JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1983, p. 72–78
0095-1137/83/010072-07$02.00/0

Vol. 17, No. 1

Enzyme-Linked Immunosorbent Assay for *Clostridium difficile* Toxin A

DAVID M. LYERLY, NADINE M. SULLIVAN, AND TRACY D. WILKINS*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 11 February 1982/Accepted 28 September 1982

Antibodies against *Clostridium difficile* toxin A were purified by affinity chromatography from antiserum prepared against crude *C. difficile* toxin preparations. The affinity-purified antibody preparation was free of detectable amounts of antibodies to other *C. difficile* antigens, as demonstrated by crossed immunoelectrophoresis, and specifically neutralized the cytotoxicity of toxin A. An indirect enzyme-linked immunosorbent assay (ELISA) was subsequently developed using the antibody preparation for the specific detection of toxin A. The ELISA, which could detect 1 ng (5 ng/ml) of toxin A, was used to quantitate the toxin in the culture supernatant fluids of strains of *C. difficile*. The ELISA values for toxin A closely correlated with the toxin A and B cytotoxic titers of the supernatant fluids. In addition, toxin A was detected by ELISA in human fecal specimens from persons with antibiotic-associated colitis, demonstrating that this toxin is produced during *C. difficile* colitis.

Clostridium difficile, which has been implicated as the major causative agent of antibiotic-associated colitis (AAC) in humans and experimental animals (3–11, 15, 19, 23, 25, 27), produces at least two toxins (2, 31, 32; N. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res. **28**:285, 1980). Both of these toxins are lethal in experimental animals (20, 31) and are cytotoxic in tissue culture assays, although one of the toxins, toxin B, is much more active than the other, toxin A, against all types of tissue culture cells tested to date (13). In addition to their apparent differences in cytotoxic activity, these toxins differ in other biological activities. For example, toxin A elicits a hemorrhagic fluid response in the rabbit intestinal loop assay, whereas toxin B is consistently negative (24, 32). Toxin A also causes a much greater fluid accumulation in the suckling mouse assay than toxin B, even though toxin B is more lethal in the assay (24).

Although toxin A and toxin B can be quantitated by their activity against tissue culture cells, the greater activity of toxin B interferes with the detection of toxin A by this assay; the two toxins have to be separated before the assay to determine the cytotoxic titer of toxin A (31). The different activities of these toxins in the rabbit intestinal loop and suckling mouse assays indicate that these assays might be useful in detecting toxin A; however, these assays are tedious and not very sensitive. The present report describes an enzyme-linked immunosorbent assay (ELISA) that is specific for toxin A and does not require the initial separation of toxins A and B. We examined culture supernatant fluids of *C. difficile* strains by the ELISA for the presence of toxin A, and our results demonstrated that the concentration of toxin A (by ELISA) closely correlated with the cytotoxic titers of toxins A and B in the supernatant fluids. In addition, the presence of toxin A in fecal specimens from persons with AAC was demonstrated by the ELISA.

MATERIALS AND METHODS

Determination of protein. Protein was estimated by the method of Lowry et al. (22) with bovine albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard, and by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as the standard.

Preparation of toxin A-Sepharose. A preparation of toxin A (ca. 1.5 mg of protein in 10 ml of 0.1 M $NaHCO_3$–0.5 M NaCl buffer, pH 8) which was purified to homogeneity by sequential ion-exchange chromatography and precipitation in acetate buffer as previously described (31) was added to 10 ml of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been activated with 3 g of CNBr (12). The suspension was gently mixed overnight at 4°C, and the uncoupled material was removed by washing the gel with 1 bed volume of $NaHCO_3$-NaCl buffer. More than 99% of the protein was bound by the activated Sepharose. The remaining active groups on the Sepharose gel were blocked by adding 1 bed volume of 1 M ethanolamine (pH 8) and mixing the gel for 4 h at room temperature. The gel, designated toxin A-Sepharose,

was washed four times with alternating volumes (2 bed volumes per wash) of 0.1 M sodium acetate–0.5 M NaCl buffer (pH 4) and $NaHCO_3$-NaCl buffer (pH 8) and stored at 4°C in the $NaHCO_3$-NaCl buffer.

