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Purified *Clostridium difficile* Cytotoxin Stimulates Guanylate Cyclase Activity and Inhibits Adenylate Cyclase Activity

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Antibiotic-associated pseudomembranous colitis has been linked with *Clostridium difficile* toxin. We examined the effect of toxins from four strains of *C. difficile* isolated from patients with pseudomembranous colitis on colonic adenylate (EC 4.6.1.1) and guanylate cyclase (EC 4.6.1.2) activities. Partially purified toxins had a cytotoxic effect on hamster fibroblasts in culture at a concentration of 10 ng/ml. Likewise, these toxins enhanced colonic guanylate cyclase activity two- to threefold, with the maximal stimulation being at 10 ng/ml. These toxins also enhanced guanylate cyclase activity in ileum, cecum, and duodenum. Both the cytotoxic activity on hamster fibroblasts and the enhancement of hamster guanylate cyclase activity were inhibited by antiserum to *C. difficile* toxin. These same toxins inhibited adenylate cyclase activity at a 100-ng/ml concentration, but had no effect at 10 ng/ml. They also had no effect at any concentration on colonic Na^+-K^+ adenosine triphosphatase. To be sure that the findings were not due to a contaminant, a purified *C. difficile* cytotoxin was used, and the same findings were found with the pure cytotoxin (at a 100-fold-lower concentration). The data suggest that activation of guanylate cyclase may be a factor in the pathogenesis of antimicrobial-associated pseudomembranous colitis.

Pseudomembranous colitis has been reported secondary to a number of antimicrobial agents, most often in relation to therapy with ampicillin (19), clindamycin (5), lincomycin (2), and the cephalosporins (7). One prospective study of Tedesco et al. (23) revealed a 10% incidence of pseudomembranous colitis and a 21% incidence of diarrhea (more than five loose stools per day) in 200 consecutive patients receiving clindamycin. Evidence suggests that toxin-producing *Clostridium difficile* is responsible for the pseudomembranous lesions (1, 10, 15). Cyclic nucleotides have been implicated in the mechanism of action of enterotoxins from two other bacteria (8, 20). Thus, cholera toxin stimulates adenylate cyclase (EC 4.6.1.1) activity (20), whereas heat-stable *Escherichia coli* toxin stimulates guanylate cyclase (EC 4.6.1.2) activity (8). The present investigation was designed to determine if *C. difficile* toxin might have its mechanism of action through stimulating adenylate cyclase or guanylate cyclase activity. The effect of *C. difficile* toxin on Na^+-K^+ adenosine triphosphatase (ATPase) was also studied. Since Taylor et al. (N. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res. **28**:285A, 1980) have recently reported separating an enterotoxin produced in vitro from the cytotoxin of *C. difficile*, a pure *C. difficile* cytotoxin (18) was used in the present investigation to help differentiate cytotoxic versus enterotoxic effects on cyclic nucleotide metabolism. The present data indicate that the partially purified toxin from four strains of *C. difficile* recovered from documented human cases of pseudomembranous colitis and the pure *C. difficile* cytotoxin stimulated colonic guanylate cyclase activity, inhibited colonic adenylate cyclase activity, and had no effect on colonic Na^+-K^+ ATPase.

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MATERIALS AND METHODS

Preparation of *C. difficile* toxin. Four strains of *C. difficile* isolated from patients with pseudomembranous colitis (A-179, A-180, A-181, and A-230) were kindly provided by V. R. Dowell, Jr., Centers for Disease Control, Atlanta, Ga. These four strains were grown in reduced brain and heart infusion broth for 72 h. The filtrates were harvested, ultracentrifuged, membrane filtered (0.45 μm), dialyzed against Earle

salts, and partially purified by ammonium sulfate precipitation. The toxins purified 6- to 10-fold by these procedures were found to have their major activity in the 40 to 45% ammonium sulfate fractions. It was these fractions of the partially purified *C. difficile* toxin that were used for the studies reported herein.

