

Neutralization of *Clostridium difficile* Toxin by *Clostridium sordellii* Antitoxins

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Neutralization of *Clostridium difficile* toxin by *Clostridium sordellii* antitoxin was studied by cytotoxicity assay in tissue culture. The sources of toxin were stools from two patients with pseudomembranous colitis and a culture filtrate of *C. difficile* isolated from one of the patients. *C. sordellii* antitoxin was available either in monovalent form or as gas gangrene polyvalent antitoxin. The potency of antitoxins against *C. difficile* determined by cytotoxicity assay did not correlate with the established values reported for mouse protection tests against *C. sordellii* toxin. An equivalent zone of optimal neutralization was demonstrated for stool toxin, and a slightly different one for culture toxin. The rate of neutralization appeared to be instantaneous, either at 24 or at 37°C. The efficacy of antitoxin in preventing cytotoxicity in cultured cells preexposed to toxin decreased rapidly with preexposure time. The union between toxin and antitoxin could be readily dissociated by simple dilution or by ammonium sulfate precipitation followed by dilution. Continued incubation of toxin-antitoxin mixture did not increase the firmness of the union; on the contrary, more dissociation occurred. The unusual looseness of the toxin-antitoxin union is probably related to lack of serological specificity or affinity. Based on these observations, a practical diagnostic method for antibiotic-induced colitis is outlined.

Antibiotic-induced pseudomembranous colitis has been shown to be causally related to toxin (1, 4-6, 8, 10) that is produced by *Clostridium difficile* (1, 4-6, 8). The toxin can be readily neutralized by gas gangrene antitoxin or monovalent *Clostridium sordellii* antitoxin (4, 8, 10). Although the exact nature of the cross-reaction between *C. difficile* toxin and *C. sordellii* antitoxin is unknown, the toxin-neutralization test has been used as a confirmatory diagnostic procedure for pseudomembranous colitis (1, 8, 10). In this report, we describe the kinetics of neutralization, factors influencing the rate of reaction, and the nature of toxin-antitoxin union. Based on these observations, a diagnostic procedure is outlined.

MATERIALS AND METHODS

Toxins. Watery stools (no. 121A and 46) from two patients with a proven pseudomembranous colitis were centrifuged at $27,000 \times g$ for 20 min. The supernatants were divided into small portions and stored at -70°C until used. A strain of *C. difficile*, isolated from one of the patients' stools, was grown in chopped-meat-glucose broth for 5 days. It was then centrifuged at $2,000 \times g$ for 20 min, and the supernatant was adjusted with $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation. The precipitate was dissolved in phosphate-buffered saline to

1/20 of the original volume and passed through a 45-nm membrane filter (Millipore Corp., Bedford, Mass.). This filtrate, divided into small aliquots and stored at -70°C , is the crude toxin or culture filtrate used in subsequent experiments.

Antitoxins. Gas gangrene polyvalent antitoxin (lot no. 374-371) was obtained from a commercial source (Lederle Laboratories, Pearl River, N.Y.). Monovalent *C. sordellii* antitoxins were obtained from the Center for Disease Control (CDC), Bureau of Biologics (BBS), and Virginia Polytechnic Institute. The CDC and BBS antitoxins came from a common source. The one from Virginia Polytechnic Institute was an equine immune serum against *C. sordellii*; all others were immunoglobulins prepared from equine immune serum, either as the monovalent form against *C. sordellii* or polyvalent against gas gangrene organisms.

Titration. Toxin titration was carried out by making serial 10-fold dilutions in phosphate-buffered saline at pH 7.2. One tenth of each dilution was inoculated into three WI-38 (human embryonic lung) cell cultures. Cytopathic effects were read after 24 h of incubation at 37°C. The titer was expressed as 50% tissue culture dose (TCD₅₀), as calculated by the method of Reed and Muench (9). The cell culture was purchased from a commercial source (Flow Laboratories, Rockville, Md.) and was maintained in 5% fetal calf serum in minimal Eagle medium.

Demonstration of equivalent zones. Neutralization was carried out by mixing equal amounts (0.2

ml) of varying dilutions of the crude toxins and serial dilutions of the individual antitoxins. The crude toxins were serially diluted 10-fold, and the antitoxin dilutions were made in phosphate-buffered saline in two-fold increments ranging from 1:10 to 1:5,120. After varying periods of incubation, the mixtures were incubated with the WI-38 cell culture. After 24 h of incubation at 37°C, these cultures were examined for cytotoxic changes.

Effect of time and temperature on neutralization. The effect of time and temperature on neutralization was studied by mixing 1 ml of a toxin dose of 100 times the TCD₅₀ (stool no. 121A) with 1 ml each of serial twofold dilutions of *C. sordellii* antitoxin (BBS). One set was incubated at 37°C and the other at 24°C. At 10-min intervals, each mixture was tested for residual toxin by inoculation of WI-38 cell cultures.