Purification of toxin A antibodies. Goat antiserum (1 ml) against crude *C. difficile* toxin preparations (14) was dialyzed against 1 liter of $NaHCO_3$-NaCl buffer overnight at 4°C. The dialyzed material was applied to a column (1 by 12 cm) of toxin A-Sepharose, and the column was washed with the $NaHCO_3$-NaCl buffer until there was no measurable absorbance at 280 nm. Antibodies bound to the gel were eluted by applying 2 ml of 3.5 M KSCN (pH 6.8) (28) to the column and washing with $NaHCO_3$-NaCl buffer. About 2 bed volumes of eluate were collected and concentrated to 1 ml with a Minicon-B15 concentrator (Amicon Corp., Lexington, Mass.). The eluate, designated toxin A antibody preparation, was washed twice (4 ml per wash) on a Minicon-B15 concentrator with physiological saline (pH 7.4).

Crossed IEP. Crossed immunoelectrophoresis (IEP), which was used to demonstrate the monospecificity of the toxin A antibody preparation, was performed on glass slides (5 by 5 cm) with the LKB 2117 Multiphor apparatus and IEP kit, using the general methodology presented in the quantitative IEP manual of Axelsen et al. (1). Wells (1 mm in diameter) were cut in gels composed of 1.2% (wt/vol) low electroendosmotic agarose (Sigma Chemical Co.) in 0.025 M Tris–Tricine buffer (pH 8.6). For the first dimension, samples (4 μl) were placed in the wells and subjected to electrophoresis at 9 to 10 V/cm for 45 min at 10°C. In the second dimension, the upper part of each gel (11.6 cm^2) was composed of a 1.2% agarose gel (3.4 ml) containing 50 μl of antiserum or toxin A antibody preparation. Electrophoresis was performed at 2 V/cm for 18 h at 4°C. Gels were pressed, washed, stained, and destained as previously described (1).

In addition, samples (4 μl containing 20 μg of protein) of homogenous toxin A preparations (31) were subjected to electrophoresis under the conditions described above for the first dimension. The first-dimension gels were sliced into 3-mm sections, and each section was homogenized in 1 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5). The supernatant fluids were examined for cytotoxicity as described below, and the first-dimension migration pattern of toxin A was compared with the crossed IEP pattern obtained with the toxin A antibody preparation.

The class of the antibody against toxin A was determined by crossed IEP. Samples of the toxin A antibody preparation were subjected to electrophoresis in the first dimension as described above and then in the second-dimension agarose containing rabbit anti-goat serum or rabbit anti-goat immunoglobulin G (IgG) (Cappel Laboratories, Inc., Cochranville, Pa.).

Cytotoxicity and neutralization assays. Microtiter plates containing Chinese hamster ovary cells (CHO-K1 cells) were prepared as previously described (14). Samples (20 μl) of toxin A and B preparations (31) were added to the wells, and the minimum amount of toxin necessary to cause 100% rounding (1 TCD_{100}) of the cells was determined as previously described (14).

Neutralization titers were determined by incubating equal volumes of twofold serial dilutions of *C. difficile* antiserum (14) or toxin A antibody preparation (in 0.05 M Tris-hydrochloride buffer, pH 7.5) with 20 TCD_{100} of toxin for 1 h at room temperature and then adding samples (20 μl) of the incubation mixtures to wells such that each well received 1 TCD_{100} of toxin. The neutralization titer was expressed as the reciprocal of the dilution which completely inhibited the rounding of the cells.

ELISA for toxin A. Rabbit antiserum against a crude *C. difficile* toxin preparation (14) was diluted 1/1,000 in carbonate buffer (pH 9.6), and 0.3 ml of the diluted antiserum was added to each well of microtiter plates (Immulon type 2; Dynatech Industries, Alexandria, Va.). The wells on the outer perimeter gave inconsistent results and elevated background readings; therefore, these wells were avoided in the assay. The plates were incubated overnight at 37°C and emptied, and each well was washed once with 0.3 ml of phosphate-buffered saline–0.05% (vol/vol) Tween 20 (PBS-T). Test samples of antigen (0.2 ml) were then added to the wells, and the plates were incubated for 1 h at 37°C. After incubation, the wells were washed four times with PBS-T (the third wash was incubated in the wells for 2 min at room temperature), and 0.2 ml of a 1/500 dilution (in PBS-T containing 0.1% [vol/vol] neutral rabbit serum) of toxin A antibody was added to each well. After incubation for 1 h at 37°C, the wells were washed as described above, and 0.2 ml of a 1/500 dilution (in PBS-T containing 0.1% neutral rabbit serum) of rabbit anti-goat IgG-alkaline phosphatase conjugate (Sigma Chemical Co.) was added. The plates were incubated for 1 h at 37°C and washed as described above, and 0.2 ml of a 1 mg/ml solution (in diethanolamine buffer, pH 9.8) of *p*-nitrophenyl phosphate (Sigma Phosphatase 104 substrate) was added to each well. The plates were incubated for 30 min at room temperature, and the reaction was terminated by the addition of 20 μl of 5 N NaOH to each well. The contents of each well were added to 0.8-ml volumes of deionized water, and the absorbance at 405 nm was measured. Controls included (i) coating the wells with neutral rabbit serum (Pel-Freez Biologicals, Rogers, Ark.) in place of rabbit antiserum, (ii) adding PBS-T in place of the test sample of antigen, and (iii) adding PBS-T in place of the toxin A antibody preparation.