Cytotoxicity testing. Serial dilutions of partially purified *C. difficile* toxin were tested for cytopathic effects according to the methods of Larson and Price (13) with the modification of using 24-h cultures of hamster lung fibroblasts. Antiserum (lot no. 1) to *C. difficile* toxin (kindly provided by T. Wilkins, Virginia Polytechnic Institute [6]) was used at a dilution of 1:1,000 and was added to the cell culture simultaneously with the toxin. Results were read after 24 h of incubation.

Purified *C. difficile* cytotoxin. *C. difficile* cytotoxin was isolated from ATCC strain 9689 and purified by ultrafiltration (100,000 nominal molecular weight cutoff), precipitation with 75% $(NH_4)_2SO_4$, and chromatographic separation using Bio-Gel A5m followed by ion-exchange chromatography on a diethylaminoethyl–Sephadex A-25 column as described previously (18). The synthetic basal medium described previously (18) was used for purification of the cytotoxin because it contained only low-molecular-weight (less than 100,000) substances. The purified toxin which displayed only one band on polyacrylamide gel electrophoresis was heat labile, and approximately 170 pg was cytopathic for human amnion cells (18). This isolated toxin increased vascular permeability in rabbits and caused ileocecitis without death in hamsters when injected intracecally (18).

Guanylate cyclase assay. Tissues used in these experiments were obtained from male Syrian golden hamsters weighing 60 to 70 g that had been maintained ad libitum on Purina laboratory chow. The various whole tissues were homogenized with a Polytron homogenizer, using 10-s bursts in cold 0.03 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.6, and then centrifuged at 37,000 × *g* for 15 min (4°C). After centrifugation, 95% of the guanylate cyclase activity was found in the supernatant. The supernatant and particulate fractions were assayed for 10 min at 37°C for guanylate cyclase activity as previously described (4, 24). The reaction mixture consisted of 20 mM Tris-hydrochloride, pH 7.6; 4 mM $MnCl_2$; 2.6 mM cyclic guanosine monophosphate (GMP) (used to minimize destruction of ^{32}P-labeled cyclic GMP); a guanosine triphosphate-regenerating system (5 mM creatine phosphate and 11.25 U of creatine phosphokinase [EC 2.7.3.2]); 100 μg of bovine serum albumin; 20 mM caffeine; and 1.2 mM [α-^{32}P]guanosine triphosphate (approximately 5 × 10^5 cpm). The enzyme preparation contained 0.1 to 0.2 mg of protein. The volume of the supernatant and particulate fractions used was 25 μl, and the final volume of the cyclase assay, which includes either the supernatant or particulate fractions, the reaction mix, and the radioactive isotopes, was 75 μl. After a 10-min incubation, the reaction was terminated by the addition of 10 μl of 0.1 M ethylenediaminetetraacetic acid, pH 7.6, containing about 30,000 cpm of ^3H-labeled cyclic GMP (to estimate recovery in the subsequent steps) and boiling for 3 min. After cooling in an ice bath, the ^{32}P-labeled cyclic GMP formed was isolated by sequential chromatography on Dowex-50-H^+ (200 to 400 mesh) and alumina, using the modification described in detail previously (24). The overall recovery of the cyclic GMP after the two-stage chromatographic procedure was 95%. Cyclic GMP formation was linear with time for at least 20 min and with added protein from 50 to 400 μg. All of the ^{32}P-containing material was identifiable as cyclic GMP as determined by thin-layer chromatography on polyethyleneimine-cellulose (Brinkmann Instruments Inc., Westbury, N.Y.), using 1 M LiCl as the solvent, and on Chromar sheets (Mallinckrodt, St. Louis, Mo.) developed with absolute alcohol and concentrated NH_4OH (5:2, vol/vol). Each of the *C. difficile* toxins was added to the supernatant without any preincubation. Each assay was conducted in triplicate on supernatant and particulate tissue fractions of three animals run separately, with the results confirmed in three separate experiments. Protein was determined by the method of Lowry et al. (14).