Delayed effect of antitoxin. WI-38 cultures were exposed to either 400 or 4,000 TCD₅₀ of the crude *C. difficile* toxin (0.1 ml). *C. sordellii* antitoxin (BBS) was diluted twofold serially, starting at 1:10. Each set of the antitoxin dilutions (0.1 ml each) was added to toxin-treated cell cultures at 10-min intervals up to 60 min. The 0-h portion of the experiment was done by adding antitoxin dilution immediately before the addition of the toxin.

Dissociation of the toxin-antitoxin union. The toxin-antitoxin mixtures (1 ml of each), which had been incubated at room temperature for 30 min, were diluted fivefold serially in phosphate-buffered saline, and each was tested for toxicity by inoculation of cell cultures. Both the stool and culture filtrate were tested at 1:10 dilution against *C. sordellii* antitoxin at 1:40 dilution. The amounts of each component of the toxin-antitoxin mixture were selected from the equivalent zone: i.e., a dilution of antitoxin that demonstrated complete neutralization of the toxin dilution used. Similar toxin-antitoxin mixtures also were tested for toxicity after precipitation with ammonium sulfate. After the toxin-antitoxin mixtures had been incubated for 30 min, (NH₄)₂SO₄ (at 65% saturation) was added, and the resulting precipitate was dissolved in phosphate-buffered saline. The dissolved precipitate was then diluted and inoculated into cell cultures.

The stability of toxin-antitoxin union was further studied by daily observation of neutralization in tissue culture. Serial twofold dilutions (starting at 1:10) of both gas gangrene polyvalent antitoxin and *C. sordellii* antitoxin (BBS) were mixed with two doses of each of the two toxins, one from the stool no. 121A and the other from the culture filtrate.

RESULTS

Titration of toxin. TCD₅₀ per 1 ml was titrated in WI-38 cultures for the two stools and the *C. difficile* culture filtrate. The values, given in log₁₀ numbers, were: stool no. 121A, 5.25; stool no. 46, 5.6; and culture filtrate, 3.6. These represent reciprocal dilutions of 1.8 × 10⁵, 4 × 10⁵, and 4 × 10³, respectively.

Demonstration of equivalent zones. Toxin from the two stools and the *C. difficile* culture filtrate was tested against monovalent *C. sor-*

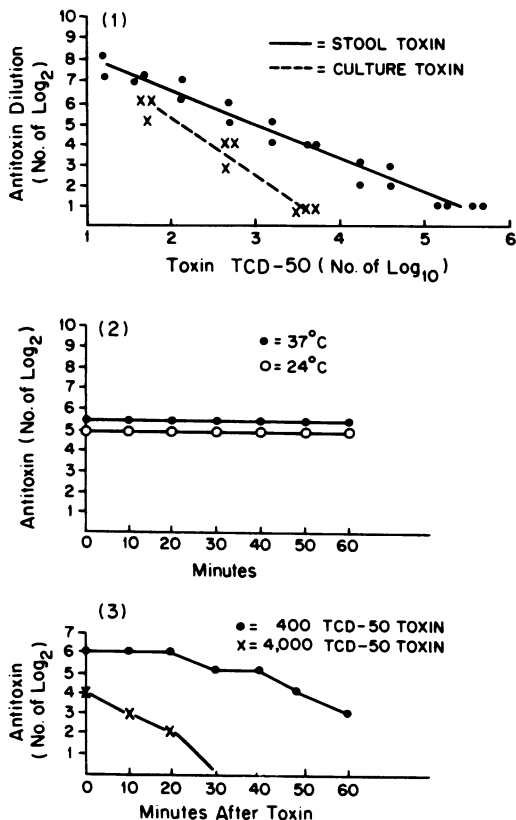


FIG. 1. Toxin-antitoxin reaction: demonstration of equivalent zone. Toxin from three sources was tested against *C. sordellii* antitoxin. The initial antitoxin dilution was 1:10. The straight line represents toxin from stools, and the broken line represents toxin from culture filtrate. Points in the figure represent the highest dilution of antitoxin showing neutralization.

FIG. 2. Effects of temperature and time of exposure on the extent and rate of neutralization. The ordinate represents twofold dilutions of *C. sordellii* antitoxin, starting at 1:10; the abscissa represents time of exposure. Neutralization starts immediately and shows no increase as the incubation of toxin-antitoxin mixture continues.