Production of toxin A by strains of *C. difficile*. All of the strains examined for toxin A production were obtained from the Virginia Polytechnic Institute and State University anaerobe collection (Blacksburg) and were identified by L. V. Holdeman and W. E. C. Moore.

Dialysis tubes were prepared which were similar to the dialysis flasks developed by Sterne and Wentzel (30) for the production of botulinum toxin. Dialysis tubing (1 cm in width) containing ca. 2 ml of saline was suspended in ca. 30 ml of brain heart infusion. The tubes were inoculated with actively growing cultures of *C. difficile* by adding 0.1 ml of 1/10 dilutions (in Virginia Polytechnic Institute salts dilution blanks [17]) of the cultures to the saline. After incubation for 3 days at 37°C, the cells were removed by centrifugation (9,000 × *g* for 15 min), and the culture supernatant fluids were passed through 0.45-μm membranes. The culture supernatant fluids were examined for toxin A by ELISA and for cytotoxicity by tissue culture assay. The cytotoxic titer of the supernatant fluids, representing the toxin B activity, was determined as previously described (31). Because toxin B cytotoxicity masks the cytotoxicity of toxin A in culture superna-

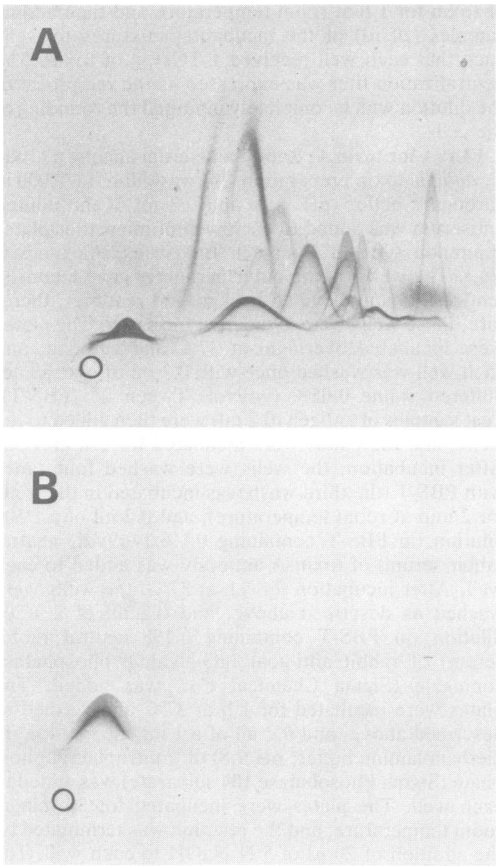

FIG. 1. Analysis of affinity-purified toxin A antibody preparation by crossed IEP. The well in each plate initially contained 22 μg of *C. difficile* strain 10463 culture supernatant fluid (31). (A) The upper portion of the gel contained 50 μl of goat antiserum against crude *C. difficile* toxin preparation (14). At least 20 immunoprecipitin arcs are visible. (B) The upper portion of the gel contained 50 μl of toxin A antibody preparation purified from goat antiserum by affinity chromatography on toxin A-Sepharose. Only one immunoprecipitin arc is visible.

tant fluids, toxin A was separated from toxin B. Samples (0.25 ml) of the supernatant fluids were applied to columns (total bed volume of each column, ca. 2.5 ml) of DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N.J.), and toxin A was eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.25 M NaCl. The results of preliminary studies performed with toxin A and B preparations of known cytotoxicities demonstrated that this procedure effectively separated toxins A and B with less than 10% loss of toxin A cytotoxicity. In addition, the results of neutralization studies demonstrated that the cytotoxicity eluted with 0.25 M NaCl was neutralized by antiserum against partially purified preparations of toxin A, but not by antiserum against partially purified preparations of toxin B (21).

