Comparison of Two GM1-Erythrocyte Assays To Detect Heat-Labile Escherichia coli Enterotoxin in Stool Specimens

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Two erythrocyte immunoassay techniques to detect the presence of Escherichia coli heat-labile enterotoxin (LTh) in stool supernatants and cell-free culture supernatants were compared. In the competitive assay, GM1 ganglioside was coated onto V-shaped-well microdilution plates and enterotoxin was coupled to sheep erythrocytes. As little as 0.8 ng of LTh per ml was detected by this method, which was based on the competition between the LTh of the test sample and the sensitized erythrocytes. The second assay made use of chimera antibody prepared by coupling polyclonal anti-LTh antibody to a monoclonal antibody specific for sheep erythrocytes. In this case, LTh, which was specifically bound to a GM1 ganglioside-coated plate, was detected by successively adding the chimera antibody and sheep erythrocytes. The limit of detection of the chimera antibody erythrocyte immunoassay was 0.2 ng/ml. Stool samples were collected from 167 infants hospitalized for diarrhea in the hospital of Noumea, New Caledonia. False-negative reactions due to proteases present in the stool samples were avoided by the addition of phenylmethylsulfonyl fluoride.

Enterotoxin-producing Escherichia coli (ETEC) is a well-recognized etiological agent of acute gastroenteritis and causes watery diarrhea in people of all ages (11, 29, 30). ETEC may produce a heat-labile enterotoxin (LTh), a heat-stable enterotoxin (STA) (1, 27, 28), or both. Toxigenic tests to demonstrate LTh directly in stool supernatants have been developed during the last 5 years (23, 24). Although various tests have been described to detect LTh in stool specimens, several difficulties have hampered their widespread adoption, particularly by poorly equipped laboratories (8, 23, 24). Immunoenzymatic methods (enzyme-linked immunosorbent assays [ELISAs]) for the detection of LTh seem to be more easily applied than other methods in such laboratories. Nevertheless, in tropical countries the instability of substrates at high environmental temperatures renders these methods difficult. We investigated the possibility of using sheep erythrocytes as markers (14, 15, 25) in two methods to detect LTh in stool specimens. One was a competitive GM1 erythrocyte assay employing a V-shaped-well microdilution plate coated with GM1 and LTh-coupled sheep erythrocytes. The second was a sandwich-type erythrocyte immunoassay in which a GM1-coated plate and anti-LTh and anti-sheep chimera antibodies were used (9a).

Protease activity, which is known to be present in fecal material (16, 20), is responsible for false-negative reactions, as it may cause proteolysis of the antibody used in solid-phase immunoassays or of the protein antigen in the sample (20, 26). To avoid such proteolysis, we examined the possibility of detecting LTh directly in stool specimens by employing the known affinity of this toxin for the cholera toxin (CT) receptor ganglioside GM1 (17) and by inhibiting proteolysis in stool samples with a protease inhibitor.

MATERIALS AND METHODS

Specimens. Stool specimens were obtained from 167 children under 3 years of age who were hospitalized for acute watery diarrhea in the pediatric section of the territory hospital of Noumea, New Caledonia, between January and December 1985. All the feces were collected after natural evacuation. Portions of each sample were used for microbiological screening (34) and frozen immediately at −80°C for storage.

E. coli strains and cultivation. E. coli strains were obtained by plating the 167 fecal samples directly onto MacConkey agar. From each culture, five characteristic lactose-fermenting colonies were selected at random. The strains were cultivated as described previously (29) in Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract (CAYE) medium. CAYE medium was supplemented with 0.25% glucose and 45 μg of lincomycin per ml; polymyxin B sulfate was added 45 min before the end of the culture at a final concentration of 2,000 IU/ml. Strains were tested for the production of LTh by the ganglioside immunosorbent assay (GM1 ELISA) (10) and CHO-K1 assay (12) and for the production of STa by the suckling mouse assay (5). LTh-positive supernatants were titrated by GM1 ELISA (10) before and after treatment with polymyxin B.

Purified enterotoxin. Strain H10407 (kindly provided by B. Rowe, Central Public Health Laboratory, Division of Enteric Pathogens, London, England) was used for the production of LTh. Purified LTh was obtained through Bio-Gel ASM (Bio-Rad Laboratories, Richmond, Calif.) affinity chromatography, followed by gel filtration on Sephacryl S-200 (Pharmacia, Uppsala, Sweden) (5). The purity of LTh was checked by polyacrylamide gel electrophoresis (21), and the amount of protein was determined with the Folin phenol reagent (22).

