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Differential Detection of Cholera Enterotoxin and *Escherichia coli* Heat-Labile Enterotoxin by Enzyme-Linked Immunosorbent Assays with Antibodies Specific to the Two Toxins

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Enzyme-linked immunosorbent assays (ELISAs) with antibodies specific to either cholera enterotoxin (CT) of *Vibrio cholerae* or heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* were developed to detect LT and CT, respectively. With these ELISA systems, LT and CT could be detected only with the respective specific antibody. Both antibody ELISA and ganglioside ELISA were used for differential detection of LT and CT, but the former method seemed to be more specific. By this ELISA, as little as 0.1 ng of purified LT or CT could be detected per ml. The type of toxins in fluids in intestinal loops of experimental animals challenged with living cells of either *V. cholerae* or LT-producing *E. coli* was identified correctly by this ELISA. These results suggest that the ELISA systems reported here could be used to detect and differentiate CT and LT in unknown samples; it could also be used for assaying toxins in stool specimens for the diagnosis of diarrhea due to *V. cholerae* or LT-producing *E. coli* directly, without or before bacterial isolation.

Vibrio cholerae and enterotoxigenic (ET) *Escherichia coli*, which induces a diarrhea clinically similar to that of *V. cholerae* (9), are the most important causative organisms of diarrhea, especially in developing countries (7). The two organisms produce structurally, biologically, and immunologically similar enterotoxins (7); that is, *V. cholerae* produces cholera enterotoxin (CT) and ET *E. coli* produces heat-labile enterotoxin (LT). The presence of CT and LT in patient stools containing *V. cholerae* and LT-producing ET *E. coli*, respectively, has been demonstrated by sensitive assay methods, such as enzyme-linked immunosorbent assay (ELISA) (1, 6, 10). These findings seem to be important, especially with respect to clinical diagnosis, because they imply that a diagnosis can be made by direct detection of toxins in the stools without or before isolation of the organisms. The methods reported previously cannot, however, differentiate CT from LT because of the antigenic similarity of the two and thus cannot be used for differential diagnosis of diarrheas caused by *V. cholerae* and LT-producing ET *E. coli*.

Recently, we have reported the isolation of monospecific antibodies to CT and LT, respectively, as well as an antibody common to CT and LT, by a combination of immunoaffinity column chromatographic methods (5). Using these monospecific antibodies, we developed ELISA systems to detect and differentiate CT and LT, as described in this report. By these ELISAs, rapid differential diagnosis of diarrhea caused by *V. cholerae* or LT-producing ET *E. coli* by direct detection of toxins in patient stools should be possible without or before isolation of the organisms.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* A-38 and X-21596 were isolated from patients in Bangladesh. *E. coli* 61, which produces both LT and heat-stable enterotoxin, and strain 31, which produces LT (3, 4), were isolated from stools of patients with traveller's diarrhea at the Osaka Airport Quarantine Station, Osaka, Japan.

Antibodies. LT was isolated and purified as described previously (13). CT was purchased from Sanko Junyaku Co., Tokyo, Japan. Antisera were raised in rabbits as described previously (5). Anti-LT monospecific immunoglobulin (anti-LT_S), which can recognize LT but not CT, anti-CT monospecific immunoglobulin (anti-CT_S), and antibody common to CT and LT were isolated by a combination of immunoaffinity column chromatographic methods as described previously (5).

One-antibody ELISA. The common antibody to CT and LT was labeled with alkaline phosphatase essentially as described previously (T. Honda, M. Sato, U. Kongmuang, and T. Miwatani, J. Diar. Dis. Res., in press). ELISA was performed as described in Table 1. For precoating ELISA microplates with the immunoglobulins, anti-LT_S (or anti-CT_S) was diluted to 2 μg/ml with 0.05 M carbonate buffer (pH 9.6), and 0.175-ml volumes of the solution were introduced into the wells of flat-bottomed, polyvinyl microtiter plates (Micro test III; BD Labware, Oxnard, Calif.). Appropriate dilutions and concentrations of alkaline phosphatase-immunoglobulin conjugate were determined by checkerboard titrations. The ELISA was usually performed in duplicate. The optical density at 405 nm of the color reaction was measured in a Titertek Multiskan MC apparatus (Flow Laboratories, Inc., Rockville, Md.).

