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Effects of *Clostridium difficile* Toxins A and B in Rabbit Small and Large Intestine In Vivo and on Cultured Cells In Vitro

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***Clostridium difficile* is recognized as the major cause of antibiotic-associated colitis. *C. difficile* produces two toxins, A (enterotoxin) and B (cytotoxin), that are implicated in the pathogenesis of the colitis. We examined the dose responses, time course, and synergism of these two toxins in ligated rabbit intestinal loops and in tissue culture. In rabbit small intestinal loops, toxin A caused histologically demonstrable intestinal tissue damage as early as 2 h. The secretory response ≥8 h was similar to that of a cholera toxin control. The effect of toxin A on tissue damage or secretion was seen even if toxin was removed after 5 min. Purified toxin A caused significant net accumulation of sodium, chloride, potassium, and total protein and slightly increased osmolality of the fluid content at 6 h; these effects were similar to those caused by crude *C. difficile* culture filtrates containing toxins A and B. Crude *C. difficile* toxin caused fluid accumulation with a delayed time course in the rabbit large intestine, and in contrast to its effect in small intestine, crude toxin caused net accumulation of bicarbonate and increased pH. In tissue culture, toxin A caused a rounding up of CHO and T-84 colonic carcinoma cells. A monoclonal antibody (PCG-4) that has no effect on tissue culture cytotoxicity with toxins A and B completely inhibited the secretory and tissue-damaging effects in the intestine. Toxins A and B were synergistic in the gut only at high doses of toxin B (≥10 μg/ml), and they were additive in tissue culture. The cytopathic effect in tissue culture was not consistently associated with trypan blue uptake. The cytopathic effect of toxin A in tissue culture did not appear to involve inhibitable Ca^{2+}-dependent or prostaglandin synthesis pathways or intact microfilament or microtubule function for its activity and was not inhibited by reducing or lysosomotropic agents. Our results suggest that toxins A and B have independent and distinct effects in vivo and in vitro.**

The major recognized cause of antibiotic-associated colitis is cytotoxigenic *Clostridium difficile* (3, 6). *C. difficile* produces two toxins (A and B) which are implicated in the pathogenesis of the colitis (1, 6, 29, 31). *C. difficile* toxin A has both enterotoxic and cytopathic activities, and toxin B is a potent cytotoxin (1, 20). Toxin A causes fluid accumulation associated with mucosal damage in several animal models: rabbit ileal and colonic loops (1, 27; A. A. M. Lima et al., Abstr. Clin. Res. **34**:442A, 1986), hamster cecal segments (24), and mouse and rat intestine (24). Toxin B has no enterotoxic activity (1, 20, 24) but is a more potent cytotoxin than toxin A in tissue culture lines by approximately 1,000-fold (1, 20). Both toxins cause erythematous and hemorrhagic lesions in rabbit and guinea pig skin (1, 20), and both toxins are lethal to mice, with toxin A having about the same potency as toxin B when injected intraperitoneally (23). In addition, toxin A is lethal to rabbits when high doses are placed in ligated ileal loops (24). The mechanism by which toxin A causes intestinal secretion and cell damage is unknown, and the relevance of the cytopathic activities of toxins A and B to fluid secretion in the intestine is unclear.

The purpose of these studies was to compare the dose responses, time courses, and synergism of purified *C. difficile* toxins A and B in ligated rabbit small and large intestine and in tissue culture cells. In addition, the cytopathic effect of toxin A seen in tissue culture cells in vitro was explored to examine the possible mechanisms of the cytopathic effect of this toxin, using potential pharmacologic blockers.

(Some of this work was presented at the 1986 national meeting of the American Federation for Clinical Research, Washington, D.C.)

MATERIALS AND METHODS

***C. difficile* crude filtrate and preparation of toxins A and B.** *C. difficile* (VPI 10463) was grown anaerobically in dialysis tubing suspended in brain heart infusion broth. Growth conditions and preparation of filtrates have been described previously (30). Culture filtrates dialyzed (Spectrapor membrane; M_r cutoff, 12,000 to 14,000; Scientific Products, McGaw Park, Ill.) against 0.05 M Tris hydrochloride buffer (pH 7.5) had the same effect as nondialized culture filtrates; the latter were used in most experiments. Sterile filtrates were stored at 4°C until used. Crude toxin was a 1:10 dilution of the sterile filtrate.

Toxin A and toxin B were purified to homogeneity from culture filtrate of *C. difficile* VPI 10463 as previously described (23). Briefly, toxin A was purified by ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose CL-6B, and precipitation at pH 5.6. Toxin B was purified by ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose CL-6B, and immunoaffinity chromatography. Both toxins were homogeneous as shown by crossed immunoelectrophoresis and polyacrylamide gel electrophoresis.

