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Enzyme-Linked Immunosorbent Assay to Detect Shiga Toxin of *Shigella dysenteriae* and Related Toxins

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A sandwich enzyme-linked immunosorbent assay (ELISA) was developed for detection of Shiga toxin. Four species of *Shigella*, *Escherichia coli*, and *Vibro parahaemolyticus* were tested for production of Shiga or Shiga-like toxin by ELISA and Vero cell bioassay. In the ELISA, most strains of *S. dysenteriae* and some strains of *E. coli* isolated from traveler's diarrhea were positive. These ELISA-positive strains were positive by Vero cell bioassay without exception. Some *E. coli* strains and most *V. parahaemolyticus* strains were toxic to Vero cells, although they were negative in the ELISA. Much of the cytotoxic activity was not neutralized by anti-Shiga toxin antiserum. The newly developed sandwich ELISA is specific and can be a substitute for the cumbersome Vero cell bioassay.

Shigella dysenteriae, a bacterium in the family *Enterobacteriaceae*, is known to cause shigellosis. The high virulence of *S. dysenteriae* may be due to two properties of the organism, invasiveness and toxin production (10, 11). The bacteria can invade and multiply in colonic epithelial cells, causing colitis and mucous, bloody stools. In addition, *S. dysenteriae* produces Shiga toxin which exhibits several biological activities such as lethality to rabbits and mice, cytotoxicity to various cell lines including Vero cells, and enterotoxicity when injected into rabbit ileal loops (3, 4, 10, 14).

A bioassay using cultured cells such as Vero cells is available for detecting Shiga toxin (4, 15). However, this technique requires tissue culture facilities and is expensive, time consuming, and labor intensive. Although very recently a colony blot assay for this toxin has been reported (18), a simpler immunological test can be a better substitute. An enzyme-linked immunosorbent assay (ELISA) for use in diagnostic laboratories had been developed to detect many kinds of toxin such as cholera toxin (7), *Escherichia coli* heatlabile enterotoxin (6, 7), and thermostable direct hemolysin of *Vibrio parahaemolyticus* (8). However, this technique has not been used to detect Shiga toxin.

In this paper we report the development of a sandwich ELISA procedure to detect Shiga toxin of shigellae and Shiga-like toxin(s) produced by other organisms.

MATERIALS AND METHODS

Bacterial strains. Thirty-eight strains of shigellae were obtained from the Clinical Diagnostic Laboratory, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. Twenty-four strains of *V. parahaemolyticus* were supplied by the Laboratory for Culture Collections, Research Institute for Microbial Diseases, Osaka University. All 608 strains of *E. coli* were isolated from stools of patients with traveler's diarrhea at the Osaka Airport Quarantine Station, Osaka, Japan (1). All air passengers coming from cholera-infected areas were asked to report to the station if they had had diarrhea during their travels. These *E. coli* strains were isolated from such patients and found not to be enterotoxigenic *E. coli* (5). Three strains of *E. coli* O157:H7 were a generous gift from R. L. Kaplan, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Ill.

Medium and culture. Modified syncase broth was prepared by a previously described method (13). Bacteria were grown in the broth (2 ml in a 10-ml test tube) at 37° C with rotation (30 rpm) for 24 h. The supernatants and cell pellets were separated by centrifugation at 5,000 × *g* for 20 min. The cell pellets were incubated with 10,000 U of polymyxin B (0.2 ml/per tube) at 37°C for 1 h, and cell debris of the lysates was removed by centrifugation (5,000 × *g*, 20 min). Both the culture supernatants and the supernatants of the lysates were used for the assay.

Purification of Shiga toxin. Shiga toxin was purified to homogeneity from a culture supernatant of *S. dysenteriae* (RIMD 3101010) grown in modified syncase broth (15 liters). We used the purification method described previously (19), with minor modifications. After completing ammonium sulfate fractionation and column chromatography on DEAE-cellulose and chromatofocusing, we used high-performance liquid chromatography with a Mono Q column (Pharmacia, Inc., Piscataway, N.J.) for further purification. A sample dialyzed against 0.05 M Tris hydrochloride buffer (pH 8.6) was applied to the Mono Q column equilibrated with the same buffer and eluted with a 0 to 1 M linear gradient of NaCl. The purified toxin produced a single stained band on conventional polyacrylamide disk gel electrophoresis and two bands corresponding the A and B subunits on sodium dodecyl sulfate-polyacrylamide gel slab electrophoresis (data not shown).