Detection of toxin A in fecal specimens. Before we attempted to detect toxin A in fecal specimens from persons with AAC, we wanted to demonstrate that the ELISA detected the toxin in feces. Toxin A (ca. 10 μg in 1 ml of PBS-T) was added to a fecal specimen (ca. 0.2 g) which was noncytotoxic. One milliliter of PBS-T was then added to the specimen, the specimen was mixed, and the insoluble material was removed by centrifugation (15,000 × *g* for 30 min). The supernatant fluid was passed through a 0.45-μm membrane and examined for the presence of toxin A by ELISA.

Frozen human fecal specimens from patients were obtained from the Medical College of Virginia (Richmond) and from the University of Minnesota (C. Wells and D. Blazevic, Minneapolis). Watery fecal specimens were mixed with an equal volume of PBS-T. For solid stools, 0.2 g of the specimen was mixed with 1 ml of PBS-T. The supernatant fluid of each specimen was obtained as described above and examined for cytotoxicity which was neutralized by *C. difficile* antiserum and for toxin A by ELISA. Controls for ELISA consisted of specimens incubated in wells coated with neutral rabbit serum in place of rabbit *C. difficile* antiserum.

RESULTS

Preparation of toxin A antibodies by affinity chromatography. Toxin A antibodies were specifically removed from goat antiserum raised against a crude *C. difficile* toxin preparation by passing the antiserum through a column of toxin A-Sepharose. The bound antibodies were then eluted from the gel with 3.5 M KSCN, and the eluate was concentrated to the original antiserum sample volume. The resulting antibody preparation was monospecific, based on the following findings: (i) the preparation gave a single immunoprecipitin arc against a crude toxin preparation when analyzed by crossed IEP (Fig. 1); (ii) the single immunoprecipitin arc obtained with the preparation (Fig. 1B) comigrated with the cytotoxicity in homogeneous preparations of toxin A; and (iii) the antibodies in the preparation specifically neutralized the cytotoxicity of toxin A (Table 1). The preparation gave a single immunoprecipitin arc with anti-goat serum identical to the arc obtained with anti-goat IgG, indicating that the antibody against toxin A was IgG.

ELISA for toxin A. An indirect ELISA procedure which was specific for toxin A was developed by coating wells of microtiter plates with rabbit antiserum against a crude *C. difficile* toxin preparation and using the toxin A antibody preparation as the detecting antibody. Bound toxin A antibodies were determined by sequentially adding rabbit anti-goat IgG-alkaline phosphatase conjugate and substrate.

Toxin A gave a dose-dependent response in the ELISA (Fig. 2). The ELISA detected amounts of toxin A as low as 1 ng (5 ng/ml), making the assay more sensitive than the tissue

TABLE 1. Neutralization titers of *C. difficile* crude antiserum (goat) and toxin A antibody preparation against *C. difficile* toxins A and B[a]

Sample	Neutralization Titer[b]	
	toxin A	toxin B
C. difficile crude antiserum (goat)	2560	> 8,000
toxin A antibody preparation	640	< 20[c]

[a] *C. difficile* goat antiserum was raised against a crude *C. difficile* toxin preparation as previously described (14). The toxin A antibody was purified from the goat antiserum by affinity chromatography on toxin A-Sepharose.

[b] Titers are expressed as the reciprocal of the highest dilution of antiserum or antibody preparation necessary to completely inhibit rounding of the cells (1 TCD_{100}). The toxin A preparation was homogeneous and the toxin B preparation was free of detectable amounts of toxin A (31).

[c] Below the detection limits of the neutralization assay.

culture assay, which had a lower detection limit for toxin A of 500 ng/ml. Attempts to increase the sensitivity of the ELISA by coating the wells at 4°C and room temperature and by using lower dilutions of the toxin A antibody and conjugate preparations resulted in an increase in the background. Also, the use of peroxidase-labeled conjugate instead of alkaline phosphatase-labeled conjugate did not increase the sensitivity. The ELISA was consistently negative when tested with preparations of toxin B (amounts of up to 1 μg were tested) which were free of detectable amounts of toxin A (31).

Correlation of toxin A concentration with cytotoxic titers in culture supernatant fluids of *C. difficile* strains. To examine the correlation of ELISA values with the cytotoxic titers of toxin A in culture supernatant fluids of *C. difficile* strains, we separated toxin A from toxin B by ion-exchange chromatography on DEAE-Sepharose CL-6B. After this fractionation step, the titers of toxin A were determined and compared with the toxin A values determined by ELISA. The results (Fig. 3A) indicate that the ELISA values closely correlate with the cytotoxic titers of toxin A. These findings were supported by the observation that 10-fold dilutions of a homogeneous preparation of toxin A containing concentrations of the toxin ranging from 0.1 to 1,000 μg/ml had cytotoxic titers identical to those in Fig. 3A. For example, a concentration of 10 μg/ml had a titer of 10^2.