Ganglioside ELISA. ELISA with ganglioside in the precoat layer was performed essentially as described previously (8, 11, 12) with some modification (Table 2). Briefly, 0.175 ml of ganglioside mixture (type III; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 20 μg/ml in 0.01 M phosphate-buffered saline (PBS) was added to each well to precoat the surface of the ELISA microplates. After incubation overnight at 4°C, the plates were washed three times with PBS-Tween 20 (0.05%). Additional binding sites on the microplate surface were blocked by incubating the wells with 0.2 ml of 1% bovine serum albumin (BSA) in PBS overnight at 4°C. Then, the wells were washed three times with PBS-Tween 20.

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TABLE 1. Procedure for antibody ELISA with antibody specific to CT or LT

Step no.	Procedure	Concn	Diluent	Vol/well (μl)	Incubation	
					Temp	Time
1.	Precoat well with anti-LT_s or anti-CT_s	2 μg/ml	Coating buffer (pH 9.6)	175	4°C	20 h
2.	Wash three times		PBS-Tween 20	200	Room temp	1 min × 3
3.	Cover unbound surface with BSA	1 mg/ml	PBS-Tween 20	200	4°C	20 h
4.	Wash three times		PBS-Tween 20	200	Room temp	1 min × 3
5.	Add test samples			150	37°C	1 h
6.	Wash four times		PBS-Tween 20	200	Room temp	1 min × 3
7.	Add alkaline phosphatase-labeled antibody	1:500	PBS-Tween 20	150	37°C	1 h
8.	Wash four times		PBS-Tween 20	200	Room temp	1 min × 3
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					

Samples (0.15 ml each) were introduced into duplicate ganglioside-coated wells. The plates were incubated at 37°C for 1.5 h and then washed four times with PBS-Tween 20. Indicator antibodies, either anti-CT_s or anti-LT_s, diluted to 2 μg/ml with PBS containing 1% BSA, were added in a volume of 0.15 ml to the wells, and the plates were incubated at 37°C for 1.5 h. The plates were washed four times with PBS-Tween 20, and then 0.15 ml of alkaline phosphatase-conjugated antibody against rabbit immunoglobulin G (IgG) (Tago, Inc., Burlingame, Calif.) at a 1:2,000 dilution was added, and the plates were incubated for another 1.5 h at 37°C. The plates were then washed four times with PBS-Tween 20, and 0.2 ml of *p*-nitrophenyl phosphate substrate (1 mg/ml; Wako Junyaku Co., Osaka, Japan) in diethanolamine buffer (0.05 M, pH 9.7) containing 1 mM $MgCl_2$ was added. The plates were incubated at 37°C for about 60 min, and the color that developed was measured at 405 nm as described above.

Rabbit ileal loop test. The rabbit ileal loop test was carried out essentially as described by Gorbach et al. (2). Japanese white rabbits (weight, ca. 1.7 kg) were starved for 48 h. After general and local anesthesia, 5 to 10 loops, each about 10 cm in length, were constructed. *V. cholerae* and *E. coli* strains were both cultured on tryptic soy agar plates at 37°C for 6 to 7 h and harvested with tryptic soy broth. Volumes of 1 ml of these bacterial suspensions were injected into the loops. The animals were sacrificed 18 h later, and the fluids that accumulated in the loops were collected and centrifuged at 10,000 rpm for 15 min. The supernatants with or without treatment with polymyxin B (final concentration, 10,000 IU/ml; at 37°C for 60 min) were used for the toxin assay.