Cyclic GMP radioimmunoassay. Cyclic GMP was assayed as previously described (4, 25). Cultures of hamster lung fibroblasts both with and without 1 ng of pure cytotoxin per ml were harvested at 24 h. Any reaction with the cytotoxin was stopped by the addition of 0.5 ml of 10% trichloroacetic acid and freezing in liquid nitrogen. Immediately upon thawing, the cells were sonicated and used in the assay. Approximately 2,000 cpm of cyclic [^3H]GMP was added to the supernatant to monitor recovery. The trichloroacetic acid supernatant was extracted three times with 3 volumes of water-saturated ether. The aqueous residue containing the cyclic nucleotide was purified on an AG 1 × 8 ion-exchange column (Formate form) according to the method of Frandsen and Krishna (9). The appropriate eluate for cyclic GMP was collected, lyophilized, and redissolved in 0.5 ml of 50 mM acetate buffer (pH 6.2), and the portions were assayed. Cyclic GMP samples were acetylated to improve sensitivity. ^{125}I tracer and antibody were obtained from New England Nuclear Corp. (Boston, Mass.). A portion of each sample was digested with cyclic nucleotide phosphodiesterase, and nondigestible material, if any, was subtracted from each value. Results were corrected for recovery and expressed picomoles/10^6 fibroblasts.

Adenylate cyclase assay. About 200 mg of the respective tissues was homogenized in 4.5 ml of cold sucrose and centrifuged, and the supernatant was discarded; the particles were washed with cold 0.25 M sucrose, resuspended, and rehomogenized in the cold 0.25 M sucrose. Adenylate cyclase was assayed as previously described (26), and protein was determined by the method of Lowry et al. (14). The particulate fractions containing 0.09 to 0.12 mg of protein were incubated at 37°C for 15 min with 2.5 mM [α-^{32}P]-adenosine triphosphate (ATP), 3.0 × 10^6 to 3.5 × 10^6 cpm; 8 mM theophylline; 3.0 mM $MgCl_2$; 21 mM Tris-hydrochloride, pH 7.7; 0.9 mM phosphoenolpyruvate; 0.2 mg of pyruvate kinase per ml; 26 mM potassium chloride; and 0.8 mg of bovine serum albumin per ml. The total volume of the incubation mixture was 65 μl.

Na^+-K^+ ATPase assay. Na^+-K^+ ATPase was prepared as previously described (22), with the enzyme being diluted to a final volume of 400 ml and kept frozen in 3- to 5-ml portions in capped tubes. The

average specific activity of the colonic microsomal preparations of ATPase after being thawed once was 10 μmol of inorganic phosphate liberated/mg of protein per h. Protein concentration was determined by the method of Lowry et al. (14). Enzyme incubations were carried out in a total volume of 1 ml at 37°C for 30 min. All additions before initiating the reaction were at 0°C. The reaction was initiated with the addition of ATP. The incubation mixture consisted of 50 mM imidazole, pH 7.4; 100 mM NaCl; 20 mM KCl; 3 mM $MgCl_2$, and 3 mM Tris-ATP. The reaction was terminated by addition of 0.1 ml of 40% cold trichloroacetic acid. Phosphate determination was by the method of Gomori (12).

RESULTS

Effect of *C. difficile* toxin on hamster fibroblasts. The cytotoxicity of *C. difficile* toxin was assayed at each purification step. The partially purified toxins were found to have a cytotoxic effect on hamster fibroblasts in culture at a concentration of 10 ng/ml. These cytotoxic changes consisted of cell rounding and stringing of cellular processes. These changes began in 1 to 3 h and reached a maximum at 24 to 48 h. Dose-response relationships revealed that no cytotoxic effect was present at 1 ng/ml, and a maximal effect was seen when the concentration was increased to 10 ng/ml. Similar effects were seen with pure *C. difficile* cytotoxin (Fig. 1). Dose-response relationships with the pure toxin indicated that a maximal effect was seen at 1.7 pg/ml, and no cytotoxic effect was seen when the concentration was decreased to 0.1 pg/ml. This effect in cell culture was blocked by an antiserum against *C. difficile* toxin at a 1:1,000 dilution.