Previous studies in our laboratory have indicated that the titer of toxin B in culture supernatant fluids of strains of *C. difficile* is consistently about 1,000-fold greater than the titer of toxin A. Based on this observation and the findings that the ELISA values for toxin A closely correlate with toxin A cytotoxic titers, it should be possible to estimate the cytotoxic titer of toxin B if the concentration of toxin A is known. To examine this possibility, the cytotoxic titers of toxin B in supernatant fluids were determined, and these titers were compared with the toxin A cytotoxic titers and ELISA values. Of 32 strains with detectable amounts of toxin A cytotoxicity, 28 (88%) had toxin B titers which were 1,000-fold greater than the titer of toxin A. Three of the strains had toxin B titers 10,000-fold greater than the titer of toxin A, and one strain had a 100-fold difference in the cytotoxic titers of toxins A and B. Based on this fairly consistent 1,000-fold difference in the titers of toxins A and B, one would expect toxin B cytotoxic titers to show a close correlation with the ELISA value for toxin A, and this is indeed the case (Fig. 3B).

Five strains of *C. difficile* with toxin B titers of 10^3 did not produce detectable levels of toxin A cytotoxicity (which would be expected, based on the above observations). Analysis by ELISA indicated that these strains were positive for toxin A and that the amounts of toxin A produced by these strains, ranging from 0.1 to 0.5 μg/ml, were at or below the lower detection limits of the tissue culture assay.

Ten strains of *C. difficile* were examined which were negative by tissue culture assay and by ELISA, indicating that these strains did not produce detectable amounts of either toxin when grown under the described conditions. These strains may produce low amounts of these toxins, however, when grown under different conditions (N. M. Sullivan, unpublished observations).

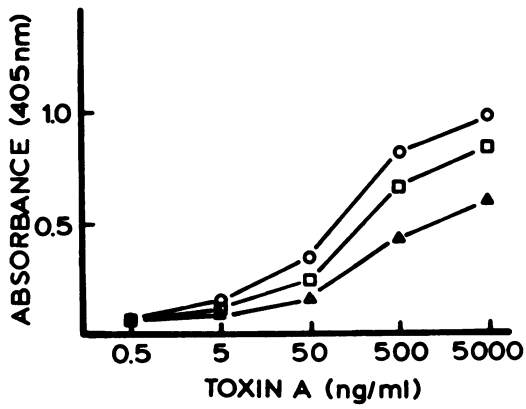

FIG. 2. Quantitation of toxin A by ELISA. The wells of a microtiter plate were initially coated with a 1/1,000 dilution of rabbit antiserum against a crude *C. difficile* toxin preparation (14), and dilutions of 1/500 (○), 1/1,000 (□), and 1/2,000 (△) of the affinity-purified toxin A antibody preparation were tested.

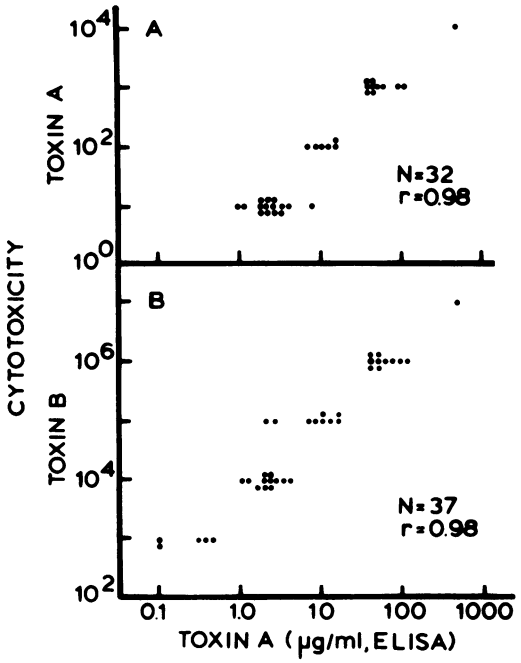

FIG. 3. Correlation of cytotoxic titers of toxins A (A) and B (B) with ELISA values of toxin A in culture supernatant fluids of strains of *C. difficile*. Cytotoxic titers are expressed as the reciprocal of the highest dilution necessary to completely round the cells. The titers of toxin B were determined directly from the supernatant fluids. The titers of toxin A were determined after the separation of toxin B from toxin A by ion-exchange chromatography. r, Correlation coefficient; N, number of strains tested.

