Inhibition of Immune Hemolysis: Serological Assay for the Heat-Labile Enterotoxin of *Escherichia coli*

DOYLE J. EVANS, JR.,* AND DOLORES G. EVANS

Program in Infectious Diseases and Clinical Microbiology, The University of Texas Medical School at Houston, Houston, Texas 77030

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Sheep erythrocytes sensitized by incubation with the heat-labile enterotoxin (LT) of *Escherichia coli* are hemolyzed in the presence of anti-LT antiserum and complement. The Microtiter (Cooke Laboratory Products) technique was used to titrate anti-LT antibody in serum by this immune hemolysis reaction. Immune hemolysis was inhibited by preexposure of the anti-LT antiserum to soluble LT before addition of the LT-sensitized sheep cells. *E. coli* mini-extract preparations were obtained by the polymyxin release technique and assayed for LT by the lysis inhibition test (LIT) and by the adrenal cell assay. All 75 adrenal cell-positive *E. coli* isolates were positive in the LIT assay. Eight of 318 adrenal cell-negative isolates tested were positive in the LIT assay, possibly indicating the presence of biologically inactive toxin.

Enterotoxigenic *Escherichia coli* have been found associated with diarrhea in many countries throughout the world (16). Diarrhea caused by these *E. coli* ranges from the familiar and bothersome travelers' diarrhea, or turista (10, 14, 18), in adults to acute dehydrating diarrhea in infants and young children (11, 12, 17). These *E. coli* may produce either an antigenic heat-labile enterotoxin (LT) or a nonantigenic heat-stable enterotoxin (ST) or both (12, 14). The true incidence of enterotoxigenic *E. coli* diarrhea remains unknown mainly because the only methods currently available for identifying these pathogens are based on detection of biologically active enterotoxin. These assay systems involve either experimental animals (1, 4, 6) or tissue culture methods (2, 12) and are expensive to maintain and generally unsuitable for all but research-oriented diagnostic work. Consequently, our goal has been to purify the LT enterotoxin of *E. coli* and to develop a serological assay for this toxin.

In vitro serological assays using erythrocytes have the advantage of being highly sensitive and relatively simple to perform, and several such methods have been described for the quantitation of enterotoxins and anti-enterotoxin antibody (9, 13, 19). We recently described an in vitro assay for antitoxin (anti-LT antibody) based on passive immune hemolysis of LT-sensitized sheep erythrocytes (LT-SRBC) (D. J. Evans, Jr., D. G. Evans, and G. Ruiz-Palacios, *Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.*, 16th, Chicago, Abstr. 368, 1976). We report here an assay for the LT of *E. coli*

based on inhibition, by soluble LT, of complement-mediated lysis of enterotoxin-sensitized SRBC by anti-LT antibody. This detection system uses antiserum prepared against purified polymyxin-released LT and the homologous toxin preparation to sensitize the SRBC. Test samples consist of easily obtained polymyxin extract preparations (7).

MATERIALS AND METHODS

Bacterial cultures. *E. coli* strain H-10407, isolated from a diarrhea case in Dacca, Bangladesh, was used for the production of LT. Numerous other enterotoxigenic and non-enterotoxigenic strains of *E. coli* were assayed for enterotoxin by the lysis inhibition test (LIT) and also by tests for biological activity. Most of these were isolated in Bangladesh and have been previously described (3, 5, 15). Other strains were collected during studies in Mexico and in Houston, Tex. Six ST-only swine *E. coli* strains were contributed by H. Moon. The double-blind series of assays was performed with 300 *E. coli* isolates derived from a study on the etiology of diarrhea in Mexico, the results of which are reported elsewhere (H. L. DuPont, J. Olarte, D. G. Evans, L. K. Pickering, E. Galindo, and D. J. Evans, Jr., submitted for publication). Stock cultures were maintained on 2.0% peptone-0.5% NaCl-2.0% agar slants.

Assays for enterotoxin activities. LT activity was determined by the Y-1 adrenal tumor cell tissue culture assay of Donta and Smith (2), in which the effect of LT is a morphological alteration (rounding) of the Y-1 adrenal cells. Here, an adrenal cell titer is the reciprocal of the highest dilution producing between 50 and 100% rounding of the Y-1 cells. Mini-extracts were tested at a final dilution of 1:20.

