorbance peak, with cytotoxic activity being highest at a conductivity of about 20 mmho. The toxin-containing fractions were pooled and dialyzed for 36 h at 4°C with three changes of 20T8/80 buffer. Total toxin activity was enriched eightfold with 100% recovery. All further steps were at 4°C.

Ion-exchange chromatography was performed in a 6.5- by 7-cm column at a flow rate of 3.2 ml/min. The toxin preparation (830 ml) was applied to the DEAE-Sepharose CL-6B column equilibrated with 20T8/80 buffer, followed by elution with the same buffer. Elution with a linear salt gradient from 80 to 500 mM NaCl in 20T8 buffer (Fig. 2) yielded two cytotoxic bands, designated toxins A and B in accordance with the nomenclature used previously (18). Toxin A eluted with the large absorbance peak at 12 mmho. Toxin B was detected at a conductivity of 25 mmho where there was little detectable absorbance. Pools of toxins A and B were concentrated by ultrafiltration (XM-100A membrane; Amicon Corp., Danvers, Mass.) or by dialysis against Carbowax PEG 20M. Purified toxins were stored at 4°C.

PAGE. Nondenaturing PAGE was performed in 8% rod or slab gels by the method of Davis (8). Gels were fixed in 10% sulfosalicylic acid, stained with 0.02% Coomassie brilliant blue R250 in 7% acetic acid, and destained with ethanol-acetic acid-water (25:8:67).

Immunoblotting of PAGE slabs. Immunological analysis was performed by the Western blot method of Burnette (6). Electrophoresis was performed as above in nondenaturing

TABLE 1. Purification of *C. difficile* toxins from dialyzed filtrates

Prepn	Vol (ml)	Cytotoxicity (MCD/ml)	Protein (mg/ml)	Sp act (MCD/mg)	Purification (fold)	Recovery[a] (%)
Dialyzed filtrates	2,600	1×10^{7}[a]	1.1	9×10^6	1	100
Phenyl-Sepharose CL-4B	830	6×10^{7}[a]	0.9	7×10^7	8	100
DEAE-Sepharose CL-6B						
Toxin A	30	5×10^5	3.9	1×10^5	1	
Toxin B	120	1×10^8	0.31	3×10^8	33	46
Concentration						
Toxin A, PEG 20	8.5	4×10^6	25	2×10^5	2[b]	
Toxin B, Amicon XM-100A	6.0	2×10^9	5.6	4×10^8	44	46

[a] Values for toxin B only.
[b] Value for toxin A only after DEAE treatment.

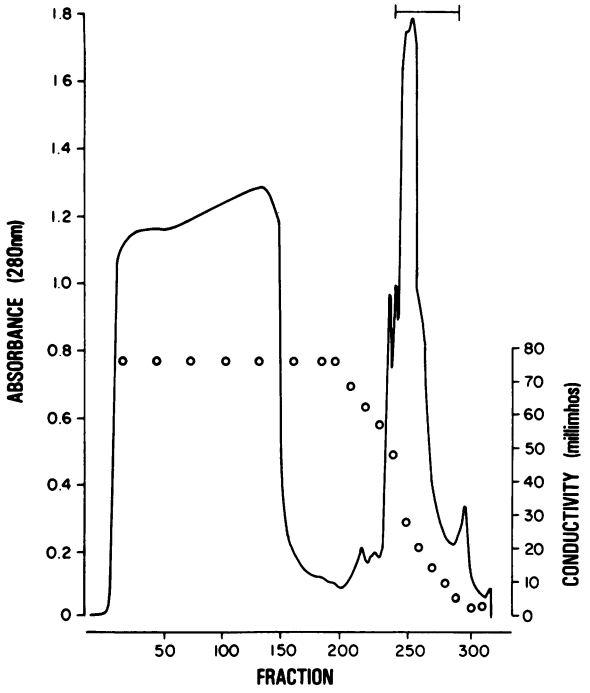

FIG. 1. Phenyl-Sepharose chromatography of toxin-containing ultrafiltrate. A total of 2.9 g of protein was applied to the column, and elution was carried out as described in the text. The eluant was monitored for absorbance at 280 nm. The conductivity of every 10th fraction was measured (○) with a type CDM2 conductivity meter (Radiometer America, Westlake, Ohio). Every second fraction was assayed for cytotoxicity in the HeLa cell microtiter assay (fivefold serial dilutions). Toxin-containing fractions, as indicated by the bar, were pooled for further processing. Fraction size was 22 ml.

