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Monoclonal and Specific Polyclonal Antibodies for Immunoassay of *Clostridium difficile* Toxin A

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Monoclonal antibody, affinity-purified antibody, and monospecific antiserum against toxin A were produced. The monoclonal antibody was an immunoglobulin G2a kappa chain isotype that immunoprecipitated toxin A, as shown by crossed immunoelectrophoresis. These antibodies were compared by counterimmunoelectrophoresis, latex agglutination, and indirect enzyme-linked immunosorbent assay for their sensitivity in detecting toxin A. Our findings indicate that these antibodies may be useful as immunodiagnostic reagents for *Clostridium difficile* disease.

Clostridium difficile, the etiological agent of pseudomembranous colitis in humans, produces two toxins (2, 15, 16). Both of the toxins, designated toxin A and toxin B, are cytotoxic for tissue-cultured mammalian cells; however, toxin B is ca. 1,000-fold more active than toxin A in the assay (11). In addition to its cytotoxicity, toxin A also possesses enterotoxin activity and causes a fluid response when injected into ligated rabbit intestinal loops (10, 16). At present, the role of these toxins in the disease process is poorly understood. We have found that hamsters must be vaccinated against both toxins to be protected against *C. difficile* cecitis (9); both toxins therefore appear to be of importance. All of the strains of *C. difficile* which we have examined produce either both toxins or neither toxin (11), and so an immunodiagnostic test could be directed against either toxin.

The most commonly used test in the diagnosis of *C. difficile* colitis is the detection of toxin B in fecal specimens of patients by tissue culture assay and neutralization of the toxin by *C. difficile* antiserum. The assay is extremely sensitive because of the high specific activity of toxin B against mammalian cells (15). However, many clinical laboratories are not equipped for tissue culture assays. In addition, the methodology and the type of tissue culture cell used vary greatly among laboratories and the assay requires overnight incubation. Other types of assays, such as counterimmunoelectrophoresis (CIE) and enzyme-linked immunosorbent assays (ELISAs), have been proposed as immunodiagnostic tests for *C. difficile* colitis, but most of the work has been done with antisera which are not specific for the toxins of *C. difficile* (6, 8, 12, 13, 17–20). We recently described an indirect ELISA with affinity-purified antibody against toxin A (11). In the following study, we compare monoclonal antibody, affinity-purified polyclonal antibody, and monospecific antiserum against toxin A by CIE, latex agglutination (LA), and ELISA. These antibodies may be suitable as immunodiagnostic reagents for *C. difficile* disease, but their relative sensitivity differs among the various assays.

MATERIALS AND METHODS

Determination of protein. Protein was estimated with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) by the method of Bradford (3). Bovine gamma globulin was the standard.

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Preparation of toxin A. Toxin A was purified to homogeneity from culture filtrates of *C. difficile* 10463 as previously described (15). The toxin was inactivated with 0.4% Formalin.

Production of monoclonal antibody. (i) Immunization. BALB/c mice (Dominion Laboratories, Dublin, Va.) were immunized with toxoid A mixed 1:1 with incomplete Freund adjuvant. The mice each received 0.1 mg of toxoid intraperitoneally once every 2 weeks for 10 weeks. Each animal received 0.5 mg of toxoid A without adjuvant intraperitoneally 4, 3, and 2 days before splenectomy.

(ii) Fusion. Spleen cells from immunized mice were fused with SP2/O myeloma cells by using the polyethylene glycol fusion method (5). Hybrid cell lines were selected in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing hypoxanthine-aminopterin-thymidine (5), and their culture supernatant fluids were analyzed for toxin A antibody by the screening ELISA described below. Hybrids which were positive for toxin A antibody were subcloned in soft agar (5).

(iii) Production of ascites fluid. About 1×10^6 to 2×10^6 toxin A antibody-producing hybrid cells of each selected clone were injected intraperitoneally into BALB/c mice primed with pristane. Ascites fluid was clarified by centrifugation and brought to 50% saturation by the dropwise addition of a solution of 100% saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in phosphate-buffered saline (PBS) to the original sample volume, and dialyzed against PBS. The preparation was passed through a 0.45-μm membrane and stored at 4°C.