Effect of *C. difficile* toxin on guanylate cyclase activity. *C. difficile* toxins from the four strains (A-230, A-179, A-180, and A-181) enhanced hamster colonic guanylate cyclase activity two- to threefold at a concentration of 10 ng/ml (Fig. 2). With respect to the ability to stimulate guanylate cyclase activity at all concentrations, the following order was observed: A-181 > A-180 > A-179 > A-230. Dose-response relationships revealed that all of the *C. difficile* toxins had their maximal stimulation of colonic guanylate cyclase activity at a concentration of 10 ng/ml and decreased to nonstimulable levels at 0.01 ng/ml (Fig. 2). These toxins also enhanced guanylate cyclase activity in hamster ileum, cecum, and duodenum (Table 1). Similar data were seen with the pure toxin in that there was a threefold enhancement at the 10-ng/ml concentration (Fig. 2). The pure toxin also enhanced guanylate cyclase activity in ileum, cecum, and duodenum as well as in colon (Table 1). Dose-response relationships revealed that the purified toxin could enhance guanylate cyclase activity at 100-fold-lower concentrations (0.01 ng/ml) (Fig. 2). With the pure toxin, maximal enhancement of guanylate cyclase activity was seen at 10 ng/ml, with significant ($P < 0.001$) enhancement being present at 1.7 pg of the pure toxin per ml. These effects of the *C. difficile* cytotoxin on stimulating guanylate cyclase were blocked by the antiserum against *C. difficile* toxin. Thus, basal colonic guanylate cyclase was increased to 1,683 ± 24 pmol of cyclic GMP/mg of protein per 10-min incubation with 1 ng of pure cytotoxin per ml, whereas with the addition of antiserum (at a 1:1,000 dilution) to the cytotoxin at time zero, only 634 ± 11 (control = 618 ± 17) pmol of cyclic GMP/mg of protein per 10-min incubation was formed. *C. difficile* toxin appeared to be heat labile, since all enhancement of guanylate cyclase activity was abolished with heating the toxins at 100°C for 5 min (data not shown). We also attempted to measure *C. difficile* partially purified and pure cytotoxin effects on particulate guanylate cyclase activity and could find no effect on the particulate enzyme fractions.

Effect of varying the guanylate cyclase cofactor manganese on *C. difficile* activation of guanylate cyclase activity. It has recently been reported that certain agents have no effect on guanylate cyclase activity under normal circumstances, but have an effect only when Mn^{2+} is absent from the incubation mixture (16). A series of experiments was done to determine whether the concentration of the guanylate cyclase cofactor manganese affected *C. difficile* toxin activation of colonic guanylate cyclase in any way. Varying the concentration of $MnCl_2$ did have an effect on *C. difficile* toxin (1 ng/ml) enhancement of guanylate cyclase activity, with a lower maximal stimulation being observed without any manganese present as compared with results obtained with 2 and 4 mM manganese. Similar findings were found with 1 ng of pure *C. difficile* cytotoxin per ml (Fig. 3).

Effect of *C. difficile* toxin on cyclic GMP levels. The pure *C. difficile* cytotoxin (1 ng/ml) incubated for 24 h with cultures of hamster lung fibroblasts increased cyclic GMP levels to 0.29 ± 0.02 pmol/10^6 fibroblasts versus 0.13 ± 0.01 pmol of cyclic GMP/10^6 fibroblasts in the cultures without *C. difficile* cytotoxin.

Effect of *C. difficile* toxin on adenylate cyclase activity. *C. difficile* toxins A-179, A-180, A-181, and A-230 and pure *C. difficile* cytotoxin inhibited hamster colonic adenylate cyclase activity from a basal level of 424 ± 9 pmol/mg of protein per 10-min incubation to 228 ± 13, 263 ± 8, 231 ± 14, 268 ± 10, and 251 ± 16 pmol/