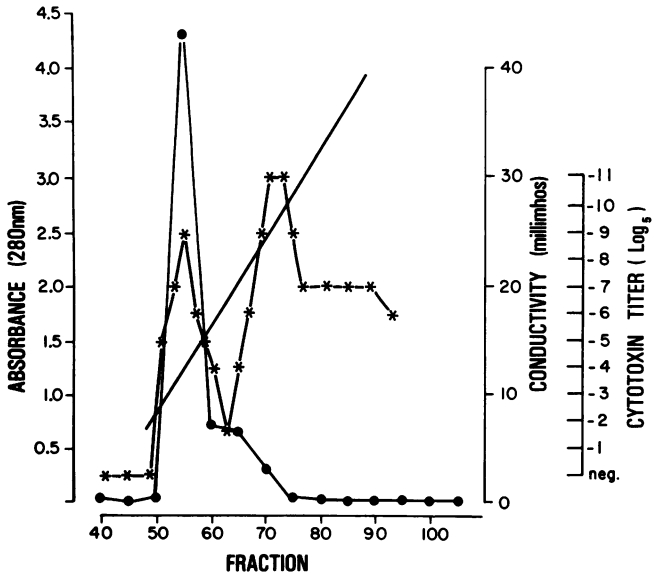

FIG. 2. DEAE-Sepharose chromatography of toxin-containing phenyl-Sepharose fractions. A total of 750 mg of protein was applied to the column, and elution was carried out as described in the text. Conductivity (straight line) and absorbance at 280 nm (●) of fractions were measured, and cytotoxicity was assayed (*). The first peak of cytotoxicity was toxin A; the second was toxin B. Fractions 53 through 56 and 70 through 74 were pooled for further processing. Fraction size was 25 ml.

8% slab gels. The gels were cut in half, and one of the halves was stained with Coomassie blue. Proteins from the other half were transferred electrophoretically to nitrocellulose and processed as previously described (4).

Mouse lethality assays. The lethality of toxin preparations for mice was assayed by intraperitoneal injections of 0.5 ml of various concentrations (at least six) of toxin into 15- to 20-g WR strain mice (five mice for each amount of toxin). For the assay, toxin was serially diluted with 20T8/100 buffer, and 0.5 ml of the buffer was injected for negative controls. From cumulative deaths for 7 days, 50% lethal doses were calculated by the method of Reed and Muench (14).

Protein synthesis. To determine whether *C. difficile* toxins inhibit protein synthesis in intact HeLa cells, [^{14}C]leucine incorporation into trichloroacetic acid-precipitable material was measured as previously described (17). Cell growth medium was replaced with incorporation medium (without leucine) without serum; at the appropriate times, toxin solutions (0.1 ml) containing 0.5 μCi of [*U*-^{14}C]leucine (347 mCi/nmol) were added. Incorporation was terminated by chilling the plate in an ice slurry. The cell monolayers were washed with cold medium, solubilized, and extracted with ice-cold 5% trichloroacetic acid. Radioactivity in the trichloroacetic acid-precipitable material reflects protein synthesis. The isotope was from New England Nuclear Corp., Boston, Mass.

Intracellular K^+ content. HeLa cells were inoculated into Costar 24-well tissue culture dishes (9.6×10^4 cells per well) and incubated overnight. Toxin solutions (0.6 ml) were added, and cell monolayers were incubated for various time periods. Cell monolayers were washed with K^+-free phosphate-buffered saline, drained, and lysed with 0.6 ml of distilled water and three cycles of freezing and thawing. Control experiments were conducted by fixing and staining intoxicated monolayers after the washings and showed that cells were still attached. Residual K^+ was measured by flame photometry (5).