Determination of isotype of monoclonal antibodies. Hybridoma culture supernatant fluid was concentrated 10-fold on a B125 Minicon unit (Amicon Corp., Danvers, Mass.). Samples (10 μl) of the concentrated fluid were tested by Ouchterlony double immunodiffusion for immunoreactivity against samples (10 μl) of heavy-chain- and light-chain-specific rabbit anti-mouse antisera (Litton Bionetics, Inc., Charleston, S.C.).

ELISAs. (i) Screening ELISA. Wells of microtiter plates (Immulon type 1; Dynatech Laboratories, Inc., Alexandria, Va.) were each coated with 0.5 μg of toxin A in carbonate buffer (pH 9.6). The plates were incubated overnight at 37°C, and each well was washed once with 0.25 ml of PBS–0.05% Tween 20–0.1% bovine albumin. Samples (0.1 ml) of culture supernatant fluid from the wells with hybridoma cells were added, and the plates were incubated for 1 h at 37°C. The

plates were washed five times with PBS–0.05% Tween 20, and 0.2 ml of rabbit anti-mouse immunoglobulin G (IgG)-alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1/500 in PBS–0.05% Tween 20 was added. After incubation for 1 h at 37°C, the plates were washed five times with PBS–0.05% Tween 20, and 0.2 ml of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate; Sigma Chemical Co., St. Louis, Mo.) in diethanolamine buffer (pH 9.6) was added to each well. The plates were incubated for 30 min at room temperature and examined for positive reactions. Controls contained RPMI 1640 medium or SP2/O culture supernatant fluid in place of hybridoma culture supernatant fluid.

(ii) Indirect ELISA. An indirect ELISA for the detection of toxin A was done as previously described (11), with monoclonal antibody, affinity-purified antibody, or monospecific antiserum for the detection of toxin bound by the solid-phase antiserum.

Neutralization assay. The neutralization titer of toxin A antibody preparations was determined as previously described (4).

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was done as previously described (11).

Preparation of affinity-purified toxin A antibody. Toxin A antibody was purified by immunoaffinity chromatography as previously described (11). The antibody was used at a concentration of 1 mg/ml.

Preparation of monospecific toxin A antiserum. A 1-year-old Alpine Nubian male goat was injected with toxoid A mixed 1:1 with incomplete Freund adjuvant. The goat received ca. 1 mg of toxoid A subcutaneously once each week for 4 weeks; it was then injected with active toxin A bound to Affi-Gel 10 (Bio-Rad Laboratories). The goat received 1 ml of a 10% suspension of the toxin A-gel (ca. 0.1 mg of toxin A protein) once each week for 6 weeks. The antiserum was brought to 50% saturation with a solution of 100% saturated $(NH_4)_2SO_4$. The precipitate was collected, dissolved in PBS to the original sample volume, and dialyzed against PBS. The preparation was passed through a 0.45-μm membrane and stored at 4°C.

CIE. CIE was done as previously described (18).

LA. Samples (0.5 ml) of latex beads (10% suspension; Difco Laboratories, Detroit, Mich.) and antibody diluted in glycine-NaCl buffer (pH 8.2) (14) were mixed and incubated for 2 h at 37°C with occasional shaking. After incubation, 0.25 ml of glycine-NaCl buffer containing 0.5% bovine albumin and 0.1% NaN_3 was added to each mixture and the sensitized beads were stored at 4°C. The LA assay was performed by mixing samples (20 μl) of toxin A diluted in PBS and sensitized beads on a ring slide for 5 min at room temperature and observing the suspension for macroscopic agglutination.

RESULTS AND DISCUSSION

One of the current diagnostic tests for *C. difficile* colitis is the detection of toxin B in fecal specimens by tissue culture assay and its neutralization by antiserum. Immunodiagnostic tests such as CIE and ELISA have been described for *C. difficile*, but most of these tests involved the use of antisera which were not specific for toxigenic *C. difficile* (6, 8, 12, 13, 17–20). In this report, we compare an immunoprecipitating monoclonal antibody, an affinity-purified polyclonal antibody, and a monospecific antiserum against toxin A by LA, CIE, and indirect ELISA to determine their sensitivity in detecting toxin A.