Detection of toxin A in human fecal specimens by ELISA. Studies were initially performed to demonstrate that the ELISA specifically detected toxin A in fecal specimens. Toxin A was mixed with a negative specimen, and extracts of the specimen were examined by ELISA for the presence of the toxin. Tenfold dilutions of the extracts elicited a dose-dependent response similar to the responses obtained with homogeneous preparations of toxin A (Fig. 2), indicating that the ELISA detected toxin A in feces.

Control (noncytotoxic) fecal specimens and specimens from persons suspected of having *C. difficile* colitis were then analyzed by tissue culture assay and by ELISA. Specimens which had cytotoxic titers of 10^3 or greater (due to toxin B) were consistently positive for toxin A by ELISA (Table 2). The specimens which had titers of 10^6 gave the highest ELISA reading, followed by specimens with titers of 10^5, 10^4, and 10^3, indicating that the toxin was quantitatively detected. There were no false-positives with the control specimens.

DISCUSSION

At the present time, no procedures are available which directly detect *C. difficile* toxin A in the presence of toxin B. Although investigators have described counterimmunoelectrophoresis and ELISA procedures for the detection of *C. difficile*, these procedures are not specific for the toxins produced by this bacterium (26, 29, 34–36). Our report describes a specific and sensitive ELISA which uses affinity-purified toxin A antibodies for the detection of toxin A. The ELISA can be used to determine the concentration of toxin A in culture supernatant fluids of strains of *C. difficile* or to detect toxin A in fecal specimens from persons suspected of having *C. difficile* colitis.

We previously have described a procedure for the preparation of affinity-purified antibodies against toxin A (D. M. Lyerly, S. E. H. West, and T. D. Wilkins, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 717, 1981). This procedure involves the sequential adsorption of *C. difficile* antiserum with a nontoxigenic strain of *C. difficile*, boiled cell washes from a toxigenic strain of *C. difficile* coupled to Sepharose, and a partially purified preparation of toxin A coupled to Sepharose. The antibodies bound to the partially purified toxin A-Sepharose are eluted with KSCN. Although this approach resulted in anti-

TABLE 2. Detection of toxin A in human fecal samples by ELISA

number of specimens tested[a]	cytotoxic titer[b]	number of specimens positive by ELISA[c]
31	0	0
9	10^1	1
6	10^2	2
7	10^3	7
4	10^4	4
1	10^5	1
2	10^6	2

[a] Watery fecal specimens were mixed with an equal volume of PBS-T. For solid stools, 0.2 g of the fecal specimens was mixed with 1 ml of PBS-T. The supernatant fluid of each specimen was obtained by centrifugation, passed through a 0.45-um membrane, and examined by tissue culture assay and ELISA.

[b] Cytotoxic titers are expressed as the reciprocal of the highest dilution of specimen which completely rounded the cells. All of the specimens which were cytotoxic were neutralized by *C. difficile* antiserum. The cytotoxicity observed in these specimens is due to toxin B.

[c] A specimen was considered positive for toxin A if the absorbance value at 405 nm was at least 50% higher than the value of the negative control.

bodies which were specific for toxin A, the procedure was less efficient than the one-step affinity purification scheme described in this paper.

Our results demonstrate that the ELISA can be used to determine the concentration of toxin A in samples and indicate that this assay may be useful in examining the production of toxin A (e.g., the presence of intracellular toxin and the kinetics of the release of the toxin into the supernatant fluid) by strains of *C. difficile*. At the present time, all of the toxigenic strains of *C. difficile* which we have examined by tissue culture assay and by ELISA produce both toxin A and toxin B. We have not found any strains which produce only one of the toxins. Based on this observation and the close correlations observed between toxin A and B cytotoxic titers and the ELISA values, it should be possible to estimate the cytotoxic titer of either toxin A or toxin B in culture supernatant fluids if the concentration of toxin A is known. For example, from the data presented in Fig. 3, if the concentration of toxin A is 10 μg/ml, as determined by ELISA, the toxin A and toxin B titers would be ca. 10^2 and 10^5, respectively. However, more strains of *C. difficile* need to be examined to determine whether these observations are consistent.