RESULTS

Characterization of purified toxins. The final toxin A and toxin B preparations had cytotoxic activities of 2×10^5 and 4×10^8 MCD/mg of protein, respectively. The specific activity of toxin A is more than 1,000-fold lower than that of toxin B and could not be measured until the toxins were separated (18). Toxin B activity was enriched 44-fold with a combined recovery of 46%. The 50% lethal dose for mice of the starting material was 200 ng. The 50% lethal doses of the final toxin A and toxin B preparations were 3.2 and 45 ng, respectively.

When toxins A and B were analyzed by electrophoretic and immunochemical techniques, toxin A was found to be free of contaminating toxin B and to be nearly homogeneous. The toxin B preparation, on the other hand, still appeared to contain some toxin A as well as a number of other minor proteins. The first step in the analysis was identification of the cytotoxic proteins. Discontinuous buffer PAGE was carried out in 8% gel rods. Gels were sliced in half lengthwise, and one half was Coomassie blue stained. Toxin A showed a single protein band (R_f of 0.074), whereas toxin B showed one major band (R_f of 0.16) and two additional minor bands (Fig. 3). To determine which bands possessed cytotoxic activity, the remaining half of each gel rod was sliced into 1-mm slices. The slices were eluted overnight with 20T8/100 buffer–0.02% sodium azide for cytotoxicity assays. As shown by the elution profiles, each of the proteins detected in the gels showed cytotoxic activity. In toxin A, all detectable activity comigrated with the single protein band. In

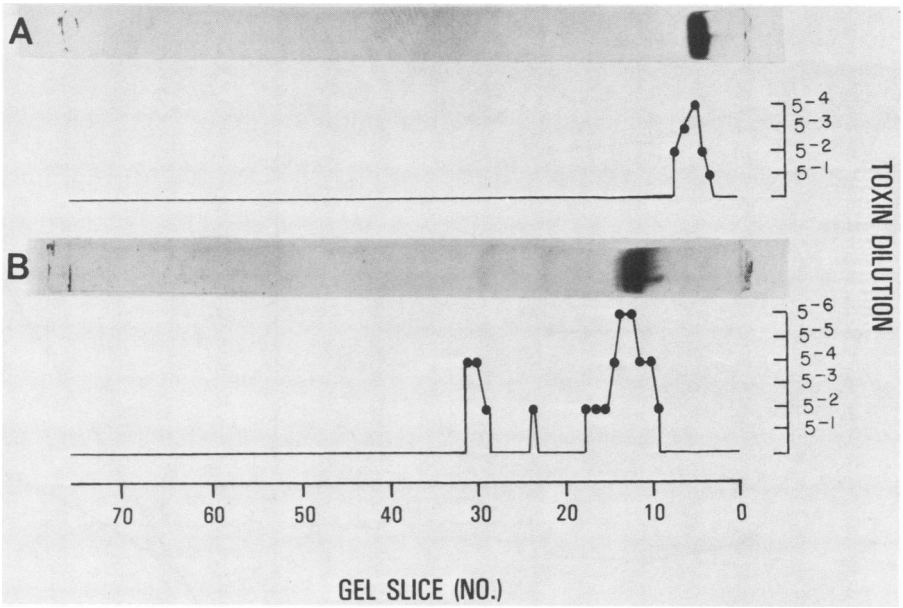

FIG. 3. Discontinuous PAGE of partially purified *C. difficile* toxin A and toxin B with elution profiles. PAGE was performed in 8% polyacrylamide gel rods. Post-DEAE toxin preparations were analyzed at 30 μg per gel. The dye front is shown by injected insoluble dye. Photographs are shown of the stained half of the gel, and the elution profiles show regions where cytotoxic activity was detected by slicing the other half of the gel and assaying eluted fractions in the HeLa cell microtiter assay.

toxin B, the majority of the cytotoxicity comigrated with the major protein band. No cytotoxic components in the toxin B preparation migrated to the toxin A position. Because the two minor, rapidly migrating bands in the toxin B preparation contained cytotoxic activity, it is possible that limited proteolysis of the native toxin molecule may have occurred during purification.