Preparation of anti-Shiga toxin rabbit antiserum. Purified Shiga toxin was converted to toxoid by Formalin treatment. Purified toxin (containing 40 μg of protein) was treated with 0.4% Formalin and incubated at 37°C for 1 week. Antisera were raised by immunizing rabbits (New Zealand White) via the foot pads with Shiga toxoid (20 μg) emulsified with an equal volume (1 ml) of Freund complete adjuvant. Booster immunization was done 1 month later with 20 μg of Shiga toxoid emulsified with an equal volume (1ml) of Freund incomplete adjuvant. The antibody titer was checked against pure toxin by an Ouchterlony test essentially as described previously (16). We used 20 μl of pure Shiga toxin (50 μg/ml) and 20 μl of serially diluted antibody in the test.

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TABLE 1. Procedure for detection of Shiga toxin by sandwich ELISA

Step no. and procedure	Concn or dilution	Diluënt	Vol (μl)/ well	Incubation conditions	
				Temp	Time
1. Precoat well with anti-Shiga toxin (immunoglobulin)	4 μg/ml	Coating buffer, pH 9.6	150	4°C	20 h
2. Wash three times		PBS-Tween 20	200	Room temp	1 min × 3
3. Cover unbound surface with BSA[a]	10 mg/ml	PBS	200	37°C	2–3 h
4. Wash three times		PBS-Tween 20	200	Room temp	1 min × 3
5. Add test samples			150	37°C	2 h
6. Wash four times		PBS-Tween 20	200	Room temp	1 min × 4
7. Add alkaline phosphatase-labeled antibody	1:1,000	PBS-Tween 20	150	37°C	2 h
8. Wash four times		PBS-Tween 20	200	Room temp	1 min × 4
9. Add substrate (*p*-nitrophenyl phosphate)	1 mg/ml	Diethanolamine buffer, pH 9.8	200	37°C	30–60 min
10. Read optical density at 405 nm in colorimeter					

[a] BSA, Bovine serum albumin.

Immunoaffinity column chromatography for preparation of antibody specific to Shiga toxin. Activated CH-Sepharose 4B (2 g; Pharmacia) was coupled with 2 mg of purified Shiga toxin per manufacturer specification. Anti-Shiga toxin serum (2 ml at a titer of 1:32) was applied to the column of CH-Sepharose 4B coupled with Shiga toxin. The column was washed with phosphate-buffered saline (PBS; pH 7.4) until the optical density of the eluate at 280 nm reached 0. The immunoglobulin bound to the column was eluted with 0.2 M glycine hydrochloride buffer (pH 2.7) containing 0.5 M NaCl. The eluted fractions were immediately neutralized (the pH was around 7.5) by adding Tris powder (Trizma Base; Sigma Chemical Co., St. Louis, Mo.). The fractions which showed precipitin lines in Ouchterlony tests (16) were pooled and concentrated on an Amicon PM-10 membrane.

Sandwich ELISA. The anti-Shiga toxin immunoglobulin purified by immunoaffinity column chromatography was labeled with alkaline phosphatase (Sigma; type VII-S) with glutaraldehyde as reported previously (6). The sandwich ELISA procedure used is summarized in Table 1. A flat-bottom, polyvinyl microplate (Falcon; Becton Dickinson Labware, Oxnard, Calif.) was precoated 150 μl of 4 μg/ml anti-Shiga toxin immunoglobulin diluted in 0.05 M carbonate buffer (pH 9.6). For assaying Shiga toxin, 150-μl volumes of the test samples were applied to the wells. Appropriate dilutions and concentrations of alkaline phosphatase-immunoglobulin conjugate were determined by checkerboard titrations.

Assay for cytotoxic activity. Vero cells (African green monkey kidney) were grown as monolayers in Eagle minimum essential medium supplemented with 10% calf serum and gentamicin (50 μg/ml). Cultures were maintained in plastic dishes at 37°C in a 5% CO_2 atmosphere. Cytotoxin activity was determined in 96-well microtiter plates. Samples (50 μl) were added to each well and then sterilized by exposing the plate to UV light for 30 min. A confluent monolayer of Vero cells grown for 3 to 4 days was removed from the dishes (4) 200 μl of the cell suspension (about 5 × 10^3 cells) was added to each of the 96-well microtiter plates, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for 18 to 24 h. Cell morphology was observed under a phase-contrast microscope. Shiga toxin (10 ng/ml) was used as a positive control, and 10,000 U of polymyxin B solution and modified syncase broth were used as negative controls.