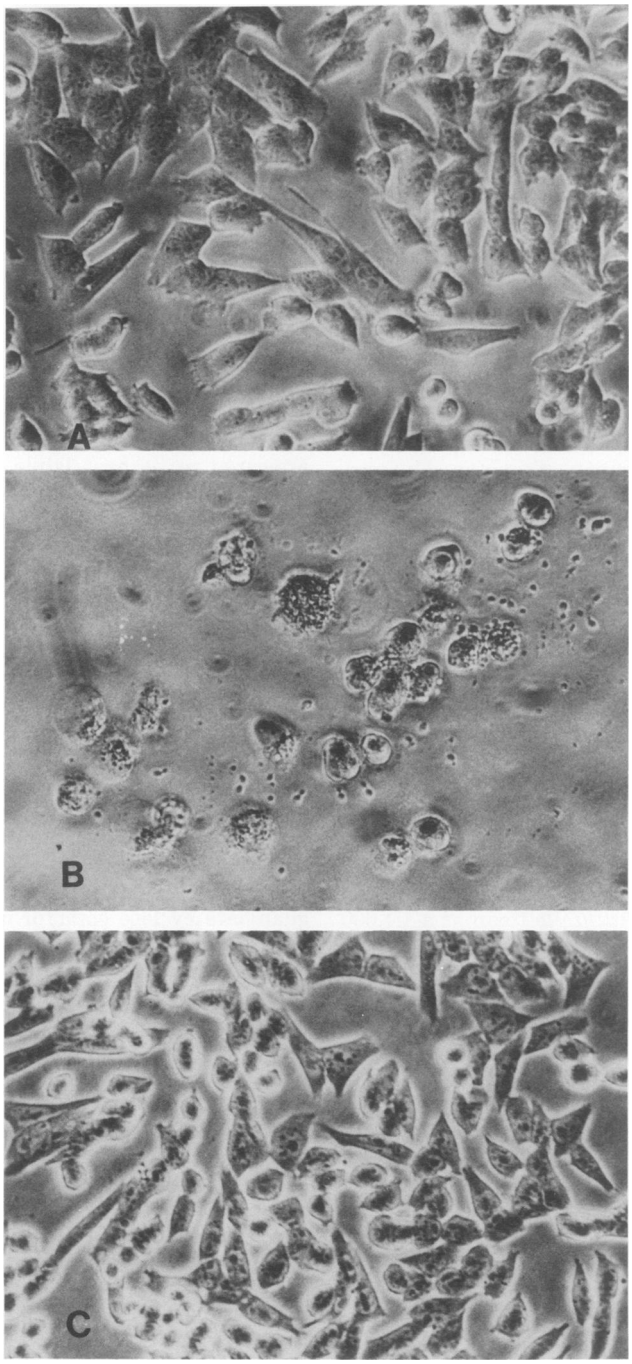

FIG. 1. *Purified C. difficile cytotoxin (2 pg/ml) cytopathic effect on hamster fibroblasts. (A) Cells without toxin at 24 h; (B) cells with toxin at 24 h; (C) cells with toxin and antitoxin (1:1,000 dilution) at 24 h.*

mg of protein per 10-min incubation, respectively, at a 100-ng/ml concentration. There was no effect on colonic adenylate cyclase activity at the 10-ng/ml concentration, and increasing the concentration of the respective toxins above 100 ng/ml had no further inhibitory effect.

Effect of *C. difficile* on Na^+-K^+ ATPase. None of the *C. difficile* toxins isolated from

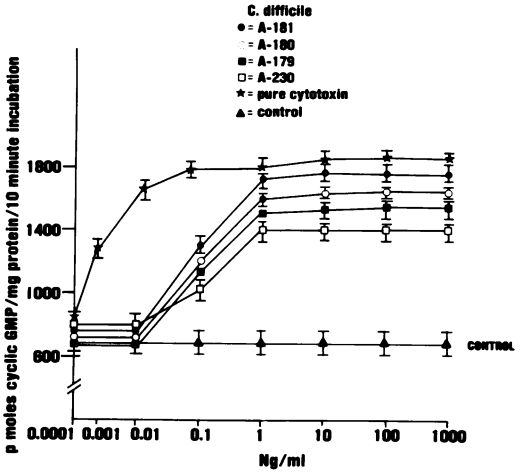

FIG. 2. *Dose-response relationship of C. difficile toxins isolated from four patients (A-179, A-180, A-181, and A-230) with pseudomembranous colitis and pure C. difficile cytotoxin on hamster colonic guanylate cyclase activity. Each value is the mean ± standard error of triplicate samples done on the supernatant of three animals in each group. Each supernatant was run separately and confirmed in three separate experiments. The values at 1 ng/ml (and higher concentrations) were significant at $P < 0.001$ compared with control values by the Student t test for unpaired values.*