Separation of toxin A and toxin B was analyzed further by immunochemical analysis (Fig. 4) by a Western blot technique after discontinuous buffer PAGE in a nondenaturing slab gel. The antiserum used in the immunoblotting was prepared with crude culture filtrates and therefore would be expected to react with both toxins and with any contaminants. The toxin A preparation (Fig. 4, lane 1) has a major protein band (R_f of 0.10) corresponding to the single cytotoxic protein in the rod gel (Fig. 3A). An additional minor, rapidly migrating protein was detected. The toxin B prepara-

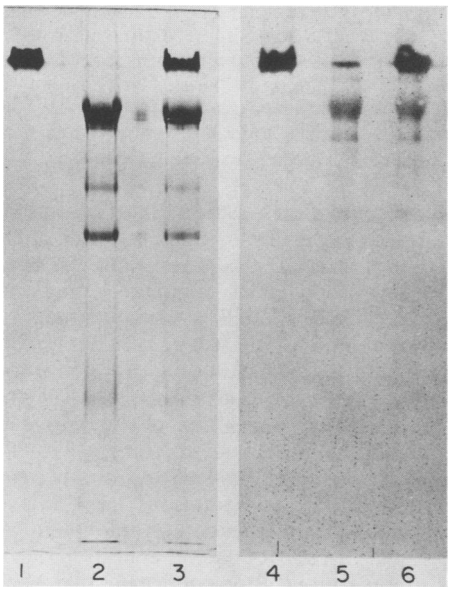

FIG. 4. Discontinuous PAGE and immunoblot analysis of partially purified *C. difficile* toxins A and B. PAGE of toxin A (30 μg, lanes 1 and 4), toxin B (30 μg, lanes 2 and 5), and toxin A plus toxin B (15 μg each, lanes 3 and 6), was performed in an 8% slab gel, and one-half of the gel was stained with Coomassie blue (lanes 1 and 3). Electrophoretic transfer onto nitrocellulose was carried out for 16 h with the other half. The nitrocellulose was incubated with a 1:65 dilution of neutralizing antibody raised against culture filtrates. Lanes 4 through 6 show a 7-h autoradiograph.

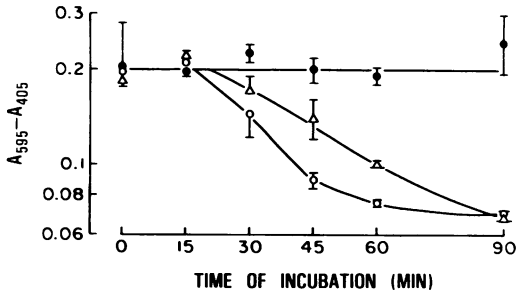

FIG. 5. Cytotoxicity in intact HeLa cells versus time. Solutions of toxin A (○) (4×10^4 MCD, 200 μg) and toxin B (△) (4×10^4 MCD, 0.1 μg) used in studies on leucine incorporation and intracellular K^+ levels were added to HeLa cell monolayers. Medium controls (●) were added concurrently. At times indicated, cells were fixed and stained as in the overnight cytotoxicity assay described in the text. The absorbance values were measured in a microtiter plate reader. Each data point is the mean of four determinations ± standard deviation; each control point is the mean of six determinations ± standard deviation. The standard deviations of the toxin A values at 0 and 15 min, which were omitted for visual clarity, are 0.012 and 0.015, respectively.