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Cholera toxin was purchased from Schwarz/Mann (Spring Valley, N.Y.). PCG-4 monoclonal antibody raised against purified toxin A was prepared by standard methods (22) and is an immunoglobulin G2a(κ) isotype. This antibody has been reported to neutralize the enterotoxic activity of toxin A (21). For these studies, the PCG-4 monoclonal antibody in ascites fluid of BALB/c mice was used at 1:10 dilution (22). Ascites fluid containing P8H3 monoclonal antibody was used in our studies as a negative control reagent. The P8H3 antibody was obtained against a different clostridial species and does not react with *C. difficile* or its antigens (C. Phelps and T. Wilkins, unpublished data).

Ligated rabbit small and large intestine assays in vivo. Male new Zealand White rabbits (1.5 to 2.5 kg) were used in all studies. Animals were fasted overnight but were given water ad libitum.

Ligated rabbit small intestinal segments (5 cm) were injected with 1 ml of crude filtrate toxin, highly purified toxin A or B from *C. difficile*, or control material (Ringer solution or heat-inactivated toxin). After 2 to 18 h, animals were sacrificed and the volume (milliliters)-to-length (centimeters) ratio for each loop was determined. Fluid recovered from small intestinal segments treated for 6 or 8 h with crude filtrate toxin, toxin A, cholera toxin, or control was assayed for electrolytes, total protein, albumin, osmolality, and pH with a sequential multiple analyzer with a computer (Technicon Instruments Corp., Tarrytown, N.Y.).

For colonic loop studies, rabbits were fasted for 48 h with water ad libitum. Colonic segments (≈25 cm) were washed at least three times with warm Ringer solution. Ligated colonic segments (≈2 cm) were then injected with 1 ml of the toxin or control solution. At 12 and 18 h, the fluid was removed from the segments and assayed for electrolyte content, total protein, albumin, osmolality, and pH as described above and the volume (milliliters)-to-length (centimeters) ratios of the segments were measured.

For histologic studies, segments of ligated rabbit loops were taken at each time point and fixed in 10% Formalin. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for examination by light microscopy.

Tissue culture studies in vitro. Stock cultures of Chinese hamster ovary (CHO) cells were grown in F12 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum in 6% CO_2 at 37°C and were passaged by trypsinization. For morphologic studies, a suspension containing approximately 10^3 cells in 0.2 ml of F12 medium plus 1% fetal calf serum was plated in each well of 96-well microtiter plates. Crude filtrate toxin, toxin A and B, or buffer control and drugs were added. Phase-contrast microscopic examination was made after 24 or 48 h to determine the percentage of cells that had become rounded. Each datum represents the mean percentage of rounded CHO cells among 400 cells counted by phase-contrast microscopy in at least three to four different studies.

Stock cultures of human colonic tumor cell line (T-84 cells), kindly provided by K. Dharmsathaphorn (9), were grown in a 1:1 (vol/vol) solution of Dulbecco modified Eagle medium and Ham's F12 medium supplemented with 15 mM sodium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5) in 6% CO_2 at 37°C and were passaged by trypsinization. For morphologic studies, a suspension containing approximately 10^3 cells in 0.2 ml of the same medium was plated for 12 to 24 h in each well of microtiter plates and *C. difficile* toxin test or control filtrates were added. Phase-contrast microscopic examination was made 24 to 48 h after toxin or control was added, and the percentage of cells that had become rounded was determined. Each datum represents the mean percentage of rounded T-84 cells among 400 cells counted in at least three to four different studies.

All statistical comparisons were done by the *t* test or, when appropriate, the paired *t* test. Probability values of $P < 0.05$ were considered significant.

RESULTS

Small intestinal loop studies. The dose response of *C. difficile* toxin A-induced secretion in ligated small intestinal loops at 6 h is shown in Fig. 1. At 1 μg/ml, toxin A produced hemorrhagic secretion with a ratio of 0.35 ml/cm, while 10 μg/ml of toxin A produced a ratio of 1.65 ml of hemorrhagic fluid secretion per cm ($P < 0.001$). Heat treatment (100°C for 30 min) abolished the secretory effect. The secretory effect was also completely blocked by prior incubation of the toxin for 30 min at 37°C with a monoclonal antibody (PCG-4, 1:10 dilution) specific for the toxin.

Figure 2 shows the time course of *C. difficile* toxin A-induced secretion in ligated small intestinal segments. Toxin A at 10 μg/ml was studied at 2 to 18 h postinoculation. After 2 h, hemorrhagic secretion (0.33 ml/cm) occurred and steadily increased over 18 h (ratio of volume/length, 2.18 ± 0.17 ml/cm; range, 1.86 to 2.45 ml/cm) to a range comparable to that seen at 18 h with 1 μg of cholera toxin per ml (ratio of volume/length, 2.27 ± 0.19 ml/cm; range, 1.41 to 2.91 ml/cm). The onset of fluid secretion with toxin A was slightly faster than with cholera toxin as it approaches maximum as early as 6 h (Fig. 2). When we examined for synergistic intestinal effects with toxins A and B, significant secretory effect was seen only at high doses of toxin B (≥10 μg/ml) (Table 1).