TABLE 1. *Enhancement by C. difficile toxin of soluble guanylate cyclase activity in hamster ileum, cecum, and duodenum*

Addition	Concn (ng/ml)	Cyclic GMP[a] (pmol/mg of protein per 10-min incubation)		
		Ileum	Cecum	Duodenum
None		610 ± 12	513 ± 10	464 ± 11
A-179	10	1,240 ± 11	1,034 ± 14**	926 ± 13**
	1	602 ± 15	501 ± 17[ns]	432 ± 18[ns]
A-180	10	1,303 ± 10	1,009 ± 13**	936 ± 12**
	1	616 ± 14	519 ± 22[ns]	444 ± 19[ns]
A-181	10	1,249 ± 13	1,109 ± 11**	952 ± 14**
	1	608 ± 17	511 ± 19[ns]	471 ± 20[ns]
A-230	10	1,228 ± 16	1,062 ± 15**	929 ± 9**
	1	614 ± 19	508 ± 21[ns]	419 ± 24[ns]
Pure toxin	10	1,347 ± 24	1,426 ± 23**	968 ± 22**
	1	1,208 ± 18	1,313 ± 20**	939 ± 21**

[a] Each value is the mean ± standard error of triplicate samples of tissue supernatants of three animals in each group. Each supernatant was run separately and confirmed in three separate experiments ($n = 9$). All values for 10-ng/ml concentrations of *C. difficile* toxins were significant at $P < 0.001$ compared with control by the Student t test for unpaired values; all values for 1-ng/ml concentrations were not significant. Values for pure toxin at both 1 and 10 ng/ml were significant at $P < 0.001$.

strains A-179, A-180, A-181, and A-230 nor the pure cytotoxin had any effect on colonic Na^+-K^+ ATPase (data not shown).

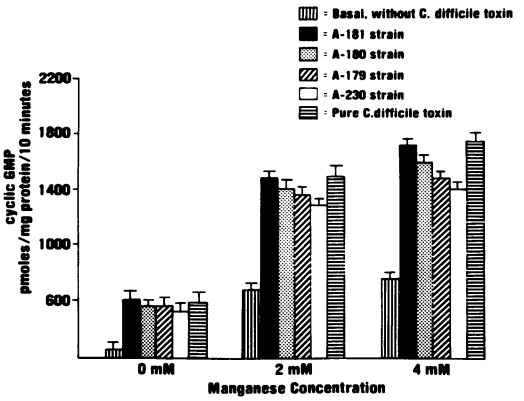

FIG. 3. *Effect of varying the manganese concentration on C. difficile toxin's (at a concentration of 1 ng/ml) enhancement of hamster colonic guanylate cyclase activity. Varying the concentration of the guanylate cyclase cofactor manganese had some effect on the toxin's activation of guanylate cyclase, since a higher maximal activation was seen at the 2 and 4 mM concentrations versus the 0 mM concentration. Each bar is the mean ± standard error of triplicate samples done on the supernatant of three animals in each group run separately in each experiment and confirmed in three separate experiments ($n = 9$). At each concentration of manganese (and even when no cofactors were present), the C. difficile toxin's activation was significant ($P < 0.001$) compared with control values by the Student t test for unpaired values.*

DISCUSSION

In this investigation, pure heat-labile *C. difficile* cytotoxin produced cytotoxicity in hamster fibroblast monolayer cultures and stimulated hamster guanylate cyclase activity. These effects occurred at or below those concentrations of toxin which increased vascular permeability in rabbits and caused ileocecitis in hamsters when injected intracecally. It should be noted that the pure *C. difficile* cytotoxin produced cytopathic effects in hamster fibroblast monolayer cultures similar to those shown previously for human amnion cell cultures (18). These effects were completely neutralized by antiserum to *C. difficile* toxin. Although antitoxin to *C. sordellii* (10, 13, 17) has been shown to block the cytotoxicity of *C. difficile* toxin, Chang and colleagues have shown this antigen-antibody union to be readily reversible, suggesting a cross-reaction rather than a high-affinity specific union (3).