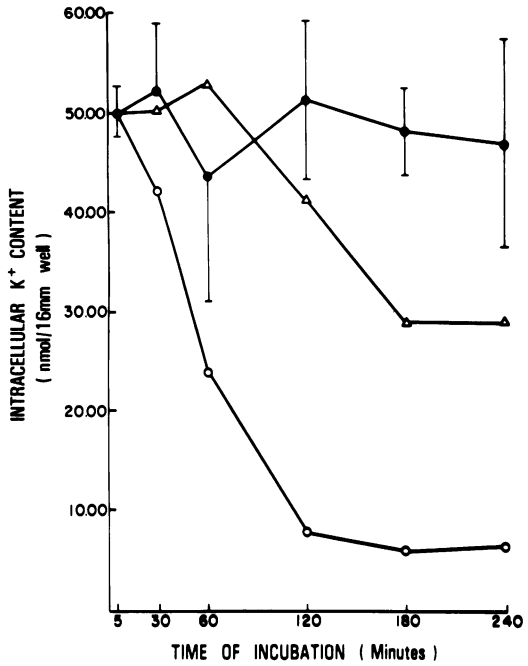

FIG. 6. Decrease in intracellular K^+ levels after exposure to *C. difficile* toxin A and toxin B. A 0.6-ml sample of a toxin solution (4×10^5 MCD/ml) was added to each well (surface area, 2 cm^2). The final toxin A concentration was 1 mg/ml, and the final toxin B concentration was 0.0005 mg/ml. Each control data point (●) represents the mean of three (5 and 30 min) or four determinations (with standard deviations). Toxin A (○) and toxin B (△) data points represent the means of two values. The ranges of the values from 5 to 240 min are as follows: (toxin A) 4.75, 6.30, 8.55, 1.10, 1.80, and 1.30 nmol per well; (toxin B) 5.45, 4.85, 20.05, 9.15, 5.20, and 4.95 nmol per well.

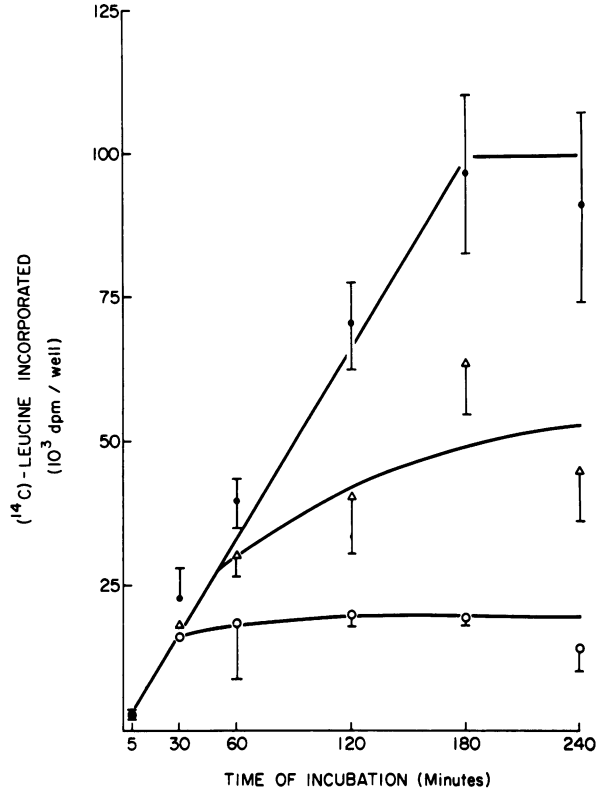

FIG. 7. Inhibition of [^{14}C]leucine incorporation by HeLa cells into trichloroacetic acid-precipitable material after exposure to *C. difficile* toxin A and toxin B. A 0.1-ml sample of a toxin solution (4×10^5 MCD/ml) was added to each well (surface area, 0.3 cm^2). The final protein concentrations of toxin A and toxin B solutions were 1 and 0.0005 mg/ml, respectively. Each control data point (●) represents the mean of six determinations ± standard deviation. Toxin A (○) and toxin B (△) data points represent the means of three values ± standard deviations. Standard deviations (5 and 30 min) are given for only control data samples for visual clarity. Standard deviations for 5 and 30 min are as follows: (toxin A) 924 and 1,287 dpm per well, respectively; (toxin B) 659 and 1,041 dpm per well, respectively.

tion had a major protein band (R_f of 0.20) and two rapidly migrating proteins corresponding to the cytotoxic proteins in the rod gel (Fig. 3B). Additional minor noncytotoxic proteins were stained; one comigrated with toxin A. After they were mixed, the toxin A and toxin B preparations could be resolved (Fig. 4, lane 3) into two major bands, demonstrating that no interaction between the molecules is interfering with their electrophoretic mobility.