The results of previous studies have indicated that toxin A and toxin B are both involved in the pathogenesis of *C. difficile* colitis. For example, Libby et al. (20) have reported that the tissue damage caused by these toxins is similar to the damage observed in hamsters with AAC and that vaccination against both toxins is necessary to protect hamsters against lethal AAC. In addition, other studies in our laboratory have indicated that strains of *C. difficile* which are not detectably cytotoxic when grown in vitro do not produce lethal AAC in germfree rats and mice (M. Ehrich, R. Van Tassell, T. Wilkins, and E. Balish, unpublished data). The production of toxin B during the disease has been indirectly demonstrated by the results of studies which have shown that cytotoxicity in fecal specimens from persons with *C. difficile* colitis is neutralized by *C. difficile* antiserum (16). However, because toxin B cytotoxicity masks the cytotoxicity of toxin A, these studies did not demonstrate the presence of toxin A. The presence of toxin A has been confirmed by ELISA in our study, demonstrating that toxin A is also produced during the disease.

The cytotoxicity test that we use for toxin B is more sensitive than the toxin A ELISA test in detecting the presence of *C. difficile* toxins. In practice this difference many not be important for the following reasons. (i) Fecal specimens often contain other toxic material that makes the cytotoxicity test useless for specimen dilutions of 1:10 to 1:100. This observation is supported by findings in our laboratory (unpublished data) and in the laboratories of other investigators (3, 5) who have noted cytotoxicity at these dilutions in fecal specimens from healthy persons and from persons with diarrhea and that the cytotoxicity is not neutralized by antisera. (ii) The methodology varies greatly among laboratories, with the result that low titers of cytotoxicity often are not reproducible. In addition, laboratories use different cell lines, and it has been reported that different cell lines vary in their sensitivity to *C. difficile* toxins (13). Therefore, the cytotoxicity observed at low dilutions may not be apparent, depending on the cell line used. (iii) The confirmed cases of pseudomembranous colitis that we have examined had cytotoxic titers of 10^3 or greater and were positive in the ELISA. We therefore believe that the ELISA test will prove useful for the clinical detection of *C. difficile*-induced colitis in patients.

Methods for increasing the sensitivity of the toxin A ELISA are currently being tested in our laboratory. We are attempting to directly label the affinity-purified antibody with various enzymes and to use the labeled conjugate in direct ELISA procedures. In addition, investigators in our laboratory have recently succeeded in developing hybridoma cell lines for the production of monoclonal antibodies to toxin A (D. K. MacDonald, J. M. Libby, and T. D. Wilkins, unpublished data). These monoclonal antibody preparations will be tested singly and in conjunction with each other in the indirect and direct ELISA procedures.

Although our results to date indicate that toxigenic strains of *C. difficile* produce both toxins A and B, the ELISA may be useful in detecting any strains, after chemical mutagenization, which produce only toxin B or which produce immunologically reactive but biologically inactive toxin A. Such strains would be useful in further evaluating the roles of toxin A in the disease. In addition, the availability of immunologically reactive but biologically inactive toxin A would permit the initiation of studies on the structure and function of toxin A that use approaches similar to those used for studying diphtheria toxin (18, 33).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI15749-03 from the National Institute of Allergy and Infectious Disease and by the Commonwealth of Virginia.

We thank S. I. Richardson for technical assistance and R. L. Van Tassell for help in preparing this manuscript. We also thank Carol Wells and Donna Blazevic of the University of Minnesota at Minneapolis for the clinical specimens used in this study.