TABLE 2. Procedure for ganglioside ELISA

Step no.	Procedure
1	Precoat well with ganglioside (type III [Sigma] 20 μg/ml in PBS)
2	Wash three times with PBS-Tween 20
3	Cover unbound surface with BSA (1 mg/ml in PBS)
4	Wash three times with PBS-Tween 20
5	Add test samples
6	Wash four times
7	Add anti-LT_s or anti-CT_s rabbit antibody (2 μg/ml)
8	Wash four times with PBS-Tween 20
9	Add alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1,000 in PBS-Tween 20)
10	Wash four times with PBS-Tween 20
11	Add substrate (*p*-nitrophenyl phosphate, 1 mg/ml in diethanolamine buffer)
12	Read optical density at 405 nm in colorimeter

RESULTS

Differential detection of CT and LT by ELISA assays. When anti-CT_s or anti-LT_s antibody was used as the precoating

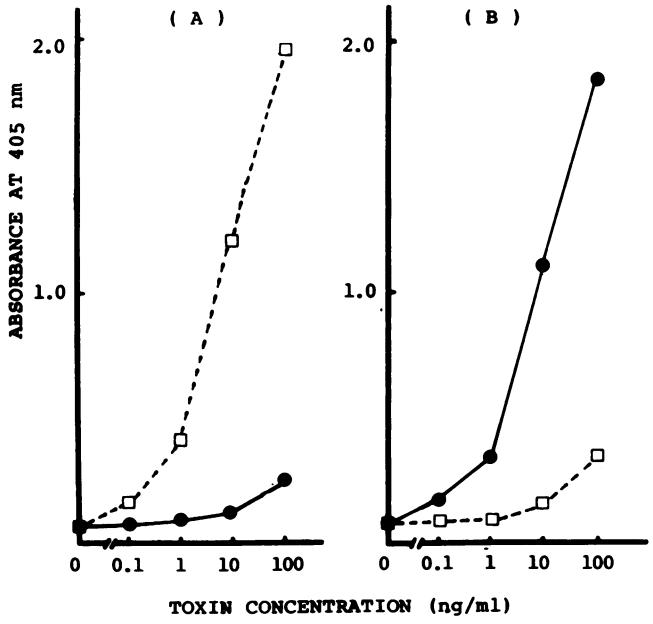

FIG. 1. Differential detection of CT and LT by antibody ELISA, with anti-LT-specific (A) and anti-CT-specific (B) antibodies used as precoating antibodies. Symbols: □, LT; ●, CT.

antibody in one-antibody ELISA, only CT and LT, respectively, could be detected (Fig. 1). Minor cross-reactions were observed with a high concentration of toxin (100 ng/ml).

Although specificity was lower than that of the one-antibody ELISA, the ganglioside ELISA system, with anti-LT_s or anti-CT_s antibody used as an indicator (second) antibody, also recognized LT and CT, respectively (Fig. 2). Cross-reactions were also observed at higher toxin concentrations than 10 ng/ml.

Identification of toxin in loop fluids induced by organisms. We used the one-antibody ELISA assays with anti-LT_s or anti-CT_s antibody to detect and differentiate the antigenic types of toxin in fluids of rabbit ileal loops challenged with living cells (about 10^8 per loop) of *V. cholerae* and LT-producing ET *E. coli*, respectively. Only CT was detected in loop fluids challenged with *V. cholerae*, and only LT was detected in loop fluids challenged with ET *E. coli* by the one-antibody ELISA (Table 3). In the case of ET *E. coli*, the amount of toxin in loop fluids was rather small, but it became higher on treatment of the fluids with polymyxin B, which was used to extract intracellular LT.

