Production of Antitoxins to Two Toxins of *Clostridium difficile* and Immunological Comparison of the Toxins by Cross-Neutralization Studies

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We prepared antitoxins specific for each of two toxins of *Clostridium difficile* and used these to demonstrate that the toxins are immunologically distinct.

Clostridium difficile has been implicated as the causative agent in many cases of pseudomembranous colitis (4, 6, 11, 12). This bacterium has been reported to produce a large toxin (3, 14, 15, 16) which is cytotoxic to a wide range of tissue culture lines (3, 8, 9, 16) and lethal to animals (3, 9, 16). The toxin is found in humans with this disease (6, 7, 12, 13) and in hamsters treated with clindamycin (1, 5–7, 10). Antibiotic-treated hamsters passively immunized with U.S. Bureau of Biologics *C. sordellii* antitoxin, a cross-reacting preparation which neutralizes the toxin of *C. difficile*, do not succumb to the effects of the toxin (2).

Recently, Bartlett et al. (6) and Taylor et al. (N. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res. **28**:285A, 1980) have suggested that two distinct toxins are produced by *C. difficile*, rather than a single toxin. This conclusion was based on their separation of two distinct activities by use of an NaCl gradient on a DEAE-Sepharose CL-6B ion-exchange column. Toxin A, which eluted at a lower salt concentration than the second toxin, toxin B, was reported to be 17 times more lethal to animals but much less cytotoxic than toxin B (Taylor et al., Clin. Res. **28**:285A, 1980). In addition to these differences, antitoxin prepared against partially purified toxin A failed to neutralize the toxic effects of toxin B, suggesting that the two toxins are antigenically distinct. These investigators have not been able to produce antitoxin to toxin B.

To clarify the relationship between these two toxins, we produced antitoxins specific for both toxin A and toxin B to study the cross-reactions of these preparations with each other.

The toxins were prepared by growing *C. difficile* VPI 10463 at 37°C for 48 h inside dialysis tubing containing 100 ml of saline suspended in 2 liters of brain heart infusion broth as previously described (3, 9). The contents of the dialysis tubing were then harvested by centrifugation at 8,000 × *g* for 10 min, followed by filtration with a

0.45-μm membrane filter. This culture filtrate was then diluted three times with 0.05 M Tris-hydrochloride, pH 7.5, in a thin-channel concentrator with an XM-100 filter (Amicon Corp., Lexington, Mass.). The retentate was finally concentrated to 5% of its original volume, and 35 ml of this preparation was applied to a DEAE-Sepharose CL-6B ion-exchange column (2.5 by 8 cm). Toxin A was eluted from this column with a concentration gradient of 0.14 to 0.16 M NaCl. Toxin B was eluted from this column with a concentration gradient of 0.38 to 0.42 M NaCl. The partially purified toxin preparations were stored at 4°C until used.

Antitoxin to a mixture of partially purified toxins of *C. difficile* (anti-AB) was prepared in rabbits as previously described (9) with the following modifications. Culture filtrate was concentrated with an XM-100 filter and diluted three times with 0.05 M Tris-hydrochloride, pH 7.5. The retentate was concentrated a final time to 5% of its original volume. Ten milliliters of this preparation was applied to a Sepharose 6B column (5 by 70 cm; Pharmacia Fine Chemicals, Uppsala, Sweden). The two toxins, which co-purify with this procedure, were located by cytotoxicity assay and concentrated 20-fold with an XM-100 filter. Toxoid was prepared from this preparation as previously described (9, 16).

Toxin A antitoxin (anti-A) was prepared in rabbits by the procedures previously described (9), with partially purified toxin A used for the preparation of the toxoid. Several attempts to produce antitoxin to toxin B (anti-B) by the same method failed. Antitoxin prepared to crude culture filtrates of *C. difficile*, however, contained antibodies to both toxins. We thought that a similar preparation lacking only toxin A might result in the production of antibodies to toxin B (as well as other *C. difficile* extracellular proteins) but not to toxin A. We therefore prepared a toxoid with 1.0 ml of partially purified toxin B preparation added to 9.0 ml of a dialysis

culture filtrate of *C. difficile* VPI 2037. This strain produces a large number of extracellular proteins but lacks both toxins, as determined by our cytotoxicity and mouse lethality assays. Rabbits injected with this preparation did produce anti-B.

The antitoxins were used first to test for cross-reactivity in neutralization of the cytotoxicity of toxins A and B. The toxicity for tissue culture cells was determined from the percentage of cells (Chinese hamster ovary, CHO-K1) that became round on exposure to either toxin. The CHO-K1 cells were grown and the toxin titers were determined as previously described (9, 16). The TCD_{100} was defined as the greatest dilution of toxin which would round 100% of the CHO-K1 cells in a microtiter well (9). Typical TCD_{100} values for toxin A and toxin B found in culture filtrates of *C. difficile* VPI 10463 were 10^4 and 10^7, respectively (i.e., toxin A, for example, could be diluted up to 1:10,000 and still round 100% of the CHO-K1 cells in the test well). The neutralization of the toxins in the tissue culture assay was performed as follows. Antitoxins were diluted in twofold series with 0.05 M Tris-hydrochloride, pH 7.5, and then mixed 1:1 with either toxin at a concentration 20-fold greater than the TCD_{100}. After incubation at room temperature for 1 h, each mixture was added to a final concentration of 10% to a microtiter well containing CHO-K1 cells (the concentration of the toxin in the well was the TCD_{100}). The antitoxin titer was the greatest dilution of antitoxin which neutralized the TCD_{100}.