There is a recent report by Taylor et al. (Clin Res. 28:285A, 1980) separating an enterotoxin produced in vitro from the cytotoxin of *C. difficile*. Although the partially purified toxins used in this study may have also contained enterotoxin, the purified cytotoxin did not. The evi-

dence that the pure cytotoxin is not an enterotoxin is fourfold. First, the cytotoxin had a much higher activity in tissue culture than that reported for the enterotoxin (Taylor et al., Clin. Res. **28**:285A, 1980). Second, the susceptibility to pH and trypsin corresponded to that of a cytotoxin. Third, the cytotoxin does not cause death in hamsters when injected intracecally (18), whereas the enterotoxin causes death (Taylor et al., Clin. Res. **28**:285A, 1980). Fourth, the purified cytotoxin did not cause fluid accumulation in the rabbit ileal loop assay, which would indicate that no enterotoxin was present. Although additional studies with a purified enterotoxin are necessary, it would appear that stimulation of guanylate cyclase is definitely associated with the cytotoxin produced by *C. difficile*.

The production of toxins by *C. difficile* was first observed 45 years ago, and in 1937 it was shown on subcutaneous injection into guinea pigs to cause edema, respiratory arrest, and death (21). In the same study, the toxin was found to be heat labile (21). In this regard, it is like cholera toxin, which is heat labile and also has cytotoxic effects. Cholera toxin's mechanism of action, however, involves activation of adenylate cyclase, whereas *C. difficile* enhances guanylate cyclase activity similarly to heat-stable *E. coli* enterotoxin (8, 20). It is of interest that *C. difficile* toxin inhibits adenylate cyclase activity while stimulating guanylate cyclase activity. These findings would indicate that there may be a reciprocal relationship between cyclic GMP and cyclic AMP with regard to *C. difficile* toxin. Whether the yin-yang hypothesis proposed by Goldberg et al. (11), that cyclic AMP and cyclic GMP mediate opposing and reciprocal biological changes, holds for *C. difficile* toxin cannot be determined from the present data. It should be pointed out that there are examples, however, of agents which do not observe this reciprocal relationship between cyclic AMP and cyclic GMP (26).

Certain agents have been reported to have no effect on guanylate cyclase activity under normal circumstances, but have an effect only when the guanylate cyclase cofactor manganese is absent from the incubation mix (16). A series of experiments was done to determine whether the concentration of manganese affected *C. difficile* toxin activation of guanylate cyclase in any way. Varying the concentration of Mn^{2+} did have an effect on *C. difficile* enhancement of guanylate cyclase activity, with a lower maximal stimulation being observed at the 0 mM concentration compared with the results obtained with 2 and 4 mM manganese (Fig. 3).

There was a difference in the present investigation in the ability of the toxins derived from different strains of *C. difficile* to cause cytopathic effects. The toxins from the strains which produced the more marked cytopathic effects were the same toxins which caused a larger degree of enhancement of guanylate cyclase activity. These findings at the cellular level suggest a possible explanation for the observation that some patients with clindamycin treatment have only diarrhea, whereas other patients have the more severe form of the disease with pseudomembrane formation, in that toxins from the different strains caused different maximal effects with regard to both cytopathicity and guanylate cyclase activity. It should be noted, however, that these toxins from the different strains were not completely pure and the above order could be due to a possible contaminant. One would, furthermore, expect that the differences in the severity of the clinical syndrome are almost certainly due to a combination of factors and not due solely to differences in the intrinsic activity of *C. difficile* toxins. Finally, it should be noted that *C. difficile* toxin had no effect on colonic Na^+-K^+ ATPase in the present investigation.

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