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Biological Activities of Toxins A and B of *Clostridium difficile*

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Examination of the biological activities of the two known toxins of *Clostridium difficile* revealed that one of the toxins (toxin A) elicited a hemorrhagic fluid response in rabbit intestinal loops and a positive fluid response in infant mice. The other toxin (toxin B) did not produce a significant fluid response in either model, although the toxin was more lethal in infant mice. Both toxins elicited erythematous and hemorrhagic skin reactions and increased vascular permeability in rabbit skin.

Clostridium difficile is believed to be an important causative agent of antibiotic-associated colitis (AAC) in experimental animals and pseudomembranous colitis in humans (1–4, 10, 13–16). Recently, investigators reported that the bacterium produces at least two toxins which can be separated by ion-exchange chromatography (20a; N. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res. 28:285, 1980). Bartlett et al. (5) and Taylor et al. (Clin. Res. 28:285, 1980) noted that one of the toxins elicited a positive fluid response in rabbit ileal loops and designated this *C. difficile* enterotoxin (referred to in the present study as toxin A). The other toxin was consistently negative in the ileal loop assay and appeared to be primarily a cytotoxin. Therefore, this toxin was designated *C. difficile* cytotoxin (referred to in the present study as toxin B). However, details of the study (e.g., the number of loops injected, toxin dose, volume-to-length fluid accumulation [FA] ratios) were not presented. Our study was undertaken to further assess the activity of *C. difficile* toxins A and B in ligated rabbit intestinal loops. In addition, the activity of these toxins in the rabbit skin permeability assay and infant mouse assay was examined.

Toxins A and B were purified as previously described (20a). Sufficient amounts (50 to 300 μg) of each toxin preparation were analyzed by polyacrylamide gel electrophoresis and crossed immunoelectrophoresis such that at least 0.5% of contaminating toxin A or B could be detected if present. The results of these analyses demonstrated that the toxin A preparation was homogeneous and free of detectable amounts of toxin B and that the toxin B preparation did not contain detectable amounts of toxin A. The results of subsequent studies indicated that 0.5% amounts of the highest doses of toxins A and B tested in each animal model did not produce significant responses, and therefore, any low amounts (less than 0.5%) of contaminating toxin A or B in the toxin preparations did not contribute significantly to the observed responses. Cholera toxin was obtained from the National Institutes of Health (Wyeth Laboratories, experimental lot no. 002). Protein was estimated with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.), with bovine gamma globulin as the standard.

Ligated intestinal loops (nine loops per rabbit with each loop ranging from 5 to 8 cm in length) were prepared in New Zealand white rabbits (1.5 to 1.7 kg) as previously described (7). Rabbits were fasted for 24 h before surgery, and water was provided ad lib before and after surgery. The small intestine of each rabbit was initially rinsed with 10 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.5 mg of neomycin sulfate per ml. Samples (1 ml) of the toxin preparations and diluent (PBS) were injected into the ends of the loops, and the injection site was separated from the remaining portion of the loop by ligation to prevent any leakage of the sample. Test loops in each rabbit were separated by blank loops. The rabbits were sacrificed at 10 h, and a ratio of volume to length (ml/cm) was determined for each loop. Cholera toxin (0.02%) served as the positive control, and ratios of greater than 1.0 were considered positive (21). In addition, sections were obtained from loops injected with the toxin preparations and diluent, fixed in 10% buffered Formalin (pH 7) for paraffin embedding, thick sectioned, and stained with eosin and hematoxylin for observation by light microscopy.

Samples (100 μl) of toxin preparations or

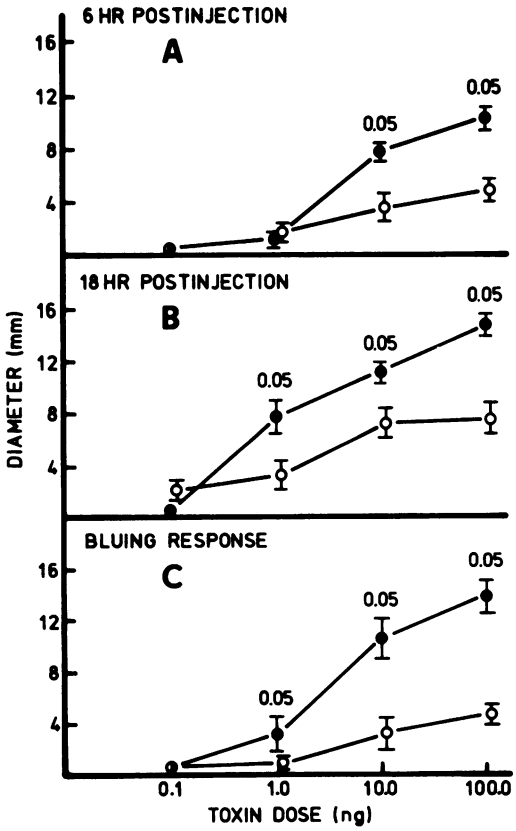
TABLE 1. Response in rabbit intestinal loops after the injection of *C. difficile* toxins A and B