ST activity was determined by the suckling mouse assay of Dean et al. (1), which measures fluid accumulation in the infant mouse intestine.

Preparation of *E. coli* enterotoxin and antitoxin. The low-molecular-weight LT of *E. coli* H-10407 was obtained by the polymyxin release technique, which has been described in detail elsewhere (7). The enterotoxin was purified by a simple ammonium sulfate back-extraction step followed by adsorption to and elution from Affi-Gel 202 (Bio-Rad, Richmond, Calif.) as recently described (8). The starting material was a polymyxin "extract" obtained from 10-liter fermenter cultures. The material precipitating between 45 and 80% saturation with ammonium sulfate was dialyzed for 18 h at 4°C against a large volume of 0.02 M phosphate buffer, pH 7.3, clarified by centrifugation at $18,000 \times g$ for 20 min, adjusted to pH 6.7, and passed through an Affi-Gel 202 column. Enterotoxin was subsequently eluted from the gel with 0.1 M tris(hydroxymethyl)aminomethane-chloride buffer, pH 8.1, containing 0.3 M NaCl.

Adult albino rabbits were hyperimmunized with Affi-Gel 202-purified enterotoxin as follows. The first dose consisted of intramuscular injections at four inguinal sites of 30 μ g of protein, emulsified in incomplete Freund adjuvant, per site. Each rabbit received five additional doses, intramuscularly at multiple sites, at 3-day intervals with enterotoxin diluted in saline. Doses were increased so that for the final injection each rabbit received a total of 400 μ g of protein. The animals were bled 14 days after the final injection, and the serum was collected, pooled, and stored at -45°C. Control serum was a pool derived from the same rabbits before immunization.

Anti-cholera toxin, a lyophilized horse antiserum, was obtained from Nobuya Ohtomo (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan).

Mini-extract preparations. Cultures of each test strain were prepared by loop inoculation of 10 ml of 2.0% Casamino Acids (Difco)-0.6% yeast extract (Difco)-salts medium (6) from the stock slants. The flasks (50 ml) were agitated at 140 rpm for 18 h at 37°C in a rotary incubator shaker. Mini-extracts were prepared from subcultures consisting of 10 ml of 2.0% Casamino Acids-0.15% yeast extract-salts medium (7) plus 1.5 ml of the overnight broth culture. After 3.5 h of incubation at 37°C as above, the mini-extract cultures were centrifuged at $12,000 \times g$ for 20 min and the supernatant fluids were discarded. Each pellet of bacteria was resuspended in 0.8 ml of 0.15 M tris(hydroxymethyl)aminomethane-chloride buffer, pH 6.6, containing 0.9% NaCl and 1.0 mg of polymyxin B per ml (Aerosporin, Burroughs Wellcome Co., Research Triangle Park, N.C.) added directly to the centrifuge tubes. The suspensions were incubated for 15 min in a 37°C water bath and then immediately centrifuged at $16,000 \times g$ for 20 min to remove the cells. The resultant supernatants, or mini-extracts, were recovered by disposable Pasteur pipettes and stored in small vials at 4°C.

Sensitization of SRBC with enterotoxin. Fresh SRBC were sensitized with enterotoxin as follows. Five-milliliter aliquots of sheep blood were washed

three times with 12 volumes of phosphate-buffered saline (0.1 M, pH 7.2) (PBS) by centrifugation at 1,800 rpm for 8 min. An LT preparation was diluted to 25 to 50 μ g of protein per ml in 0.1 M phosphate buffer, pH 6.5, and 1.0 ml of enterotoxin was added per 0.1 ml of packed SRBC. The mixture was incubated for 30 min at 37°C with intermittent shaking and then centrifuged as above. The LT-SRBC were then washed three times in PBS and finally resuspended in PBS, 1.0 ml per 0.1 ml of packed cells.

Microtiter titration of anti-enterotoxin sera. The Microtiter (Cooke Laboratory Products, Alexandria, Va.) titration system was used for the titration of anti-LT antibody by the complement-mediated immune hemolysis reaction. Twofold dilutions of the sera were prepared in PBS plus 0.02% bovine serum albumin (BSA) in U-well Microtiter plates (Linbro), and an equal volume (0.025 ml) of LT-SRBC (1.0% in PBS-BSA) was added. After incubation for 30 min at 37°C, 0.025 ml of freshly prepared guinea pig complement (1:10 in PBS-BSA) was added, and the plate was returned to the 37°C incubator for 60 min. The lytic reaction was optimal for observation 30 min after the plate was returned to room temperature.