FIG. 3. Effect of delayed addition of *C. sordellii* antitoxin on the appearance of cytotoxicity in cell cultures preexposed to toxin. The ordinate represents dilution of antitoxin, and the abscissa time after toxin exposure. Points along the lines indicate the highest dilution of antitoxin showing neutralization.

dellii antitoxin. A total of seven box titrations were performed, four with the stools and three with culture filtrate. The results are shown in Fig. 1. Each point represents the highest dilution of antitoxin that gave neutralization of various toxin dilutions. Two equivalent zones were demonstrated, one for stool toxin and the other for culture filtrate. At high toxin concentrations

(4,000 TCD₅₀), the antitoxin showed more affinity for stool toxin than for culture toxin, an eightfold difference. As the toxin concentrations decreased the difference also decreased. At the 40-TCD₅₀ level, only small differences were observed.

Effects of temperature and time of exposure. The effects of temperature and time of exposure on toxin (stool no. 121A) neutralization are shown in Fig. 2. Each point represents the highest dilution of antitoxin showing neutralization. The rapidity of neutralization was impressive in that neutralization was complete almost at the moment of contact between toxin and antitoxin (at 0 min). Further incubation of the toxin-antitoxin mixtures, either at room temperature or at 37°C, did not increase the degree of toxin neutralization. Figure 2 also shows that temperatures of incubation, either 37 or 24°C, did not affect the rate of neutralization.

Effect of delayed addition of antitoxin. The effect of delayed addition of antitoxin on cytotoxicity in tissue culture preexposed to varying amounts of toxin is shown in Fig. 3. At the toxin level of 4,000 TCD₅₀, the amount of antitoxin required for neutralization increased steadily as the time of toxin exposure increase. At 30 min, even the highest concentration of antitoxin (1:10) was ineffective. At a toxin level of 400 TCD₅₀, the effect of delayed addition of antitoxin was less pronounced. After 60 min of toxin exposure, neutralization could still be demonstrated at an antitoxin dilution of 1:40.

Neutralizing activity of clostridial antitoxins. The neutralizing activity of clostridial antitoxins from different sources against *C. difficile* toxin is shown in Table 1. Both polyvalent and monovalent (*C. sordellii*) antitoxins were tested against 4,000 and 400 TCE₅₀ of the toxin

TABLE 1. Neutralizing activity of clostridial antitoxins against *C. difficile* toxin

Antitoxin	Activity ^a at toxin level (TCD ₅₀):	
	4,000	400
Gas gangrene polyvalent antitoxin	40	320
<i>C. sordellii</i> antitoxins ^b		
CDC ^c	20	160
BBS ^c	20	160
Virginia Polytechnic Institute ^d	<10	20

^a Reciprocal of dilution.

^b Monovalent antitoxin (Burroughs Wellcome Co.) had a neutralizing titer of 1:80 against 10 TCD₅₀ of the stool (no. 121A) toxin.

^c Immunoglobulins.

^d Immune serum.

(stool no. 121A). The polyvalent antitoxin was slightly more potent than the monovalent ones from the CDC and BBS. Antitoxin from Virginia Polytechnic Institute had a low titer of activity.

Firmness of toxin-antitoxin union. The firmness of toxin-antitoxin union was examined by simple dilution and by ammonium sulfate precipitation plus dilution. Table 2 shows that toxin-antitoxin union could be readily dissociated by either method. When stool toxin (containing 1,000 TCD₅₀) or culture toxin (containing 100 TCD₅₀) was mixed with *C. sordellii* antitoxin at 1:40 dilution (undiluted in Table 2), no evidence of toxicity was detected (in the equivalent zone). Dilution of both mixtures to 1:25 or more led to the reappearance of toxicity, indicating dissociation of toxin from antitoxin. The amount of toxin released from the mixture ranged from 75 to 95%. When the mixture was treated with 65% ammonium sulfate, the precipitates contained similar amounts of active toxin. In addi-

TABLE 2. Dissociation of toxin-antitoxin^a complex

Toxin	TCD ₅₀	Dilution ^b	No. of cultures with cytotoxicity/no. inoculated		
			Toxin	Toxin-antitoxin mixture	Precipitated toxin-antitoxin mixture
Stool no. 121A	1,000	None	2/2	0/2	2/2
	200	5	2/2	0/2	0/2
	40	25	2/2	2/2	2/2
	8	125	2/2	2/2	2/2
	1.6	625	2/2	0/2	1/2
	0.32	3,125	1/2	0/2	0/2
Culture	100	None	2/2	0/2	2/2
	20	5	2/2	0/2	0/2
	4	25	2/2	2/2	2/2
	0.8	125	1/2	0/2	0/2
	0.16	625	0/2	0/2	0/2

^a Antitoxin used was 1:40 dilution of anti-*C. sordellii* antitoxin (BBS).

^b Reciprocal of dilution.