The autoradiographic pattern seen when antiserum was incubated with immobilized toxin A (Fig. 4, lane 4) was the same as the pattern seen in the stained gel, a major toxin A band and a minor noncytotoxic component migrating, however, more slowly than toxin B. In the toxin B preparation, toxin B, the major cytotoxic protein, reacted strongly with the antibody, but the minor cytotoxic bands were barely visible. The protein comigrating with toxin A reacted strongly relative to the amount of protein detected by staining. Two minor proteins were apparent which were not detectable in the stained gel. Again, the resolution of toxin A and toxin B could be demonstrated after mixing the preparations (Fig. 4, lane 6).

From Fig. 3 and 4, then, it can be inferred that the toxin A preparation contains no toxin B that can be detected by biological activity, protein staining, or immunochemistry. The rapidly moving bands in the toxin B preparation, two of which are cytotoxic, are possibly degradation products of toxin B. The protein in the toxin B preparation which comigrates with toxin A is not cytotoxic and may be a contaminant of similar physical properties. At any rate, a quantitative examination suggests that it is of no importance in the studies we report below. The activity of toxin A per milligram of protein is 1,000-fold less than that of toxin B. To measure rapidly produced effects on cell functions, we used 200 μg of toxin A per 10^4 cells. As the equivalent cytotoxicity by toxin B requires only 0.1 μg of protein per 10^4 cells, contamination by toxin A would be of no consequence.

Effects on metabolic functions. In the study of the biochemical basis of action of each toxin, we have investigated the time course of their effects on several metabolic functions. Since toxin A is 2,000-fold less cytotoxic per milligram protein than toxin B, toxin solutions were adjusted on the day of each experiment to contain equivalent amounts of cytotoxic activity. Assay of each preparation that day by the overnight cytotoxicity assay confirmed that each solution was of equivalent cytotoxic activity (data not shown). Using these preparations, we examined the changes in morphological and staining characteristics after incubation for 0 to 90 min. Observation of cell morphology by light microscopy showed that all cells in monolayer culture appeared rounded after 45 min of incubation with either toxin A or toxin B at this concentration (4×10^5 MCD/ml). To quantitate these effects, cell monolayers were fixed and stained after short