LIT for *E. coli* LT. *E. coli* LT, as in Affi-Gel 202 column fractions or mini-extracts, was determined by allowing the soluble LT to react with a standard amount of antitoxin before addition of the LT-SRBC and complement. The presence of toxin in test samples was indicated by inhibition of the immune hemolytic reaction. The standard amount of antitoxin was the highest dilution, determined by titration, that produced complete lysis of LT-SRBC. The LIT was performed as follows. Twofold dilutions of the test materials were prepared in PBS-BSA in Microtiter wells, and an equal volume (0.025 ml) of a predetermined dilution of antitoxin was added. *E. coli* toxin-treated SRBC (0.025 ml of a 1% suspension) were added after incubation of the plate at 37°C for 30 min. Guinea pig complement (0.025 ml of a 1:10 dilution in PBS-BSA) was added after another 30 min of incubation, and the plate was returned to 37°C for a final 30-min period.

For the survey of known LT-positive and LT-negative strains of *E. coli*, the mini-extract samples were diluted four Microtiter wells (to 1:16), in duplicate, such that 12 samples were tested per plate. The dilution of anti-LT serum used in this series was either 1:100 or 1:120 of the pooled rabbit hyperimmune serum. For the survey of *E. coli* isolates performed in blind fashion, samples were diluted only 1:2 and 1:4, in duplicate, so that 24 tests were performed per plate, including 2 positive and 2 negative controls per plate. The dilution of anti-LT serum used in this series was 1:80, since this dilution produced the best differentiation between the positive and negative samples.

RESULTS

Immune hemolysis of enterotoxin-sensitized SRBC. Figure 1 shows the results obtained by titrating rabbit anti-LT serum, normal rabbit serum, and a horse monospecific anti-cholera toxin serum. The titers were 1:64,

FIG. 1. Microtiter plate showing titration, in duplicate, of three sera by immune hemolysis of *E. coli* enterotoxin-sensitized SRBC. Rows 2 and 3: rabbit anti-LT serum; rows 4 and 5: normal rabbit serum; rows 6 and 7: horse anticholera serum.

1:2, and 1:512, respectively, for these sera. Other rabbit anti-LT sera, from individual animals, ranged from 1:64 to 1:512. The pooled hyperimmune rabbit serum used for the lysis inhibition test possessed a titer of 1:128. Specificity of the immune hemolysis reaction is demonstrated by lysis of LT-SRBC by anti-cholera toxin serum, which is known to contain antibody cross-reactive with *E. coli* LT.

LIT for *E. coli* enterotoxin. Hemolysis of LT-SRBC was inhibited by preincubation of the anti-LT serum with soluble LT before addition of the LT-SRBC and complement. This inhibition, via competition for anti-LT antibody, was quantitated by preparing serial dilutions of the LT-containing samples in the Microtiter system and performing the LIT as described in Materials and Methods. An example of results obtained by the LIT assay is shown in Fig. 2. Here, the LIT was used to monitor elution of LT from an Affi-Gel 202 column, one of the steps used in the purification of the enterotoxin. LIT titers agreed with values obtained for biological activity. For example, in this experiment LT titers in the adrenal cell assay were 5×10^3 , 1×10^5 , 5×10^3 , and 1×10^2 , respectively, for column fractions 18, 19, 20, and 21.

Detection of LT in mini-extract preparations by the LIT assay. Mini-extracts were

FIG. 2. Elution profile obtained by adsorbing the heat-labile enterotoxin of *E. coli* to Affi-Gel 202 in the presence of 0.02 M phosphate buffer, pH 6.7 (10-ml fractions), and eluting the enterotoxin with 0.1 M tris(hydroxymethyl)aminomethane-chloride buffer, pH 8.1, containing 0.3 M NaCl (3-ml fractions). Enterotoxin was determined by titration of each fraction in the LIT assay.

prepared from 45 enterotoxigenic (LT-positive) and 48 non-enterotoxigenic (LT-negative) strains of *E. coli* and tested by both the LIT and the adrenal cell assay techniques. All of the

preparations that were positive in the adrenal cell assay were also positive in the LIT assay. All but six LT-positive strains produced LIT titers of at least 1:16 and 80 to 100% rounding of the adrenal cells. Mini-extract preparations that produced LIT titers of 1:4 or 1:8 produced 20 to 80% rounding of the Y-1 cells. The LT-negative *E. coli* strains, including 10 known to be of the ST-only type, were all negative in the LIT assay, although some were questionably positive at the 1:2 dilution. Increasing the anti-LT serum concentration eliminated this possible source of error.