TABLE 3. Instability of toxin-antitoxin union

Antitoxin	Toxin dose (TCD ₅₀)	Antitoxin titer ^a at time of incubation (h):			
		24	48	72	96
Gas gangrene antitoxin	400	320	160	80	80
	4,000	40	40	20	10
<i>C. sordellii</i> antitoxin	400	160	80	80	40
	4,000	40	20	10	10

^a Reciprocal of antitoxin dilution.

tion, antitoxin was also present in the precipitate, either by ammonium sulfate or in combination with toxin.

The continued incubation of the toxin-antitoxin mixtures did not increase the firmness of their union. On the contrary, more dissociation took place (Table 3). As incubation continued, the amount of antitoxin required for sustained neutralization increased, indicating the instability of toxin-antitoxin union.

DISCUSSION

The cross-reaction between *C. sordellii* antitoxin and *C. difficile* toxin is interesting but somewhat perplexing. We have tested a number of *C. sordellii* strains from various sources in our tissue culture assay. None has produced a cytotoxic material. On the other hand, all strains of *C. difficile* tested in this assay have elaborated a cytotoxin. (A recent report indicated that some strains of *C. difficile* failed to produce toxin [5]. We have had similar experience on initial examination, but subsequently, on careful study, all turned out to be toxin producers.) Since antitoxin to *C. difficile* is not yet available, serological differentiation between these two species based on exotoxin characteristics of clostridia is not possible at the present time.

The antitoxin potency of clostridial antitoxins against *C. difficile* toxin as measured by cytotoxicity assay in tissue culture does not correlate with the established protective potency in mice against the homologous *C. sordellii* toxin(s). The gas gangrene polyvalent antitoxin mixture is reputed to contain 1,500 units of antitoxin against *C. sordellii* toxicity in mice, and the monovalent antitoxin (CDC, BBS) contains 20 units. In the cytotoxicity assay, however, the antitoxic titers of these materials against *C. difficile* toxin differed by only twofold (Table 1). This discrepancy suggests that the methods are measuring different toxin(s). Since a parallel mouse protection against *C. sordellii* was not done, we cannot rule out the possibility that deterioration of the antitoxins may have been a contributory factor.

The rate of combination between toxin and antitoxin is very rapid. By the tissue culture assay, there was no detectable difference in the degree of reaction between the 0-min sample and the subsequent ones taken after varying periods of incubation of the toxin-antitoxin mixture (Fig. 2).

The linear relation between toxin titer and antitoxin dilution is consistent with antigen-antibody reactions in general (Fig. 1). A zone of equivalence between the toxin and antitoxin ap-

pears to exist. It is interesting that the toxin-antitoxin equivalence was somewhat different between stool toxin and culture toxin: less antitoxin was required to neutralize stool toxin than to neutralize culture toxin. Further studies utilizing purified toxin and specific antitoxin are needed to clarify this point.

The toxin-antitoxin reaction was found to be readily reversible. Simple dilution was sufficient to dissociate the union. Addition of ammonium sulfate to 65% saturation precipitated 95% of toxin, and antitoxin as well (Table 2). The reversibility of the reaction is commonly observed in virus neutralization (7) and in certain serological reactions such as dissociation of antibody from precipitates and agglutinated cells and solution of precipitates in excess of antigen (3). The fact that the union between *C. difficile* toxin and *C. sordellii* antitoxin is easily reversible suggests that this represents a cross-reaction rather than a specific reaction which produces a firmer union. Again, the impurity of the toxin and antitoxin preparations precludes any more definite conclusions.

The degree of protection by antitoxin for toxin-treated cells declines rapidly with the time of toxin exposure (Fig. 3). Antitoxin has been shown to protect animals from death if given before toxin challenge, but we have been unable to demonstrate protection if the antitoxin is administered after the toxin (2). This observation suggests that passive immunization may not be a useful therapeutic approach in patients with this disease.

Based on the observations of the kinetics of neutralization, we have developed a rapid and practical diagnostic test for antibiotic-associated pseudomembranous colitis. A variety of cell cultures can be used: HeLa, amnion, WI-38, hamster kidney, human brain, monkey kidney, and others. When primary human amnion cell culture is used, young cultures, less than 3 weeks old, are not suitable. In this test, 0.1 ml of a 1:5 dilution of commercially available gas gangrene antitoxin (Lederle) or monovalent *C. sordellii* antitoxin is added to half of 2 to 4 cell cultures. This is followed by inoculation of all cultures with 0.1 ml of centrifuged watery stool specimen or aqueous extract of formed stool. No preincubation of the stool specimen with antitoxin is necessary. These cultures are placed in an incubator and examined microscopically 3 to 5 h later and again after overnight incubation. The test is positive if cytotoxicity appears in cultures inoculated with the stool sample alone, but not in those containing antitoxin. Because of the possibility of dissociation, incubation for more than 2 days is not recommended.

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