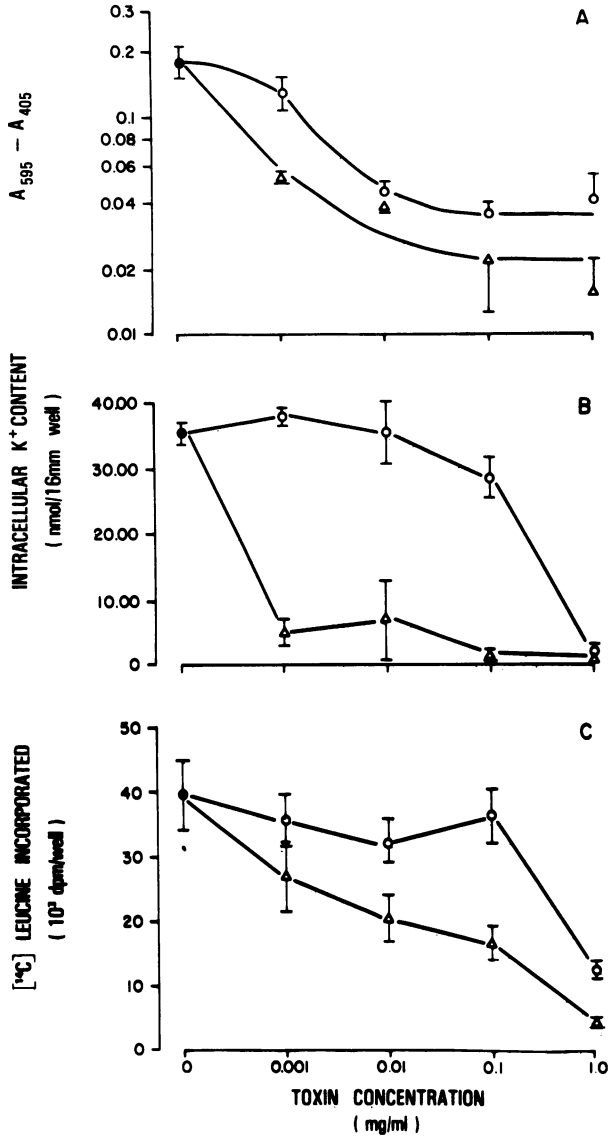

FIG. 8. Changes in HeLa cells after incubation with various concentrations of *C. difficile* toxins. Solutions of toxin A (○) and toxin B (△) were added to intact cell monolayers concurrently with medium controls (●). Incubation was for 120 min. (A) Cytotoxicity, determined as in Fig. 5; (B) decrease in intracellular K^+ levels; (C) inhibition of [^{14}C]leucine incorporation. Each data point is the mean of three determinations ± standard deviation. Control points are the means of six (A and C) or eight (B) determinations.

intervals of toxin incubation. The number of rounded HeLa cells correlates inversely with the staining intensity. By this procedure, cytotoxic effects could be detected in intact cells after incubation for 30 min with toxin A or 45 min with toxin B (Fig. 5). The effect of toxin A was greater until 90 min of incubation.

Since the rapid changes in morphological and staining characteristics suggested that the toxins inhibited membrane functions, we examined the ability of HeLa cells to retain intracellular potassium after incubation with toxin for increasing time intervals. In the presence of *C. difficile* toxins, HeLa cells quickly lost intracellular K^+ (Fig. 6). Loss of K^+ by cells incubated with toxin A was about 50% by 60 min and was complete by 120 min. Toxin B at an equivalent cytotoxic amount caused about 40% loss of K^+ by cell monolayers by 180 min. No further loss was observed at 240 min. Loss was of a lesser extent than that caused by toxin A, despite the equal cytotoxic effects evident in stained cell monolayers 90 min after incubation (Fig. 5).

To determine the effects of these toxins on protein synthesis, we examined their ability to inhibit leucine incorporation. When intact HeLa cell monolayers were incubated with equivalent cytotoxic amounts of *C. difficile* toxin A or toxin B, protein synthesis was inhibited (Fig. 7). Toxin A inhibited after approximately 30 min. Inhibition in cells treated with toxin B was seen after 60 min. However, for both toxins these amounts had caused decreased staining in cell monolayers by 45 min (Fig. 5).

Since these differences in rates of action could reflect the large difference in toxin protein concentration, we examined effects on cell monolayers exposed to equivalent protein concentrations. Cell staining, cellular K^+ levels, and protein synthesis were measured over a range of concentrations of toxin A and toxin B after 120 min of incubation. Figure 8A shows that cytotoxic changes were observed at 120 min at all but the lowest concentration of toxin A, and maximal effect was seen with toxin A and toxin B at both 0.1 and 1.0 mg/ml. In contrast, only the highest concentration of toxin A (1 mg/ml) caused a loss of K^+ and inhibition of protein synthesis at 120 min. With toxin B, all concentrations tested caused complete loss of cellular K^+ by 120 min. Inhibition of protein synthesis by 120 min varied with the amount of toxin B. There was about 30% inhibition at 0.001 mg/ml, 50% at 0.01 mg/ml, 60% at 0.1 mg/ml, and maximal inhibition at 1 mg/ml. For comparison, note that toxin protein concentrations reported in Fig. 5, 6, and 7 are 1.0 mg of toxin A per ml and 0.0005 mg of toxin B per ml. When the toxins were equivalent in protein concentration rather than in cytotoxic activity, then, toxin B was more potent than toxin A after 120 min of incubation.

DISCUSSION

The basis for cytotoxicity to intact HeLa cells by highly purified toxin A and toxin B of *C. difficile* has been investigated. The results presented here suggest that both toxin A and toxin B may exert their action on the cell membrane. With toxin A, loss of potassium and inhibition of protein synthesis occurred concurrently after 30 min of incubation. With toxin B, both potassium levels and protein synthesis were disturbed after 60 min of incubation. Such a sequence of events indicates a primary disruption of membrane permeability or energy metabolism. A concurrent decrease in protein synthesis would result immediately from the decrease in cytoplasmic potassium ion concentration.

