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# Production of *Clostridium difficile* Antitoxin

MARION EHRICH,* ROGER L. VAN TASSELL, JEFFREY M. LIBBY, AND TRACY D. WILKINS

*Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061*

We have produced antitoxin to the toxin of *Clostridium difficile* in rabbits and in goats. Antitoxin dilutions of 1/8,000 and 1/5,120 were capable of neutralizing lethal doses of the toxin in mice and in tissue culture, respectively.

Pseudomembranous colitis is a relatively rare disease that may result from the disturbance of the colonic flora by antibiotic therapy. Filtrates of stools from patients with pseudomembranous colitis are toxic for tissue culture cells (3, 4, 6–8, 10, 12). This toxicity could be neutralized by *Clostridium sordellii* antitoxin obtained from the U.S. Bureau of Biologics (1, 4–6, 12, 13), and it has been suggested that this fortuitous cross-reaction with the toxin of the true pathogen, *C. difficile* (8, 10), might be used as an important clinical tool in the diagnosis of this disease (6).

Unfortunately, the supply of this *C. sordellii* antitoxin is very limited. There are also no data available on how this antitoxin was prepared or even what strain of *C. sordellii* was used as the source of the toxin. Thus, it seemed to several investigators (5, 6) that there was an obvious need for antitoxin to the toxin of *C. difficile*. For unknown reasons, production of such antitoxin so far has been successful only in our laboratory. This note gives the details of how we immunized the animals and the titers of the antitoxin that resulted.

*C. difficile* VPI 10463 was used because it produced more toxin than the other strains of *C. difficile* that we examined. The organism was grown for 48 h at 37°C inside a dialysis sack containing 150 ml of saline suspended in 2 liters of fresh brain heart infusion broth (2, 9; T. D. Wilkins, M. Ehrich, R. Van Tassell, and E. Balish, *in* D. Eaker and T. Wadstrom (ed.), *Natural Toxins*, in press). The inoculum consisted of 1.5 ml of a 1:10 dilution of an overnight culture in brain heart infusion broth. Cells were removed by centrifugation at 8,000 × *g* for 10 min followed by filtration (0.45 μm, Millipore Corp., Bedford, Mass.). The large proteins in this filtrate were concentrated 30-fold with an XM100 filter (Amicon, Lexington, Mass.) and applied to a 5- by 30-cm AcA34 column (LKB, Bromma, Sweden). The toxin was contained in the void volume, and this was further purified by precipitation with 45% saturation of ammonium sulfate at 0 to 4°C. Toxoid was prepared by incubating this toxin (1 mg of protein per ml, Bio-Rad Protein Determination, Bio-Rad Laboratories, Richmond, Calif.) in a final concentration of 0.4% formaldehyde for 36 h at 37°C. This preparation contained the toxin as the major band seen on gradient polyacrylamide gels (molecular weight of 550,000), but several other proteins were still present (2). Toxin was also prepared from *C. sordellii* VPI 8827. This toxin preparation was the culture filtrate from cells grown in the same type of dialysis sack cultures used for *C. difficile* toxin production.

New Zealand white male rabbits, 4 to 6 kg (Pel-Freez Biologicals, Rogers, Ark.), were given two 0.5-ml injections of 1:1 toxoid with Freund complete adjuvant (Miles Laboratories, Elkhart, Ind.) in the muscles of each rear leg at weekly intervals. After three injections, the toxoid was mixed with Freund incomplete adjuvant and injected weekly for 7 more weeks. Antitoxin was detected after 6 to 10 weeks of injections and reached maximum titers within 11 weeks from the start of the injections. At that time, the toxoid-incomplete Freund adjuvant suspension was injected every other week, followed at weekly intervals by 40-ml bleedings from the ear vein. The antitoxin titers remained stable while the animals were kept on this regimen. We also used two randomly bred female goats, about 1 year old (College of Veterinary Medicine, Virginia Polytechnic Institute and State University). They were given 6 ml of the toxoid-adjuvant mix each time by multiple dorsal subcutaneous injections. The goats were injected on the same schedule as the rabbits.

We also obtained *C. sordellii* antitoxin from the U.S. Bureau of Biologics, Bethesda, Md., to test with the toxins of *C. sordellii* VPI 8827 and *C. difficile* VPI 10463.