Crude culture filtrates at 1:10 dilution produced significant ($P < 0.001$) hemorrhagic fluid secretion (volume/length =

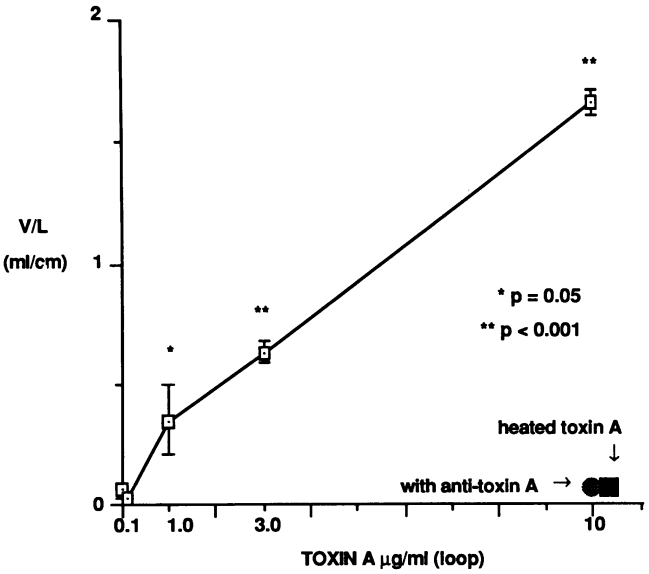

FIG. 1. Dose response of *C. difficile* toxin A-induced secretion in ligated rabbit small intestine at 6 h. Symbols: ⊡, *C. difficile* toxin A; ■, toxin A (10 μg/ml) heated for 100°C for 30 min; ⊛, toxin A incubated for 30 min with PCG-4 monoclonal antibody before injection into the intestinal loop. Bars represent standard error of the mean (SEM). V, Volume; L, length.

1.07 ± 0.09 ml/cm) and tissue damage in small intestinal segments at 6 to 8 h (Table 2). Heated (100°C, 30 min) filtrates were inactive.

Colonic loop studies. Figure 3 shows the time course of *C. difficile* crude filtrate toxin-induced secretion in rabbit colonic loops. Crude toxin (1:10 dilution) effects were studied at 8 to 18 h. There was significant fluid accumulation, but this was less hemorrhagic than seen in small intestinal segments at 12 h; at 18 h fluid accumulation to a range comparable to that seen in small intestinal segments studies occurred. The time course of secretion seen with *C. difficile* toxin in colonic loops was delayed compared with that in small intestinal loop studies (Table 2). Neither toxin A (10 μg/ml) nor toxin B (10 μg/ml) alone or together caused secretion or mucosal damage over 2 to 18 h in rabbit colonic loops, suggesting that other factors are involved in the effect of *C. difficile* in the colon. Only at ≥50 μg/ml did purified toxin A cause colonic secretion similar to that seen with crude toxins (data not shown).

Fluid secretion studies. Table 2 summarizes the intestinal fluid electrolyte content, total protein, albumin, osmolality, and pH after 6 to 8 h of fluid accumulation in small intestinal loops in vivo induced by crude culture filtrate (1:10 dilution in Ringer solution), toxin A, and cholera toxin. *C. difficile* crude toxin induced a significant net accumulation of sodium, chloride, and potassium ($P \leq 0.03$), but did not cause significant bicarbonate accumulation. In contrast to cholera toxin, crude *C. difficile* toxin showed significant net accumulation of total protein (31.4 ± 4.0 mg/cm, $P = 0.01$). The major protein found was albumin (24 ± 3.1 mg/cm, $P = 0.009$) which represented 77% (24/31 mg/cm) of the total protein content in the fluid. The osmolality was greater (328 ± 2.4 mosmol/kg, $P = 0.008$) with *C. difficile* filtrate crude toxin than with heated controls. A similar but smaller effect was seen with toxin A as well as with cholera toxin. In contrast to cholera toxin, crude *C. difficile* toxin caused a significant decrease ($P < 0.025$) in the pH of the intestinal fluid compared with the alkaline pH seen with the control heat-inactivated toxin.