We isolated several monoclonal antibodies which reacted with homogeneous preparations of toxin A. One of these antibodies, designated PCG-4, immunoprecipitated toxin A (Fig. 1B). When analyzed by Ouchterlony double immunodiffusion, the antibody gave a single immunoprecipitin line with antiserum against mouse IgG2a and mouse kappa chain. The monoclonal antibody did not react with antiserum against mouse IgG1, IgG2b, IgG3, and lambda chain and did not neutralize the cytotoxicity of toxin A.

Affinity-purified antibody was prepared from goat antiserum against culture filtrates of strain 10463 by using immunoaffinity chromatography. Monospecific antiserum was prepared in a goat by using two forms of homogeneous toxin A. The goat was initially vaccinated with toxin inactivated with Formalin and then with active toxin coupled to agarose beads. The inactive toxin served to prime the animal, and the low levels of toxin antibody elicited by these initial vaccinations helped to minimize any tissue damage caused by the active toxin on the beads. The monospecificity of the affinity-purified antibody and monospecific antiserum was shown by crossed immunoelectrophoresis (Fig. 1C, D). The neutralization titers of the affinity-purified antibody and monospecific antiserum were 1,280 and 2,560, respectively.

The antibody preparations were compared by LA, CIE, and indirect ELISA. The results of these studies are summarized in Table 1. The affinity-purified antibody and monospecific antiserum were comparable in sensitivity in the CIE assay and indirect ELISA and were more sensitive than the monoclonal antibody. In the LA assay, the monospecific antiserum was less sensitive than the monoclonal antibody and affinity-purified antibody, even though the antiserum had a higher neutralization titer. This difference in sensitivity was probably due to the high percentage of serum proteins other than toxin A antibody coating the latex beads.

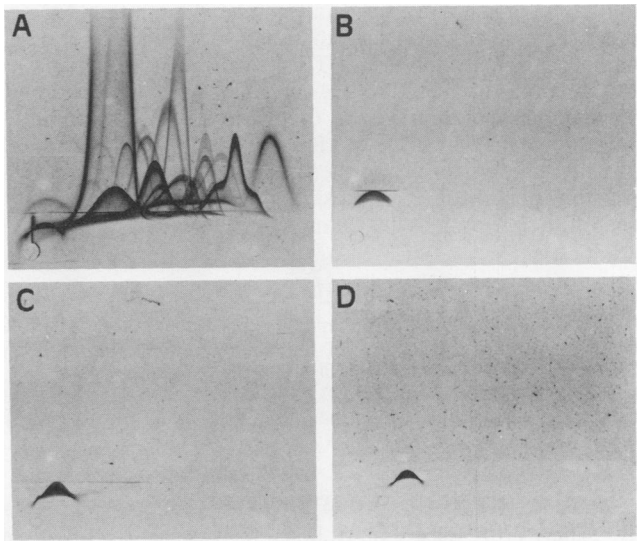

FIG. 1. Analysis of toxin A antibody preparations by crossed immunoelectrophoresis. The well in each plate contained 80 μg of *C. difficile* 10463 culture filtrate. The upper portion of the gel contained the following antibody preparations: (A) 0.1 ml of goat antiserum against *C. difficile* 10463 culture filtrate; (B) 0.1 ml of PCG-4 toxin A monoclonal antibody (ascites fluid); (C) 0.1 ml of affinity-purified toxin A antibody; (D) 0.25 ml of monospecific toxin A antiserum. At least 25 immunoprecipitin arcs are visible on plate A; plates B, C, and D contain only a single immunoprecipitin arc against toxin A.

TABLE 1. Detection of toxin A by CIE, LA, and ELISA with monoclonal antibody, affinity-purified antibody, and monospecific antiserum

Antibody prepn	Concn (μg/ml) of toxin A (ng of toxin A protein) detected by[a]:		
	CIE	LA	ELISA
Monoclonal antibody	63 (630)	1 (20)	0.02 (4)
Affinity-purified antibody	4 (40)	0.3 (6)	0.005 (1)
Monospecific antiserum	4 (40)	16 (320)	0.005 (1)

[a] Amounts shown are the lowest concentrations of toxin A detected by the assay. The number in parentheses is the actual amount of toxin A protein detected. Dilutions of each antibody preparation were tested to determine the conditions which gave the greatest sensitivity and lowest background.