Monoclonal antibodies. The 8-azaguaine-resistant mouse myeloma cell line SP2/O-Ag was kindly provided by M. Shulman (31). The cell hybrids were grown in Eagle medium reinforced by doubling the amino acids, vitamins, and glucose in the presence of 10 μM azaserine and 50 μM hypoxanthine. This was supplemented with 1 mM pyruvate—2 mM glutamine—10% (vol/vol) heat-inactivated horse serum. Cells were grown in petri dishes incubated at 37°C in a 10% CO2 atmosphere. Three-month-old BALB/c mice were injected intraperitoneally with 200 μl of sheep erythrocytes at 10% in phosphate-buffered saline (PBS). Three weeks later,
a booster injection of 200 μl of a sheep erythrocyte suspension was administered (10% in PBS). Three days after the last immunization, the mice were killed by neck dislocation, and the spleens were removed. The spleen cell suspension was washed three times and suspended in serum-free medium for fusion. Fusion of spleen cells and SP2/0-Ag cells was performed on membrane filters, as described previously by Buttin et al. (3), or by a mass fusion technique (4). In the two experiments, a 45% (wt/vol) solution of polyethylene glycol 1000 (Merck & Co., Inc., Rahway, N.J.) was used. Screening for hybridomas was done by the rosette-forming test (7). Hybrid cells secreting anti-erythrocyte antibodies were cloned by limiting dilution in flat-bottom microtiter plates (3040 F; Becton Dickinson Labware, Oxnard, Calif.), in the presence of 2.106 BALB/c fresh thymocytes as a feeder layer. The single clones selected by microscopic examination were reassayed for antibody secretion. The hybrid cells were grown as ascites in the peritoneal cavities of BALB/c mice primed with pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Ascites fluid was centrifuged at 800 x g for 15 min at 4°C, heat inactivated at 56°C for 30 min, and centrifuged at 15,000 x g. Immunoglobulins were isolated from ascites fluid by precipitation with ammonium sulfate at 50% saturation, followed by dialysis against PBS.

**Polyclonal antibodies.** Purified LTh was used as the immunizing antigen. Serum samples were taken from five rabbits (weight, 3 kg each; Fauve de Bourgogne) before immunization and were stored at −20°C. For immunization, anti-LTh antibodies were prepared as recommended by Honda et al. (19) and titrated by ELISA as described previously (9). The titer of the sera was 800.

The immunoglobulin G (IgG) fractions from rabbits were purified by chromatography on DEAE-cellulose (32). IgG specific for LTh was prepared by immunoaffinity column chromatography (18), titrated, and concentrated (9). The titer of anti-LTh IgG was 1,280.

**Preparation of chimera antibody.** Chimera antibodies were prepared by a one-step glutaraldehyde coupling procedure (2). The optimal conditions for the preparation of the chimera antibodies were determined by testing various incubation times and concentrations of either glutaraldehyde or antibodies. Polyclonal anti-LTh antibodies were coupled with monoclonal anti-sheep erythrocytes as follows. To 0.9 ml of 0.1 M phosphate buffer (pH 6.8) containing 2 mg of polyclonal anti-LTh antibodies (titer, 1,280) and 1 mg of monoclonal anti-sheep erythrocytes (titer, 128,000), 0.1 ml of a 1% aqueous solution of glutaraldehyde (TAAB Laboratories) was added with gentle stirring. Coupling was carried out for 5 h at 30°C. After the coupling reaction, 100 μl of 2 M glycine (Prolabo) was added. The preparation was further incubated for 3 h, centrifuged, and dialyzed against PBS (0.01 M phosphate buffer [pH 6.8], 0.15 M NaCl) for 12 h at 4°C and mixed with an equal volume of bidestilled glycerol (Prolabo). The resulting conjugate was stored at −20°C.

**Coupling of sheep erythrocytes with LTh.** A sheep erythrocyte suspension in sterile Alsever solution (Diagnostics Pasteur, Aulnay-sous-Bois, France) was centrifuged at 1,500 x g and washed 4 times with PBS at 4°C. Washed erythrocytes were coupled with LTh by a one-step method with glutaraldehyde (2). Briefly, 300 μg of LTh was added to 2 ml of a 2% erythrocyte suspension in PBS. Glutaraldehyde was added with gentle stirring at a final concentration of 0.25%. After incubation, erythrocytes were washed twice in PBS, followed by incubation with 0.1 M (final concentration) glycine in PBS for 2 h. Erythrocytes were then washed four times and suspended in 2 ml of PBS with 0.02% sodium azide (Sigma Chemical Co., St. Louis, Mo.) and stored at 4°C until use.