The LIT assay was next applied to *E. coli* isolates obtained via diarrhea case studies in order to determine the performance of the test under routine conditions. Mini-extracts were prepared, and the adrenal cell assay was performed independently in a double-blind fashion. Culture supernatant fluids of these isolates were also assayed by the adrenal cell method in order to confirm the efficiency of the mini-extraction procedure. The LIT assay was performed in a simple qualitative manner as described in Materials and Methods. Figure 3

shows typical results obtained by this procedure. Table 1 shows the data obtained by testing mini-extracts of 300 different *E. coli* isolates originating from 48 different study cases. Thirty isolates were positive and 262 isolates were negative according to the results of both tests. Eight isolates obtained from two separate cases were found to be positive in the LIT assay and negative in the adrenal cell assay. No isolates were positive for adrenal cell activity and negative in the LIT assay. Thus the overall result was agreement between the results of the two assay techniques in 292 of 300 (97%) tests performed. There was complete agree-

TABLE 1. Detection of enterotoxigenic (LT-positive) *Escherichia coli* by the lysis inhibition test and by the Y-1 adrenal cell assay

Lysis inhibition test	Adrenal cell assay	No. of isolates	Diarrhea cases
+	+	30	14 (1-5 per case)
-	-	262	32 (5-10 per case)
+	-	8	2 (4 of 5; 4 of 10)
-	+	0	0

FIG. 3. Microtiter plate showing results of the LIT assay for *E. coli* enterotoxin in polymyxin mini-extracts. Tests were performed in duplicate using undiluted (rows 1, 3, 5, and 7 of wells) and a 1:2 dilution (alternate rows) of samples. Lysis of enterotoxin-sensitized SRBC by anti-LT serum (1:80 dilution) was inhibited by four samples, resulting in visible pellets.

ment between the adrenal cell assays performed on culture fluids and the corresponding mini-extracts.

DISCUSSION

SRBC are readily sensitized to complement-mediated hemolysis by exposure to the *E. coli* LT under appropriate conditions. Thus, LT-SRBC provide a convenient reagent for quantitating anti-LT antibody in sera using the familiar Microtiter technique. We have used this simple and economical assay for the determination of anti-LT antibody in the serum of immunized animals, as reported here, and in human sera. Also, inhibition of complement-mediated hemolysis of LT-SRBC by soluble LT provides the basis for a serological assay for detecting and quantitating LT in a variety of test samples. The results reported here indicate that the LIT assay possesses sufficient sensitivity and specificity to differentiate between enterotoxigenic (LT-positive) and nonenterotoxigenic (LT-negative) *E. coli*. In the work reported here, a total of 75 LT-positive and 318 LT-negative *E. coli* cultures were tested. All of the LT-positive cultures produced LT-positive polymyxin "extracts" (mini-extracts) as detected by their activity in the adrenal cell assay, and all were positive in the LIT assay. Thus, strain differences were of little or no significance in the efficiency of the mini-extraction process or of the LIT assay. Of the adrenal cell-negative cultures tested, only eight isolates, representing only two separate sources, gave disparate results. These strains may have produced either biologically inactive LT antigen or LT with activity below the sensitivity of the adrenal cell assay. The results of the diarrhea case study would not have been significantly different if the isolates had been tested by the LIT assay alone. The LIT assay indicated toxigenic *E. coli* in 16 (33%) of 48 cases, whereas the adrenal cell assay indicated toxigenic *E. coli* in 14 (29%) of the cases.

One obvious drawback of the LIT assay is the requirement for purified *E. coli* LT as a reagent in the assay. Another area for improvement is the possible simplification of the mini-extraction procedure, which is an integral part of the assay system because culture supernatant fluids alone proved to be inappropriate for the LIT assay. We are currently investigating the possibility of using a direct test for LT based on passive immune hemolysis and also possible variations in the mini-extraction procedure in our efforts to facilitate identification of enterotoxigenic *E. coli* as a routine procedure.

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