Since the specific activity of toxin B is at least 1,000-fold higher than that of toxin A, we first adjusted both to equivalent cytotoxic levels to make comparison feasible after short incubation periods and within the same time frame. At equivalent cytotoxic levels, toxin A produced cell rounding more quickly than did toxin B. In toxin A-treated cells, cell rounding and effects on crystal violet staining correlated with losses of intracellular potassium and inhibition of protein synthesis. Toxin B-treated cells, on the other hand, showed maximal cell rounding before the measured biochemical changes were evident. The protein concentration of toxin A in these experiments was 2,000-fold greater than that of toxin B. When we next compared the action of both toxins at equal protein concentrations over a wide range from 1 mg/ml to 1 μg/ml, toxin A at low doses caused

cell rounding before any changes in K^+ levels or protein synthesis could be detected. On the other hand, at all concentrations, toxin B caused cytotoxicity and disrupted cell functions and K^+ levels more markedly than it inhibited protein synthesis.

The large difference in cytotoxic activity per milligram of protein between toxin A and toxin B has been observed previously (18). Trace contamination of the toxin A preparation with toxin B cannot account for the action of toxin A, since cytotoxicity in the toxin A preparation was associated totally with the toxin A protein band after PAGE. Conversely, a minor amount of toxin A protein in the toxin B preparation would have no detectable effect in these experiments since the cytotoxic activity of toxin A is 1,000-fold less than toxin B on a milligram basis. Possible explanations for this difference are that toxin B has a much higher binding affinity than toxin A, that toxin B acts catalytically and toxin A acts stoichiometrically, and that the toxin A contains 99.9% inactive molecules. Our data do not distinguish among these or other possibilities. In spite of this difference, our data clearly demonstrate that toxin A is a potent cytotoxic protein.

These investigations of the biochemistry of the toxinogenic effects of *C. difficile* required purification and quantitative fractionation of toxin A and toxin B. The procedure described here allowed separation of toxin A and toxin B, neither containing detectable amounts of the other cytotoxic activity. Other workers have shown that ion-exchange chromatography would separate the two toxins (2, 18, 19), and that these proteins are unrelated immunologically. Our use of hydrophobic interaction chromatography before ion-exchange chromatography allowed processing of large amounts of dialyzed filtrates with good recovery and resulted in the removal of large amounts of contaminating protein. As shown in Fig. 3 and 4, electrophoretic and immunochemical analysis of the toxin A preparation failed to detect either cytotoxic activity or a protein band with an electrophoretic mobility corresponding to that of toxin B. In the toxin B preparation, the minor protein band that comigrated with purified toxin A had no apparent cytotoxicity and probably represents a nontoxic protein contaminant.

The availability of relatively large amounts of both purified toxin A and toxin B has allowed us to extend our earlier observation that *C. difficile* toxins induce an inhibition of cellular membrane functions within 1 h after treatment (17). Incubation of cells with high concentrations of toxin A and toxin B resulted in rapid K^+ loss. Although Florin and Thelestam (10) reported partial inhibition of protein synthesis in 4 h, this report is the first biochemical evidence of rapid damage to cells by purified toxin A and toxin B. These workers also observed that 10 mM dinitrophenol, if added within 30 min of toxin addition, prevents the development of actinomorphic changes in toxin-treated cells, suggesting that toxin uptake is an energy-dependent process. Others have looked at the early effects of toxin on tissue cultured cells. Despite 100% HeLa cell rounding in the presence of *C. difficile* toxin, Gurwith et al. (11) found no evidence of membrane damage at 24 h with ^{51}Cr release as the criterion. The kinetics of the appearance of cytopathic effects was not studied by these investigators. Thelestam and Bronnegard (20) found no leakage of [^{3}H]uridine from prelabeled lung fibroblasts after 24 h of incubation, and they concluded that the toxin does not cause membrane damage. They did note, within 3 h, the disruption of straight microfilament bundles, an observation that has been confirmed by Wedel et al. (22). Within 40 min of the addition of toxin B to smooth muscle cells, there is extensive myofilament bundle disruption followed by eventual disappearance of thin myofilaments. These observations with differing toxin preparations from different laboratories require investigation of direct effects of highly purified toxin A and toxin B on the cytoskeleton, on cell membranes, and on energy metabolism.

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