Banno et al. (1) recently described an LA assay which detects toxin A at a concentration of 15 ng/ml. The assay was based on the agglutination rate measured at 940 nm. The type of assay we describe may be more applicable to clinical use, since the reaction is easily visible and does not require specialized equipment. Laughon et al. (7) have recently reported the development of ELISAs in which antiserum against toxin A or toxin B is used, although the monospecificity of the antisera used in their study was not documented. Their assay, which involved a 12-h incubation of antigen with wells coated with hamster antiserum against *C. difficile* culture supernatant fluid, detected 0.1-ng amounts of toxin. Our assay, which involves a 1-h incubation of antigen with wells coated with rabbit antiserum against *C. difficile* culture filtrate, detects 1-ng amounts of toxin A. If we increase the incubation time to 12 h, the sensitivity of our assay is increased ca. 10-fold (data not shown).

We have found that assays which detect toxin A in the range of 5 to 50 ng/ml can detect the toxin in fecal specimens from patients with *C. difficile* colitis (11); therefore, the ELISA may be suitable as a diagnostic test. Monoclonal antibodies can be prepared in unlimited quantities and are more reproducible reagents than affinity-purified antibodies and monospecific antisera. Therefore, we are testing the PCG-4 monoclonal antibody and affinity-purified antibody in various combinations with other monoclonal antibodies against toxin A which we have isolated in an effort to increase the sensitivity of these assays. In addition to their possible diagnostic use, these antibodies are useful as research tools for studying toxin A.

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LITERATURE CITED

1. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and T. Nozawa.** 1984. Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from *Clostridium difficile*. Rev. Infect. Dis. **6**:S11–S20.
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. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Ehrich, M., R. L. Van Tassell, J. M. Libby, and T. D. Wilkins.** 1980. Production of *Clostridium difficile* antitoxin. Infect. Immun. **28**:1041–1043.
5. **Galfre, G., and C. Milstein.** 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. **73**:3–46.
6. **Jarvis, W., O. Nunez-Montiel, F. Thompson, V. Dowell, M. Towns, G. Morris, and E. Hill.** 1983. Comparison of bacterial isolation, cytotoxicity assay, and counterimmunoelectrophoresis for the detection of *Clostridium difficile* and its toxin. J. Infect. Dis. **147**:778.
7. **Laughon, B., R. P. Viscidi, S. L. Gdovin, R. H. Yolken, and J. G. Bartlett.** 1984. Enzyme immunoassays for detection of *Clostridium difficile* toxins A and B in fecal specimens. J. Infect. Dis. **149**:781–788.
8. **Levine, H. G., M. Kennedy, and J. T. LaMont.** 1982. Counterimmunoelectrophoresis vs. cytotoxicity assay for the detection of *Clostridium difficile* toxin. J. Infect. Dis. **145**:398.
9. **Libby, J. M., B. S. Jortner, and T. D. Wilkins.** 1982. Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. **36**:822–829.
10. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.
11. **Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins.** 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**:72–78.
12. **Poxton, I. R., and M. D. Byrne.** 1981. Detection of *Clostridium difficile* toxin by counterimmunoelectrophoresis: a note of caution. J. Clin. Microbiol. **14**:349.
13. **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.
14. **Severin, W. P. J.** 1972. Latex agglutination in the diagnosis of meningococcal meningitis. J. Clin. Pathol. **25**:1079–1082.
15. **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.
16. **Taylor, N. S., G. M. Thorne, and J. G. Bartlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.
17. **Welch, D. F., S. K. Menge, and J. M. Matsen.** 1980. Identification of toxigenic *Clostridium difficile* by counterimmunoelectrophoresis. J. Clin. Microbiol. **11**:470–473.
18. **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.
19. **Wu, T. C., and J. C. Fung.** 1983. Evaluation of the usefulness of counterimmunoelectrophoresis for diagnosis of *Clostridium difficile*-associated colitis in clinical specimens. J. Clin. Microbiol. **17**:610–613.
20. **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* antigen. J. Infect. Dis. **144**:378.