Sample[a]	Vol/length ratio[b]
Toxin A	1.71 ± 0.42
Toxin B	0.44 ± 0.22
Diluent	0.03 ± 0.03
Cholera toxin	1.44 ± 0.11

[a] Samples (1 ml) of toxin A (50 μg), toxin B (50 μg), diluent (0.01 M PBS, pH 7.4), and cholera toxin (0.02%) were injected into the ligated intestinal loops of four rabbits, with each rabbit receiving an injection of each sample. The results in the table are a representative experiment, with all four rabbits being challenged on the same day.

[b] The volume/length ratios were determined 10 h after injection and are expressed as the mean ± 1 standard error. A ratio of greater than 1.0 was considered positive.

diluent (PBS containing 0.05% gelatin) were injected intradermally into the shaved backs of New Zealand white rabbits (2.3 to 3.2 kg), and the diameters of the skin reactions were measured at 6 and 18 h. The ability of the toxins to increase vascular permeability in rabbit skin was examined by the procedure of Evans et al. (9).



The rabbits were injected intravenously with a solution of Evans blue dye in 0.15 M NaCl at a dose of 40 mg per kg of body weight ca. 18 h after the intradermal injection of the samples. Bluing diameters were measured 3 h later. Cholera toxin (0.02%) served as the positive control.

Infant mice (strain CFW, 4 to 5 days old and weighing 3.0 ± 0.5 g) were inoculated intragastrically with samples (50 μl) of toxin A or B preparations or diluent (0.005 M Tris-buffered saline, pH 7.1) as previously described for the inoculation of infant mice with cholera toxin (6). After inoculation, the mice were anally occluded externally with a drop of cyanoacrylate ester glue, and 4 h later the mice were sacrificed by cervical dislocation. The FA ratio was determined for each mouse as previously described (6).

The results of the rabbit skin assay and infant mouse assay studies were examined by an analysis of variance test and the Duncan multiple range test (20). In the rabbit skin assay, a total of four rabbits were used, with each rabbit receiving multiple replicate injections of each toxin dose. Therefore, the rabbit variance was included in the statistical analysis.

Toxin A elicited a viscous hemorrhagic fluid response in rabbit intestinal loops (Table 1). Analysis by light microscopy revealed the presence of an inflammatory infiltrate and hemorrhage in intestinal loops injected with toxin A. The hemorrhagic nature of the response to toxin A is similar to the observations previously reported by Katz et al. (12), who noted that rabbits

FIG. 1. Responses in rabbit skin after the injection of *C. difficile* toxins A and B. Samples (100 μl) of toxins A and B, diluent (0.01 M PBS, pH 7.4, containing 0.05% gelatin), and cholera toxin (0.02%) were injected intradermally into rabbits, and the diameters of the skin reactions were measured at 6 h (A) and 18 h (B) after the injections. After 18 h, the rabbits were injected intravenously with Evans blue dye, and the bluing responses were measured ca. 3 h later (C). A total of four rabbits were injected, with each rabbit receiving four replicate injections of each dose. Each point represents the mean of the lesion diameter, and the bars represent ±1 standard error. At 6 h, 10-ng and 100-ng doses of each toxin elicited significant ($P < 0.05$) erythematous or hemorrhagic reactions or both, and at 18 h, doses of 1 ng or greater resulted in significant reactions ($P < 0.05$). Doses of 1 ng or greater of toxin A produced a significant ($P < 0.05$) bluing response, whereas 10 ng or greater of toxin B produced significant ($P < 0.05$) bluing responses. Cholera toxin, which served as the positive control, produced erythematous reactions which were 3.65 ± 0.83 and 6.36 ± 1.06 mm in diameter at 6 and 18 h, respectively, and produced a bluing response of 2.68 ± 1.88 mm. *P* values are expressed to denote significant differences between toxin A and toxin B responses. Symbols: ●, toxin A; ○, toxin B.

treated with clindamycin developed AAC and that cell-free extracts of the cecal contents from these rabbits produced severe, hemorrhagic ileitis in rabbit intestinal loops. In addition, the intense fluid response produced after the injection of toxin A resembles the earlier findings of Humphrey et al. (11) and Rifkin et al. (18), who reported that the filtrates of the cecal contents and feces of hamsters with AAC elicited positive fluid responses in rabbit ileal loops. Toxin B elicited a weak variable fluid response (Table 1); however, no significant hemorrhage was noted. Higher doses of the toxin B preparation were not tested because the amount of preparation injected correlated to a cytotoxicity titer of ca. 10^7, and this titer is the highest that we have observed in animals and humans with *C. difficile* colitis (unpublished data).