Neutralization test. The cytotoxic activities observed in cell lysates of various organisms were tested with anti-Shiga toxin rabbit serum to determine its cytotoxin-neutralizing activity. The diluted antiserum (1:100 in Eagle minimum essential medium) was mixed with an equal volume of cell lysate and incubated at 37°C for one h. Then, 50 μl of each mixture was used for Vero cell assay. Preimmune rabbit serum was included as a negative control. After overnight incubation, cell morphology was observed to determine neutralization of cytotoxic activity. Neutralization was considered positive if the toxin could be neutralized by anti-Shiga toxin rabbit serum but not by preimmune rabbit serum.

RESULTS

Sandwich ELISA. Various concentrations of purified Shiga toxin were tested by the sandwich ELISA to evaluate its sensitivity (Fig. 1). The ELISA was sensitive enough to

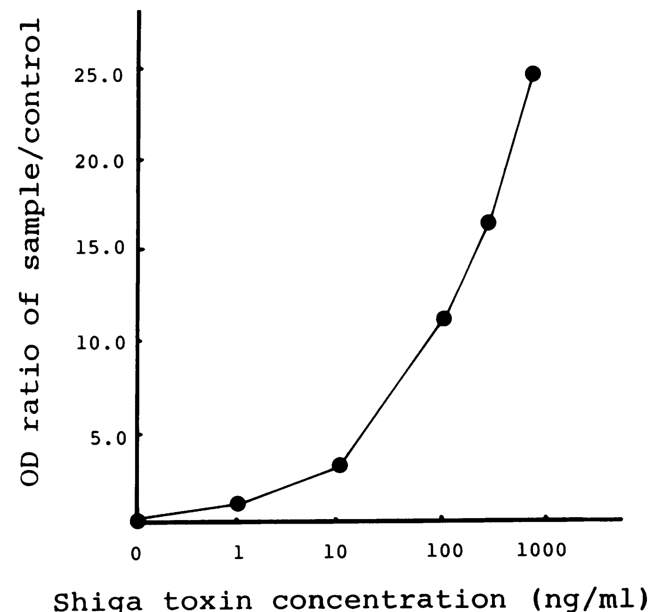

FIG. 1. Detection of Shiga toxin by sandwich ELISA.

TABLE 2. Detection of Shiga(-like) toxin by sandwich ELISA and cytotoxicity test

Test organism (no. of strains)	No. (%) of strains positive by:			
	Sandwich ELISA		Cytotoxicity test	
	Cell lysate	Culture super-natant	Cell lysate	Culture super-natant
S. dysenteriae (14)	12 (85.7)	12 (85.7)	12 (85.7)	12 (85.7)
S. flexneri				
1a (1)	0	0	0	0
1b (2)	0	0	0	0
2a (1)	0	0	0	0
2b (1)	0	0	0	0
3a (1)	0	0	0	0
4a (1)	0	0	0	0
4b (2)	0	0	0	0
6b (1)	0	0	0	0
Unknown (6)	0	0	0	0
S. sonnei 1 (2)	0	0	0	0
S. boydii 4 (6)	0	0	0	0
V. parahaemolyticus (24)	0	0	22 (91.6)	3 (12.5)
E. coli[a] (608)	4 (0.65)	0	111 (18.3)	NT[b]
E. coli O157:H7 (3)	3 (100)	3 (100)	3 (100)	3 (100)

[a] *E. coli* isolated from traveler's diarrhea.
[b] NT, Not tested.

detect several nanograms of purified Shiga toxin per milliliter. A good dose-response correlation was observed with 10 to 1,000 ng of toxin per ml. Culture supernatants and the supernatants of cell lysates of various organisms were examined by sandwich ELISA (Table 2). The ELISA data were interpreted by reading optical density at 405 nm (8). When the optical density of a test sample was more than twice that of a negative (medium) control, it was considered positive. Most (12 of 14) *S. dysenteriae* strains produced positive reactions, but *S. flexneri*, *S. sonnei*, and *S. boydii* were negative. Shiga toxin could not be found in preparations of *V. parahaemolyticus*. On the other hand, 0.65% (4 of 608 strains) of the supernatants of cell lysates of *E. coli* isolated from traveler's diarrhea were positive. Culture supernatants and supernatants of cell lysates of all three strains of *E. coli* O157:H7 tested were also positive in the ELISA.