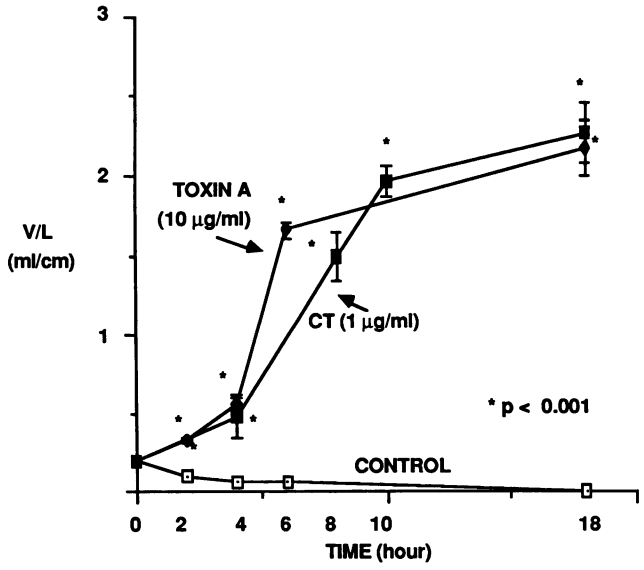

FIG. 2. Time course of *C. difficile* toxin A- and cholera toxin (CT)-induced secretion in ligated rabbit small intestinal segments. Control was with Ringer solution. Bars represent SEM. V, Volume; L, length.

TABLE 1. *C. difficile* toxin A effect in ligated rabbit small intestine at 6 h with different doses of toxin B

Toxin A (μg/ml)	Volume/length ratio (ml/cm) at the following toxin B concn (μg/ml)[a]:			
	0	1.0	10	30[b]
0	0.05 ± 0.03	0.08 ± 0.04	0.13 ± 0.03	0.17 ± 0.06
1.0	0.01 ± 0.004	0.05 ± 0.03	0.27 ± 0.08[c]	0.38 ± 0.13[c]
3.0	0.14 ± 0.03	0.19 ± 0.06	0.50 ± 0.04[d]	0.73 ± 0.19[d]

[a] Each value represents the mean ± SEM in five rabbits.
[b] 30 μg of toxin B per ml corresponds to 5×10^5 times the cytopathic dose in tissue culture cells in vitro.
[c] $P < 0.01$ for toxin A plus B versus toxin A alone.
[d] $P < 0.01$ for toxin A plus B versus toxin B alone or toxin A alone.

C. difficile toxin A (10 μg/ml), like cholera toxin (1 μg/ml), caused net accumulation of sodium, chloride, and potassium ($P < 0.025$). Neither purified toxin A nor crude *C. difficile* toxin caused bicarbonate secretion in the small intestine like that seen with cholera toxin (Table 2). However, crude *C. difficile* toxin did cause net bicarbonate secretion (with other electrolytes) in the large intestine that was significant by 12 h and increased further by 18 h (Table 3). *C. difficile* crude toxin, toxin A, and cholera toxin showed similar intestinal fluid accumulation and increased osmolality in small bowel. In contrast to the effect of cholera toxin, but similar to crude *C. difficile* toxin, purified toxin A caused a significant increase of the total protein ($P < 0.025$).

Table 3 summarizes the effects of crude *C. difficile* toxin on the intestinal fluid electrolytes, total proteins, albumin, osmolality, and pH after 12 and 18 h in rabbit colonic loops in vivo. The control consisted of the fluid just before injection into the colonic loops, since sample controls at 8 to 18 h were not obtainable in amounts adequate for analysis. At 12 h, crude *C. difficile* toxin caused net accumulation of potassium and bicarbonate, and at 18 h net accumulations of all electrolytes were significant. The total protein was significantly increased at 12 h and was further increased at 18 h. Albumin was the major secreted protein at 12 h (35 ± 3.5 mg/cm, $P = 0.01$) and at 18 h (94 ± 7.7 mg/cm, $P = 0.002$), representing 78 and 67% of the total protein, respectively. The osmolality showed significant increases at 12 and 18 h. The pH was significantly increased by 12 and 18 h compared with that of the test material before inoculation into the colonic loops.

Histologic studies. The histologic changes produced by 1 μg of *C. difficile* toxin A per ml after 2 h showed villus disruption with only some basal cells surviving (Fig. 4). Pretreatment of toxin A (10 μg/ml) with monoclonal antibody PCG-4 (1:10 dilution of ascites) completely prevented the toxin-induced intestinal damage and its secretory effect at 6 h.

To determine the time of toxin exposure required for subsequent effects, we placed toxin A (3 μg/ml) and Tris buffer control in adjacent loops for 5 min, after which they were removed and the segments were washed three times with Ringer solution. The loops were then returned to the abdomen for the full 5-h period before the animal was sacrificed and the loop histology was determined. Loops that had been treated with toxin A and subsequently washed showed secretory responses with disrupted villi after 5 h with pathology similar to that described above.