The injection of 1-ng or greater doses of toxin A or B into rabbit skin resulted in the production of erythematous or hemorrhagic lesions or both (Fig. 1A and B), and 10-ng or greater doses of either toxin resulted in an increase in vascular permeability (Fig. 1C). Toxin A produced significantly greater lesions and bluing responses than did toxin B. Rolfe and Finegold (19) reported that a cytotoxin preparation purified from *C. difficile* culture supernatant fluids caused an increase in vascular permeability in rabbit skin, although these investigators did not distinguish between the two known toxins of the bacterium and could not attribute the increased permeability to one or both of the toxins.

In the infant mouse assay, 0.25-μg or greater doses of toxin A caused a significant accumulation of fluid (Fig. 2), and there were no deaths up to the 5-μg dose. Toxin B was much less active, and 2.5 μg or greater was necessary to produce a significant fluid response. Doses of 1 to 5 μg of toxin B resulted in the death of ca. 50% of the mice (the FA ratios of mice receiving 1- to 5-μg doses of toxin B were determined from the survivors), and all 12 of the mice given 10 μg of toxin B were dead by 4 h.

Although toxin A elicits a positive fluid response, the hemorrhagic nature of this response (at least in rabbit intestinal loops) is not observed in the fluid response to well-characterized enterotoxins such as those of *Escherichia coli* and *Vibrio cholerae* (17). In addition, Donta and Shaffer (8) reported that a *C. difficile* toxin preparation which we found to contain toxins A and B produced morphological changes in mammalian cells which were not associated with the biochemical changes caused by *E. coli* and *V. cholerae* enterotoxins (i.e., did not activate adenylate cyclase or increase production of steroid). Because of these differences, we have not referred to toxin A as an enterotoxin. The ability of toxin A to produce a fluid response may be, at least in part, responsible for the diarrhea observed in AAC and pseudomembranous colitis (3, 14–16). Also, the finding that toxin B is more lethal in infant mice than toxin A indicates that toxin B may be involved in the pathogenesis of colitis caused by *C. difficile*. However, additional studies are needed to evaluate the roles of these toxins in *C. difficile*-induced AAC and pseudomembranous colitis.

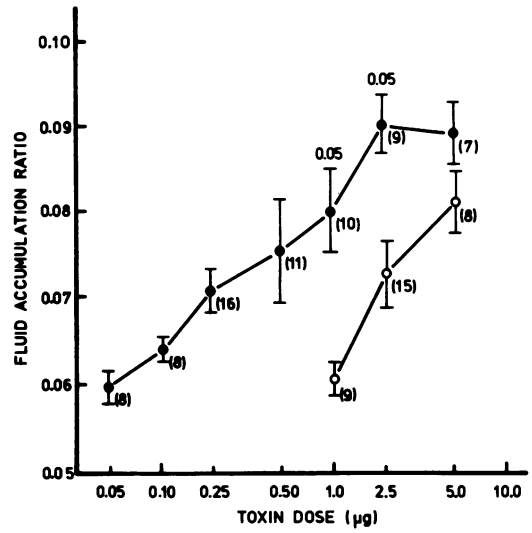


FIG. 2. Responses in the infant mouse after the administration of *C. difficile* toxins A and B. Samples (50 μl) of toxins A and B, heat-inactivated toxins A and B (5 μg of each toxin in 50 μl, heated at 100°C for 30 min), and diluent (0.005 M Tris-buffered saline, pH 7.1) were administered intragastrically into infant mice, and FA ratios were determined 4 h after challenge. The number of infant mice used to determine each data point is given in parentheses. Each point represents the mean of the FA ratio, and the bars represent ±1 standard error. The FA ratios after inoculation with diluent, heat-inactivated toxin A, and heat-inactivated toxin B were 0.0602 ± 0.0025 (11 mice), 0.0634 ± 0.0015 (9 mice), and 0.0549 ± 0.0014 (8 mice), respectively, with these values not being significantly different. Doses of 0.25 μg or greater of toxin A and 2.5 μg or greater of toxin B elicited significant responses ($P < 0.05$). *P* values are expressed to denote significant differences between toxin A and toxin B responses. Symbols: ●, toxin A; ○, toxin B.

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ADDENDUM IN PROOF

Banno et al. (Biochem. Internatl. 2:629–635, 1981) and Taylor et al. (Infect. Immun. 34:1036–1043, 1981) recently reported that the known toxins of *C. difficile*

increased vascular permeability and that one of the toxins elicited a positive fluid accumulation response in rabbit intestinal loops. These findings are consistent with our observations. However, Taylor et al. noted that both toxins were negative in their suckling mouse assay.

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