Vero cell assay. All strains tested by ELISA were also tested for cytotoxic activity by using Vero cells (Table 2). Among *Shigella* strains, only ELISA-positive strains showed a cytotoxic effect on Vero cells, whereas ELISA-negative strains did not show the cytotoxic effect. Of the supernatants of cell lysates, 91% (22 of 24 strains) of *V. parahaemolyticus* and 18% (111 of 608 strains) of *E. coli* were cytotoxic. But all of these cytotoxin-positive strains except four *E. coli* strains were negative in the ELISA. Cytotoxic activity was detected in culture supernatants and cell lysates of three strains of *E. coli* O157H7.

Neutralization of cytotoxic activity by anti-Shiga toxin serum. The cytotoxic activities observed in some of the cell lysates of various organisms were tested with anti-Shiga toxin rabbit antiserum to determine whether the serum could neutralize the cytotoxic activity. The results are shown in Table 3. The cytotoxin produced by *S. dysenteriae* and some strains of *E. coli*, including *E. coli* O157H7 (three strains) and four strains positive in the ELISA, could be neutralized with the antiserum, whereas the cytotoxic activity seen in preparations of *V. parahaemolyticus* could not be neutralized with the antiserum.

TABLE 3. Neutralization of cytotoxic activity with anti-Shiga toxin rabbit serum

Cytotoxin source	No. of strains tested	No. of strains showing neutralization	
		+	−
S. dysenteriae	12	12	0
V. parahaemolyticus	22	0	22
E. coli (traveler's diarrhea)	23	9	14
E. coli O157:H7	3	3	0

DISCUSSION

A sandwich ELISA was used to detect Shiga toxin. From cultures of *S. dysenteriae* in modified syncase broth, Shiga toxin was detected by the ELISA in both supernatants and cell extracts. Immunologically related substances (Shiga-like toxin) were detected by the ELISA in (i) cell extracts of rare strains (0.65%) of *E. coli* (the serotypes of which were not determined) which had been isolated from traveler's diarrhea and (ii) all *E. coli* O157:H7 strains. All of these ELISA positive *E. coli* strains produced cytotoxin which could be neutralized by anti-Shiga toxin serum. However, we could not detect Shiga (-like) toxin in preparations of *S. flexneri*, *S. sonnei*, *S. bodyii*, or *V. parahaemolyticus*. Among these, *V. parahaemolyticus* was the only bacterium which showed a cytotoxic effect on Vero cells. O'Brien et al. (12, 13) reported that some strains of *V. parahaemolyticus*, as well as *Shigella* species and *E. coli*, produce Shiga-like toxin. Our ELISA results, however, were negative with *V. parahaemolyticus* preparations, in spite of positive cytotoxicity. Moreover, the cytotoxicity of *V. parahaemolyticus* could not be neutralized by anti-Shiga toxin antiserum. A possible explanation for this is that the cytotoxin seen in our preparations of *V. parahaemolyticus* is antigenically different or a totally unrelated molecule, e.g., a hemolysin. This non-neutralizable toxin(s) was also demonstrated with preparations of *E. coli* (Table 3). This may be due to antigenic heterogeneity of *E. coli* verotoxins, as has been previously suggested (9, 17).

One problem of the ELISA is that some *E. coli* produced a toxin which could be neutralized by the antiserum but were negative for the ELISA. This may be because the amount of toxin produced is too little to be detected by the ELISA but is adequate for cytotoxic assay. Picograms per milliliter of toxin can be detected by cytotoxic assay, whereas nanograms per milliliter of toxin are required for positive ELISA results. In fact, our purified Shiga toxin exhibited about 10^4 50% cytotoxic doses per ng of toxin. We also should point out differences in culture protocol between O'Brien et al. (12) and this report; if we had used 500 ml of broth as did O'Brien et al., instead of 2 ml, some of these strains might have produced positive results in the ELISA. We believe, however, that our 2-ml culture system is more convenient for screening cultures which produce large amounts of Shiga (-like) toxin.

Although the ELISA method developed in this study may not be as sensitive as the bioassay using tissue culture cells, it can identify Shiga (-like) toxin-producing *S. dysenteriae* and *E. coli* O157:H7. Thus, the ELISA has advantages over the Vero cell assay in being more specific, simpler, faster, and less expensive. The colony blot ELISA recently described (18) also has may advantages over the Vero cell assay, although procedures of the colony blot ELISA are more complex than the ELISA described in this report.

Therefore, the ELISA method developed by us may be a better alternative to the cumbersome tissue culture assay.

ADDENDUM

After this report was submitted, Donohue-Rolfe et al. (2) reported an ELISA for detection of Shiga toxin with a monoclonal antibody.

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