In vitro studies. Toxin A (1 μg/ml)- and toxin B (1 ng/ml)-treated cells (CHO and T-84 cells) were viable after 48 h as determined by trypan blue exclusion. To examine possible mechanisms of the cytopathic effect of *C. difficile*

TABLE 2. Fluid accumulation alterations by *C. difficile* crude filtrate toxin, toxin A (10 μg/ml), and cholera toxin (1 μg/ml) in ligated rabbit small intestinal loops at 6 to 8 h[a]

Treatment	Fluid accumulated (ml/cm)	Electrolytes (microequivalents/cm)				pH	Total protein (mg/cm)	Albumin (mg/cm)	Osmolality (mosmol/kg)
		Sodium	Chloride	Potassium	Bicarbonate				
Control	0.06 ± 0.03	39 ± 3.4	35 ± 3.0	1.2 ± 0.1	4.6 ± 0.4	8.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	297 ± 2.6
Toxin A	1.66 ± 0.05[b]	77 ± 5.5[b]	57 ± 3.3[b]	3.2 ± 0.2[b]	5.1 ± 1.0	ND[c]	27 ± 2.6[b]	ND	308 ± 3.7[b]
Cholera toxin	1.49 ± 0.16[b]	233 ± 4.0[b]	56 ± 4.0[b]	8.9 ± 0.3[b]	60 ± 0.0[b]	8.7 ± 0.1	2.4 ± 0.75	2.0 ± 0.9	325 ± 8.2[b]
Heat-inactivated *C. difficile* toxin	0.22 ± 0.1	33 ± 17.0	12 ± 6.0	2.5 ± 0.9	10 ± 4.3	8.9 ± 0.0	5.7 ± 1.3	1.7 ± 1.0	305 ± 3.0
Active *C. difficile* crude toxin	1.07 ± 0.09[b]	120 ± 12.9[b]	85 ± 8.5[b]	6.8 ± 0.5[d]	14 ± 3.2	7.5 ± 0.1[b]	31.4 ± 4.0[b]	24.1 ± 3.1[b]	328 ± 2.4[b]

[a] Each value represents the mean ± SEM of four to six rabbit ileal loops in at least three different animals.
[b] $P < 0.025$ for active toxins versus heat-inactivated or control solution.
[c] ND, Not done.
[d] $P = 0.03$.

toxin A, we examined reducing agents, calcium antagonists, prostaglandin synthesis inhibitors, microfilament and microtubule inhibitors, and lysosomotropic agents for their effects with toxin A on CHO cells (Table 4). The dose of toxin A used was 0.5 μg/ml (the minimum dose of toxin which consistently caused rounding of 100% of CHO cells). The potential pharmacologic blockers were added 30 min before toxin A. All experiments were done in duplicate wells, in at least three different studies with serial log dilutions of each drug at 10^{-3} to 10^{-6} M. The concentrations of the drugs shown in Table 4 are the maximum doses that produced no morphologic changes in CHO cells alone. Of all the pharmacologic agents tested, none blocked the cytopathic effect of toxin A when compared with Tris buffer controls or drugs alone.

To determine the minimum time required for toxin A to cause cytopathic effect on CHO cells, we incubated toxin A on CHO cells for 5 min, after which it was removed and each well was washed two times with phosphate-buffered saline. Each well was filled with F12 medium supplemented with 1% fetal calf serum and returned to the incubator at 37°C for 24 h. The cytopathic effect of toxin A on CHO cells was rinsable in that two changes with fresh medium after a 5-min exposure to toxin A resulted in a 100-fold reduction in toxin effect. However, this cytopathic effect was not altered or reversed by rinsing after 24 h of toxin exposure. A similar cytopathic effect on the T-84 human colonic carcinoma cells was observed.

We also examined for any additive or synergistic cytopathic effect on CHO cells with toxins A and B (Fig. 5); toxin A at ≥0.5 μg/ml or toxin B at ≥0.2 ng/ml caused rounding of CHO cells, an effect that was additive with subeffective doses of the other toxin.

To evaluate further the possible correlation of toxin A effects on the intestine in vivo and in tissue culture in vitro, we used the same monoclonal antibody (PCG-4) that completely blocks secretory and intestinal mucosa-damaging effects of toxin A. Toxin A or B was treated with monoclonal antibody for 30 min at 37°C before the toxin-antibody mixture was added to CHO cells in vitro. The cytopathic effect caused by toxin A or toxin B at different doses was not altered by the PCG-4 monoclonal antibody when compared with an unrelated P8H3 monoclonal antibody or phosphate-buffered saline control.