DISCUSSION

In this report, we described two ELISA systems to detect and differentiate CT and LT with monospecific antibodies to the two toxins purified by immunoaffinity chromatography. As discussed previously (Honda et al., in press), one-antibody ELISA has the following advantages: (i) fewer steps are necessary than in the ganglioside ELISA (8, 11, 12) because the toxin in the samples is recognized directly with an antitoxin-alkaline phosphatase conjugate and (ii) this simpler procedure avoids the possibility of nonspecific reactions and gives quicker results. The only disadvantage of this ELISA is that the antitoxin-alkaline phosphatase conjugate must be prepared in the laboratory. Thus, one-antibody ELISA was simpler and more specific than ganglioside ELISA for detection and differentiation of CT and LT. The minimum detectable concentration of toxin in the ELISA was about 0.1 ng/ml, and a good dose-response relationship was observed with 1 to 100 ng of toxin per ml (Fig. 2). However, some cross-reaction between the two toxins was observed; therefore, in practice, for identification of CT or LT in test samples, it is necessary to treat the samples with both anti-LT_s and anti-CT_s and compare the optical densities of the resulting reactions.

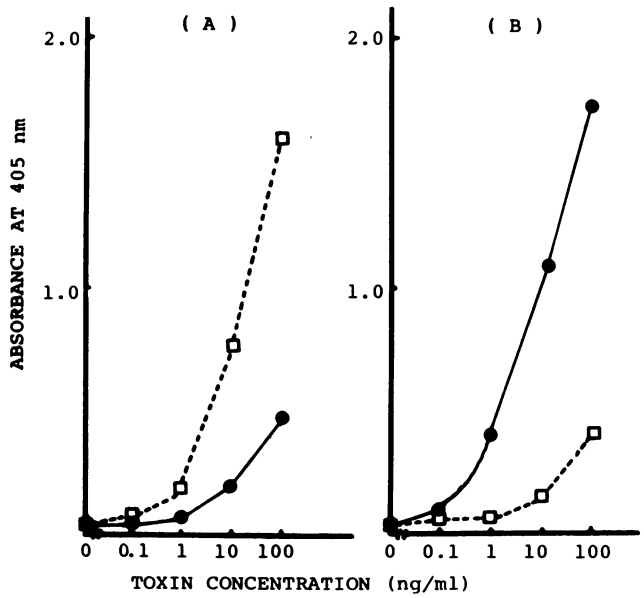

FIG. 2. Differential detection of CT and LT by ganglioside ELISA with anti-LT-specific (A) and anti-CT-specific (B) antibodies as indicator (second) antibodies. Symbols: □, LT; ●, CT.

TABLE 3. Antigenic analysis of enterotoxin by antibody ELISA in intestinal fluids of rabbit ileal loops induced by living organisms

Bacterial strain	ELISA reaction		Amount of toxin in loop fluid (ng/ml)
	Anti-LY_s	Anti-CT_s	
V. cholerae			
A-38	−	+	16–27
X-21696	−	+	40–129
E. coli			
61	+	−	0.9–2.1 (590–830)[a]
31	+	−	0.2–0.3 (108–710)[a]

[a] Fluid was tested after polymyxin B treatment.

The successful application of the ELISA for differentiation of toxins in rabbit intestinal fluids induced by challenge with living organisms (Table 3) suggests that the two antigenic types of toxin could be detected and differentiated in diarrheal stools with the ELISA system described here. We found that the amount of toxin produced in intestinal fluids can be increased by treating the fluids with polymyxin B. So, in practice, it seems better to assay toxin in stools after polymyxin B treatment. Although data obtained in animal experiments cannot be directly applied to humans, there have been several reports (6, 10) that state that LT or CT can be detected directly in human diarrheal stools by ELISA assays. Thus, it should be possible to use the ELISA systems reported here directly as novel methods for assaying toxins in stool specimens and differential diagnosis of diarrhea due to *V. cholerae* or LT-producing ET *E. coli*, without or before bacterial isolation. Thus, this technique should be useful for the rapid determination of the cause of diarrhea.

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