To obtain neutralization titers, we used both death of tissue culture cells and mice as bioassay systems. Toxicity for tissue culture cells was determined from the percentage of cells (Chinese hamster ovary, CHO-K1) that became round on exposure to the toxin. The CHO-K1 cells were grown in F12 medium with 2% fetal calf serum in the wells of microtiter plates to yield about 1,000 cells per well. Toxin was added to the wells as 0.02 ml of serial twofold dilutions,

TABLE 1. *Neutralization of the toxins of C. difficile and C. sordellii by C. difficile antitoxin and by C. sordellii antitoxin from the U.S. Bureau of Biologics*

| Toxin | Tissue culture titer | | | | Mouse titer | | | |
|---|---|---|---|---|---|---|---|---|
| | *C. difficile* antitoxin | | U.S. Bureau of Biologics | | *C. difficile* antitoxin | | U.S. Bureau of Biologics | |
| | Goat | Rabbit | Lot 33 | Lot 34 or 35 | Goat | Rabbit | Lot 33 | Lot 34 or 35 |
| *C. difficile* | 1:5,120 | 1:1,280 | 1:640 | 1:200 | 1:8,000 | 1:1,024 | 1:256 | 1:64 |
| *C. sordellii* | Not cytotoxic | | | | 1:128 | 1:16 | 1:512 | 1:128 |

and the plate was then incubated at 37°C in 5% $CO_2$ for 18 h. After incubation, the cells were fixed with methanol and then stained with crystal violet. The 50% tissue culture dose was the dilution of toxin yielding 40 to 60% round cells, and the 100% tissue culture dose was the dilution that resulted in 100% round cells. Antitoxin titers were measured by incubating twofold serial dilutions of antitoxin (in 0.01 M potassium phosphate buffer, pH 6.9) with the 100% tissue culture dose of the toxin for 1 h at room temperature and then adding 0.02 ml to each well. The antitoxin titer was the dilution that prevented all of the cells from becoming round. All tests were done at least in duplicate.

Toxicity in mice was determined as the number of mice dead 16 h after intraperitoneal administration of 0.15 ml of twofold serial dilutions of toxin. Each dilution of toxin was given to eight mice, and the lethal doses for 50 and 100% were determined graphically from integrated data (11). Antitoxin titers were determined by mixing twofold serial dilutions of antisera with the 100% lethal dose of the toxin. After incubation for 1 h at room temperature, 0.3 ml of the mixture was administered to mice by intraperitoneal injection. The titer of the antitoxin was the dilution at which all mice survived. This was the assay of choice for *C. sordellii* toxin since this toxin was not cytotoxic.

Table 1 gives the highest dilutions of *C. sordellii* antitoxin which could neutralize *C. difficile* toxin in the two assay systems. The titer of the antitoxin produced in goats was higher than that produced in rabbits. There was only a very small amount of cross-neutralization of *C. sordellii* toxin by *C. difficile* antitoxin. The antitoxin diluted more than 1:1,000 protected mice from *C. difficile* toxin, although dilutions greater than 1:16 failed to protect mice given *C. sordellii* toxin.

Using a standard procedure for neutralization experiments (9), we observed that the *C. difficile* antitoxin did not protect mice from the toxins of several other species of clostridia, including *C. histolyticum*, *C. perfringens* A, *C. perfringens* E, *C. novyi*, and *C. septicum*.

In double diffusion tests, the antitoxin to *C. difficile* produced several bands against *C. difficile* culture filtrate or *C. sordellii* culture filtrate. Multiple bands were also produced when *C. sordellii* antitoxin from the U.S. Bureau of Biologics was tested against *C. difficile* or *C. sordellii* toxins. It should be remembered that this antitoxin was produced against a partially purified preparation, and, therefore, antibodies are present against several proteins. Evidently, some products produced by *C. difficile* and *C. sordellii* are immunologically similar. No bands were seen on double diffusion of *C. difficile* antitoxin with the toxins of *C. histolyticum*, *C. perfringens* A, *C. perfringens* E, *C. novyi*, or *C. septicum*.

We are not sure why we have been successful in obtaining antitoxin to *C. difficile* when others have not. Perhaps it is due to the strain of *C. difficile* that we are using or to the amount of toxin injected. Our antitoxin can be used at higher dilutions than the *C. sordellii* antitoxin, since it has a much higher titer for *C. difficile* toxin. For tissue culture assay we dilute the antitoxin 1:50. We have large amounts of the antitoxin so the dwindling supply of *C. sordellii* antitoxin should no longer be a problem.

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