DISCUSSION

C. difficile is now recognized as the major cause of antibiotic-associated colitis (2, 5, 6, 11). A substantial body of data implicate a role for toxin A and toxin B in the colitis and diarrhea seen with *C. difficile* infection (5, 12, 18, 19, 24, 27). Immunization against both toxins but not against either alone protects against systemic disease in experimental animals (12, 19). Both toxins are consistently detected in fecal specimens from humans and experimental animals; thus, both toxins are produced during the disease (25). *C. difficile* toxin also correlates well with antibiotic-associated pseudomembranous colitis (4). The disease is usually associated with gross and histologic damage to the colonic mucosa as well as watery diarrhea (90 to 95%), less frequent bloody diarrhea (5 to 10%), and hypoalbuminemia (4). A cytopathic effect of both *C. difficile* toxins has also been shown in tissue culture (10, 31). However, the correlation between the tissue culture cytopathic effect and enterotoxic effect of *C. difficile* toxin and severity of the disease as well as the role of intestinal tissue-damaging effects on the secretory process are unclear. The CHO cell tissue culture assay for toxin is the preferred method to establish the

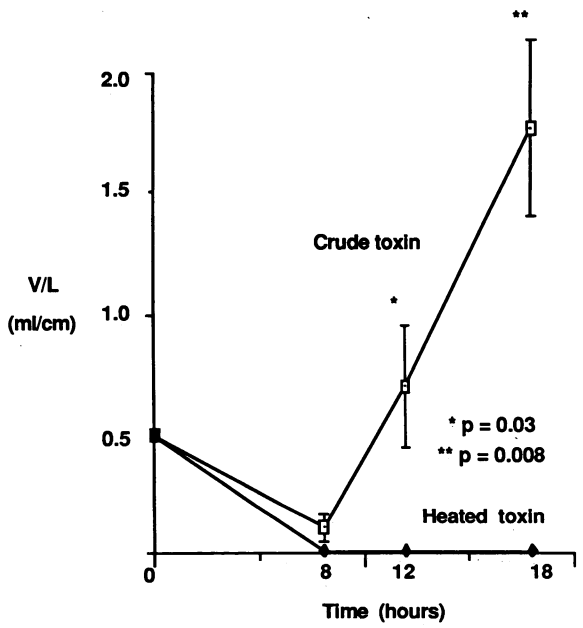

FIG. 3. Time course of crude *C. difficile* culture filtrate toxin-induced secretion in rabbit colonic loops (three different animals). Symbols: ⊡, crude culture filtrate toxin; ◆, crude culture filtrate toxin after heating to 100°C for 30 min. Bars represent SEM. V, Volume; L, length.

TABLE 3. Fluid accumulation alteration by *C. difficile* crude filtrate toxin at 1:10 dilution in rabbit large intestine at 12 and 18 h[a]

Treatment	Fluid accumulated (ml/cm)	Electrolyte (microequivalents/cm)				pH	Total protein (mg/cm)	Albumin (mg/cm)	Osmolality (mosmol/kg)
		Sodium	Chloride	Potassium	Bicarbonate				
Control	0.2 ± 0.0	73 ± 0.0	61 ± 0.0	3.1 ± 0.1	7.8 ± 0.2	7.25 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	295 ± 1.7
Crude toxin at 12 h	0.7 ± 0.25[c]	126 ± 28.9	80 ± 19.5	6.5 ± 0.8[b]	17 ± 2.5[c]	7.52 ± 0.08[b]	45 ± 7.9[b]	35 ± 3.5[b]	349 ± 7.0[b]
Crude toxin at 18 h	1.4 ± 0.37[b]	235 ± 42[c]	134 ± 18[c]	21 ± 0.8[b]	59 ± 15.2[c]	7.77 ± 0.06[b]	140 ± 9.9[b]	94 ± 7.7[b]	345 ± 18.9[b]

[a] Each value represents the mean ± SEM of three to four rabbit colonic loops in at least three different animals.
[b] $P \leq 0.025$.
[c] $P \leq 0.036$.

diagnosis of *C. difficile*-induced diarrhea or colitis (4), even though there is no good correlation between fecal *C. difficile* toxin titer and severity of the disease (7).

In this study, we found that *C. difficile* toxin A initially causes a viscous (at 2 h) and then hemorrhagic fluid secretion that steadily increases over 18 h in ligated rabbit small intestinal segments. These segments usually showed macroscopically hemorrhagic mucosa by 6 h. In contrast, cholera toxin caused clear watery fluid with no gross or microscopic mucosal damage. The amount of secretion caused by 10 μg of toxin A per ml was, however, comparable to that seen with 1 μg of cholera toxin per ml after 18 h. On the basis of their relative molecular weights (toxin A, ≈450,000; cholera toxin, ≈82,000), the molar amount of toxin A (10 μg/ml or 2.2×10^{-8} mol) required for a striking effect in rabbit small intestinal segments was similar to that of cholera toxin (1 μg/ml or 1.22×10^{-8} mol).

Toxin A caused intestinal tissue damage as early as 2 h; thus, we saw no fluid secretion without mucosal cell damage. From these results, toxin A causes secretion only at doses and times when histologic intestinal tissue damage was also seen. This is in contrast to a report by Hughes et al. (15), who saw secretory effects in Ussing chambers in vitro by 90 min without tissue damage. More recently, other workers (27, 29) implicate an association of intestinal tissue damage with net fluid secretion caused by *C. difficile* enterotoxin (toxin A).