The results of the neutralization of the cytotoxic activities are given in Table 1. Anti-A at a dilution of 1:640 neutralized toxin A in the tissue culture assay, but failed to neutralize toxin B. The converse was true for anti-B. As anti-B did not neutralize a TCD_{100} of toxin A, this was the first demonstration that the cytotoxic activity found in the toxin A preparation is not due to contamination with toxin B. Of the three antitoxins tested, anti-B and anti-AB neutralized a TCD_{100} of a culture filtrate of *C. difficile*; anti-A did not neutralize this cytotoxic activity. Toxin B is much more cytotoxic than toxin A, accounting for over 99% of the cytotoxic activity in the culture filtrate; thus, anti-A would not be expected to neutralize detectably the cytotoxic activity of the culture filtrate.

Toxicity in mice was determined by the number of mice (male ICR, 18 to 22 g; Flow Laboratories, Inc., Rockville, Md.) dead after intraperitoneal administration of 0.15 ml of twofold serial dilutions of either toxin (9). The LD_{100} was defined as the greatest dilution of toxin which would kill 100% of the mice tested within 16 h.

We also tested whether there was any cross-reactivity of the antitoxins in regard to protecting mice from the lethal effects of the toxins. Twofold serial dilutions of the antitoxins were mixed with the LD_{100} of each toxin, and after incubation at room temperature for 1 h, 0.3 ml of each mixture was administered to mice by intraperitoneal injection. The titer of each antitoxin was determined to be the highest dilution of antitoxin at which all mice survived the LD_{100}.

Mice were also passively immunized to determine whether in vivo neutralization would show any cross-reactivity. We thought that one toxin might be converted into the other in the animal. In two separate experiments, mice were administered 0.3 ml of the undiluted antitoxins by subcutaneous interscapular injection. At 2 or 6 h after this injection, each antitoxin group was divided and administered an LD_{100} of either toxin preparation by intraperitoneal injection. We have found that mice receiving a subcutaneous injection of 10-fold or greater dilutions of either antitoxin were not protected from the homologous toxin, and for this reason we administered the antitoxins at full strength. Control mice received either toxin preparation but had not been previously immunized with antitoxin.

The results of the first animal neutralization assay are given in Table 1. Anti-A protected the mice from toxin A at a dilution of 1:2,048 and appeared to neutralize toxin B to a very slight extent (1:16). Anti-B did not protect the mice from toxin A at any dilution tested but did protect the mice against toxin B. Neither anti-A nor anti-B protected mice effectively (titers of 1:4 and 1:32, respectively) from the lethal effects of the culture filtrate containing both toxins, but anti-AB did protect the mice at a dilution of 1:256. Thus, neutralizing only one of the toxins did not protect the animals from death due to the culture filtrate injection except at high concentrations of the antitoxins; therefore, there is enough of either toxin in the culture filtrate to kill mice. This experiment provided the only evidence that we have observed suggesting a small amount of cross-reaction between these antitoxins and the nonhomologous toxins. A small degree of antigenic similarity may exist between these two toxins, although the data point to a greater degree of antigenic dissimilarity.

Of the mice receiving a subcutaneous injection of anti-A followed by an intraperitoneal injection of toxin A, 10 of 10 survived. Of those receiving an LD_{100} of toxin B, however, 0 of 10 survived. Of the mice passively immunized with anti-B, 11 of 11 survived an LD_{100} of toxin B, but 0 of 12 survived an LD_{100} of toxin A. Injecting antitoxin either 2 or 6 h before toxin administration made no difference to the survival of these animals.

Passive immunization of mice with specific

TABLE 1. Antitoxin titer for neutralization of the TCD_{100} and LD_{100} of *C. difficile* toxins

Toxin prepn	Neutralization titer with antibody prepn:		
	Anti-A	Anti-B	Anti-AB
Animal assay			
Toxin A	1:2,048	>1:2[a]	1:2,048
Toxin B	1:16	1:256	1:256
Culture filtrate	1:4	1:32	1:256
Tissue culture assay			
Toxin A	1:640	>1:20[a]	1:1,280
Toxin B	>1:20[a]	>1:640	1:5,120
Culture filtrate	>1:20[a]	1:5,120	1:5,120

[a] Limit of assay (no neutralization detectable).

antitoxins thus protected the animals against the homologous toxins but not against the heterologous toxins, supporting the evidence suggesting that the two toxins are immunologically unrelated. Neither toxin was processed by the animals during the course of these experiments to an active form unrecognizable by the homologous antitoxin or recognizable by the heterologous antitoxin. As observed before, either toxin administered intraperitoneally was able to kill mice. However, the importance of either of these toxins in the gastrointestinal tracts of humans with pseudomembranous colitis has not been established.

The relationship between these two toxins remains unclear. Both toxins are large, cytotoxic, lethal to animals (6; Taylor et al., Clin. Res. **28**:285A, 1980), neutralized by U.S. Bureau of Biologics *C. sordellii* antitoxin, and always present in the same ratio (unpublished data). However, the results presented by Bartlett et al. (6), by Taylor et al. (Clin. Res. **28**:285A, 1980), and here clearly demonstrate that these two proteins are immunologically distinct and therefore must be considered separate toxins.

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