LITERATURE CITED

1. **Axelsen, N. H., J. Krøll, and B. Weeke (ed.).** 1973. A manual of quantitative immunoelectrophoresis. Methods and applications. Universitetsforlaget, Oslo, Norway.
2. **Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2**:629–635.
3. **Bartlett, J. G.** 1979. Antibiotic-associated pseudomembranous colitis. Rev. Infect. Dis. **1**:530–539.
4. **Bartlett, J. G.** 1981. Laboratory diagnosis of antibiotic-associated colitis. Lab. Med. **12**:347–351.
5. **Bartlett, J. G., T. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N. Engl. J. Med. **298**:531–534.
6. **Bartlett, J. G., T. Chang, N. S. Taylor, and A. B. Onderdonk.** 1979. Colitis induced by *Clostridium difficile*. Rev. Infect. Dis. **1**:370–378.
7. **Bartlett, J. G., N. Moon, T. Chang, N. Taylor, and A. B. Onderdonk.** 1978. Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology **75**:778–782.
8. **Bartlett, J. G., A. B. Onderdonk, R. L. Cisneros, and D. L. Kasper.** 1977. Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in hamsters. J. Infect. Dis. **136**:701–705.
9. **Bartlett, J. G., N. S. Taylor, T. Chang, and J. Dzink.** 1980. Clinical and laboratory observations in *Clostridium difficile* colitis. Am. J. Clin. Nutr. **33**:2521–2526.
10. **Bartlett, J. G., F. J. Tedesco, S. Schull, B. Lowe, and T. Chang.** 1980. Symptomatic relapse after oral vancomycin therapy of antibiotic-associated pseudomembranous colitis. Gastroenterology **78**:431–434.
11. **Chang, T.-W., J. G. Bartlett, S. L. Gorbach, and A. B. Onderdonk.** 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect. Immun. **20**:526–529.
12. **Cuatrecasas, P., and C. B. Anfinsen.** 1971. Affinity chromatography. Methods Enzymol. **22**:351–354.
13. **Donta, S. T., N. M. Sullivan, and T. D. Wilkins.** 1982. Differential effects of *Clostridium difficile* toxins on tissue-cultured cells. J. Clin. Microbiol. **15**:1157–1158.
14. **Ehrich, M., R. L. Van Tassell, J. M. Libby, and T. D. Wilkins.** 1980. Production of *Clostridium difficile* antitoxin. Infect. Immun. **28**:1041–1043.
15. **George, W. L., E. J. C. Goldstein, V. L. Sutter, S. L. Ludwig, and S. M. Finegold.** 1978. Aetiology of antimicrobial-agent-associated colitis. Lancet **i**:802–803.
16. **Gilligan, P. H., L. R. McCarthy, and V. M. Genta.** 1981. Relative frequency of *Clostridium difficile* in patients with diarrheal disease. J. Clin. Microbiol. **14**:26–31.
17. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
18. **Holmes, R. K.** 1976. Characterization and genetic mapping of nontoxinogenic (*tox*) mutants of corynebacteriophage beta. J. Virol. **19**:195–207.
19. **Larson, H. E., A. B. Price, P. Honour, and S. P. Borriello.** 1978. *Clostridium difficile* and the aetiology of pseudomembranous colitis. Lancet **i**:1063–1066.
20. **Libby, J. M., B. S. Jortner, and T. D. Wilkins.** 1982. Effect of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. **36**:822–829.
21. **Libby, J. M., and T. D. Wilkins.** 1982. Production of antitoxin to two toxins of *Clostridium difficile* and immunological comparison of the toxins by cross-neutralization studies. Infect. Immun. **35**:374–376.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Lusk, R. H., R. Fekety, J. Silva, R. A. Browne, D. M. Ringler, and G. D. Abrams.** 1978. Clindamycin-induced enterocolitis in hamsters. J. Infect. Dis. **137**:464–475.
24. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Williams.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.
25. **Onderdonk, A. B., R. L. Cisneros, and J. G. Bartlett.** 1980. *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **28**:277–282.
26. **Poxton, I. R., and M. D. Byrne.** 1981. Detection of *Clostridium difficile* toxin by counterimmunoelectrophoresis: a note of caution. J. Clin. Microbiol. **14**:349.
27. **Price, A. B., H. E. Larson, and J. Crow.** 1979. Morphology of experimental antibiotic-associated enterocolitis in the hamster: a model for human pseudomembranous colitis and antibiotic-associated diarrhoea. Gut **20**:467–475.
28. **Ruoslahti, E.** 1976. Antigen-antibody interaction, antibody affinity, and dissociation of immune complexes, p. 3–7. *In* E. Ruoslahti (ed.), Immunoadsorbents in protein purification. University Park Press, Baltimore.
29. **Ryan, R. W., I. Kwasnik, and R. C. Tilton.** 1980. Rapid detection of *Clostridium difficile* toxin in human feces. J. Clin. Microbiol. **12**:776–779.
30. **Sterne, M., and L. M. Wentzel.** 1950. A new method for the large scale production of high-titre botulinum Formol-toxoid types C and D. J. Immunol. **65**:175–183.
31. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.
32. **Taylor, N. S., G. M. Thorne, and J. G. Bartlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.
33. **Uchida, T., A. M. Pappenheimer, Jr., and R. Greany.** 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. J. Biol. Chem. **248**:3838–3844.
34. **Welch, D. F., S. K. Menge, and J. M. Matsen.** 1980. Identification of toxigenic *Clostridium difficile* by counterimmunoelectrophoresis. J. Clin. Microbiol. **11**:470–473.
35. **West, S. E. H., and T. D. Wilkins.** 1982. Problems associated with counterimmunoelectrophoresis assays for detecting *Clostridium difficile* toxin. J. Clin. Microbiol. **15**:347–349.
36. **Yolken, R. H., L. S. Whitcomb, G. Marien, J. G. Bartlett, J. Libby, M. Ehrich, and T. Wilkins.** 1981. Enzyme immunoassay for the detection of *Clostridium difficile* antigens. J. Infect. Dis. **144**:378.