Toxin A (10 μg/ml) caused significant secretion of electrolytes (Table 2) by 6 h in rabbit small intestine, a response similar to that seen with cholera toxin (except for bicarbonate). However, unlike cholera toxin, toxin A caused an increase in total protein in the intestine, which further fits the close association of fluid accumulation with the tissue-damaging effect of the crude or purified toxin.

Toxin A at doses of ≥3 μg/ml plus toxin B at ≥10 μg/ml caused a synergistic secretory effect in rabbit small intestine by 6 h (five different animals) (Table 1). In the large intestine, neither toxin A (at 10 μg/ml) nor toxin B (at 10 μg/ml) showed any secretory effect separately or together at 8, 12, or 18 h (two to four loops for each point, in two different animals). Synergistic effects were seen only in the rabbit small intestine with toxin B concentrations ≥10 μg/ml, very high concentrations that are often not present in stool specimens of patients with *C. difficile* colitis. The usual cytotoxin titer of 1:100 to 1:1,000 in patient stool specimens would be equivalent to 0.06 to 0.6 μg/ml, respectively (based on a cytotoxin titer_{50} in CHO cells, an amount that rounds ≥50% of the cells, of 0.0006 μg of toxin B per ml). However,

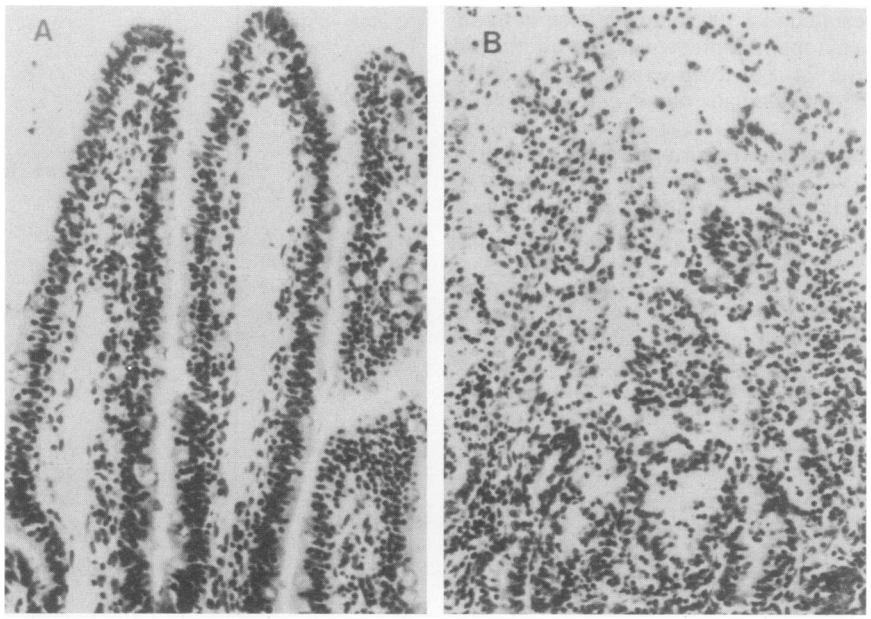

FIG. 4. (A) Control rabbit small intestinal loop after 2 h injected with Ringer solution. (B) Small intestinal loop histology after 2 h of exposure to 1.0 μg of toxin A per ml. Villi are disrupted with some intact basal crypt cells (hematoxylin and eosin, ×80).

this could be relevant in patients with very high cytotoxin titers of ≥1:10,000 in fecal specimens. At the usual cytotoxin titers seen in patients, we would expect no synergistic activity. A previous report by Lyerly et al. (24) showed that toxins A and B are synergistic only with respect to the systemic effects of lethargy and death when given intragastrically to hamsters. Necropsy of these animals showed only small amounts of mucus and edema in the intestine. In contrast to a report by Mitchell et al. (27), we found secretory and tissue-damaging effects with toxin A only at high doses (≥50 μg/ml) in the rabbit large intestine. However, we did find a time course of the secretory response using crude filtrate *C. difficile* toxin that was similar to theirs (27). Additionally, our negative results with toxin B in the large intestine agree with those of Mitchell et al. (27). The dosage differences with our findings might be explained by different *C. difficile* toxin preparations or possibly by the presence of a factor(s) in the rabbit large intestine, different from the small intestine, that might influence the effects of *C. difficile* toxin.

As with purified toxin A, our determination of the fluid secretion composition in both small and large rabbit intestine with crude filtrate toxin showed significant leakage of protein (specifically albumin) into the intestinal lumen, in association with mucosal tissue damage. This protein loss might explain in part the hypoalbuminemia seen with *C. difficile* disease (4). The physiologic pH in the rabbit small intestine also fits with extravasion or equilibration with the plasma fluid (in contrast to the alkaline pH seen in the control group). This result was not associated with changes in bicarbonate accumulation. However, rabbit large intestine showed an increase in the pH that was associated with an increased bicarbonate in the luminal fluid at 12 or 18 h. These results suggest that, in addition, net bicarbonate secretion is seen in the rabbit large intestine exposed to crude *C. difficile* toxin.

Lyerly et al. (22) have developed several monoclonal antibodies which react with homogeneous preparations of toxin A. One of these antibodies, designated PCG-4, precipitates toxin A. This antibody neutralizes the enterotoxic but not the cytotoxic activity of toxin A. We used the PCG-4 monoclonal antibody to show that it not only completely inhibits fluid accumulation in rabbit small intestinal segments, but also completely blocks the intestinal tissue damage caused by toxin A. Because PCG-4 did not inhibit the cytopathic effect of toxin A in CHO or T-84 human colonic carcinoma cells in vitro, these data suggest that the secretory response in intestinal loops in vivo seen with toxin A occurs by a different mechanism from that of the cytopathic effect in CHO and T-84 cells in vitro. These findings clearly separate the cytopathic effect of toxin A on cell cultures in vitro from the intestinal secretion seen with toxin A in vivo.

TABLE 4. Effects of potential pharmacologic blockers of toxin A cytotoxicity in CHO cells in vitro

Treatment	CHO cells (% rounded)[a]
Tris buffer control	3
Toxin A (0.5 μg/ml) alone	100
Reducing agents	
Glutathione (10^{-3} M)	100
Cysteine (10^{-3} M)	100
Calcium channel blocker (diltiazem, 10^{-4} M)	97
Trifluoperazine (10^{-5} M)	100
Indomethacin (10^{-4} M)	100
Colchicine (10^{-5} M)	100
Cytochalasin B (10^{-5} M)	100
Chloroquine (10^{-5} M)	97
Hydrocortisone (10^{-4} M)	100

[a] Percentage of rounded cells per 400 cells counted in duplicate wells in three to four different studies. Highest drug concentrations that produced no morphologic changes alone are shown.

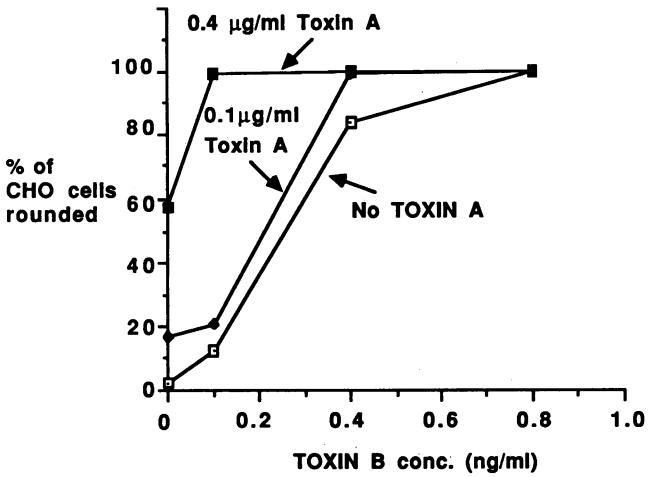

FIG. 5. Dose response of *C. difficile* toxin B on CHO cells in vitro with different amounts of toxin A. Each datum represents the mean percentage of rounded CHO cells among 400 cells counted in at least three to four different studies.

The cytopathic effects seen with toxin A and B did not appear to involve cell membrane damage as grossly examined by trypan blue exclusion in in vitro studies. The mechanism involved in the cytopathic effect of toxin A in tissue culture in vitro did not appear to involve inhibitable Ca^{2+}-dependent (8, 17, 28) or prostaglandin synthesis (14) pathways or intact microfilament or microtubule function for its activity and was not inhibited by reducing (13, 26) or lysosomotropic (16) agents. In addition, toxin A and toxin B appear to have additive cytopathic effects in tissue culture.

In summary, toxin A causes intestinal mucosal damage that precedes intestinal fluid accumulation, and these effects were time and dose dependent. Toxin B alone did not cause intestinal tissue damage or fluid accumulation and had a synergistic effect only when used at high doses (≥10 μg/ml) with toxin A (≥3 μg/ml) in the small intestine. Toxins A and B had additive cytopathic effects in tissue culture. Crude *C. difficile* toxin causes electrolyte secretion and intestinal tissue damage similar to that seen with toxin A. In addition, the effect of toxin A in the small intestine is dissociated from its cytopathic effect on tissue culture cells, and the mechanism of toxin A cytopathic effect in tissue culture does not appear to involve inhibitable Ca^{2+}-dependent or prostaglandin synthesis pathways or intact microfilament, microtubule, or lysosomal function. The full elucidation of the mechanism of the remarkable intestinal secretory and tissue-damaging effects of *C. difficile* toxin A awaits further studies.

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