**Coating of microdilution plates.** Polystyrene microdilution plates with V-shaped wells (Costar, Cambridge, Mass.) were coated by filling the wells with 100 μl of GM1 ganglioside (1.0 μg/ml; Calbiochem-Behring, La Jolla, Calif.) in PBS, and they were left at 30°C for 18 h. The plates were then washed four times with PBS containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Prolabo). Plates were stored at 4°C until use. Next, the remaining unbound reaction sites were blocked by adding 250 μl of heat-inactivated (56°C for 30 min) 10% fetal calf serum (Flow Laboratories, Inc., McLean, Va.) in PBS, and the plates were left at 37°C for 1 h. The wells were emptied and washed before use as described above.

**Competitive erythrocyte assay.** Standard CT (100 μl; Sigma), cell-free supernatants, or stool specimen supernatants diluted in PBS containing 1% bovine serum albumin (BSA) were deposited in each well. Each sample was tested in duplicate. The microdilution plate was incubated for 1 h at room temperature (25°C) and then washed three times with PBS. A 0.5% suspension (100 μl) of LTh-coated sheep erythrocytes in PBS was added to each well. The plate was gently mixed and left undisturbed at room temperature for 2 to 5 h, and the degree of erythrocyte adsorption was assessed with the naked eye as described by Guesdon and Avrameas (13).

**Erythrocyte assay with chimera antibody.** One hundred microliters each of CT standards, cell-free supernatants, and stool specimen supernatants were tested in duplicate. The microdilution plate was incubated for 1 h at room temperature and then washed with PBS-Tween 20. Then, the wells were filled with 100 μl of the chimera antibody diluted in PBS-BSA (1 μg of anti-sheep erythrocyte monoclonal antibodies per ml). The plate was left at room temperature for 2 h and then washed as described above. The wells were filled with 100 μl of a 0.05% suspension of fresh sheep erythrocytes in PBS. The microdilution plate was left undisturbed at room temperature for 5 h, and the degree of erythrocyte adsorption was assessed with the naked eye (14, 15).

**Direct LTh study.** Frozen specimens were thawed at 37°C. One portion of each stool specimen was diluted 1:5 with sterile PBS and then centrifuged at 4°C and 400 x g for 15 min. The supernatant was collected and tested by the competitive erythrocyte assay and the erythrocyte immunooassay with chimera antibody. Another portion of the stool specimen was diluted 1:5 with PBS containing 10 mM phenylmethylsulfonyl fluoride (PMSF) (a 0.1 M stock solution of PMSF was prepared in ethanol). The suspension was centrifuged as described above and then tested by both techniques. All assays were run simultaneously in duplicate for each of the 167 stool specimen supernatants. Each of the 167 stool specimen supernatants was assayed twice by both techniques. Assays were run simultaneously.

All the positive supernatants were titrated by both techniques. For titration, positive stool specimen supernatants were serially diluted from 1/2 to 1/256 (arithmetic progression).

**Control reactions.** For both methods, negative control wells were prepared in duplicate with PBS-BSA and a stool specimen negative for LTh obtained from a child hospitalized in Noumea for acute diarrhea due to an enteropathogenic strain of *E. coli* O55:B5. Positive control wells were prepared in duplicate with a solution containing 1 μg of LTh...
per ml in PBS-BSA and the diarrheal stool sample described above, with 1 μg of LTh per ml added before the test.

All the positive specimens were confirmed by a blocking assay with rabbit anti-LTh sera. A 200-μl portion of stool specimen supernatant was incubated in duplicate for 2 h at 37°C on a shaker with an equal volume of anti-LTh sera (titer, 200). One hundred microliters of the mixture was then transferred to a GM1-coated microplate and processed as described above.

To confirm the heat lability of the toxin, we heated all the positive stool specimen supernatants for 20 min at 60°C before testing the samples.

**RESULTS**

Results of the tests for enterotoxigenicity showed that 17 ETEC strains were detected by the GM1 ELISA, the CHO-K1 assay, and the suckling mouse assay. Of these, 6 produced LTh and 11 produced both LTh and STA. From the five *E. coli* isolates randomly obtained from each patient, ETEC was recorded with the following frequencies: for strains producing only LTh, four ETEC strains, one time; three ETEC strains, two times; two ETEC strains, three times; for strains producing both LTh and STA, four ETEC strains, nine times; three ETEC strains, two times. ETEC producing LTh belonged to serogroups O6 (three strains), O15 (one strain), and O78 (two strains). ETEC producing both LTh and STA belonged to serogroups O6 (four strains), O20 (three strains), and O78 (four strains).

Treatment with polymyxin B increased LTh titers only for two ETEC strains that produced both LTh and STA; one belonged to serogroup O20 and one belonged to serogroup O78. These results were then compared with those obtained with the corresponding cell-free culture supernatants and stool supernatants in the competitive erythrocyte assay and in the erythrocyte immunoassay with chimera antibodies.

**LTh detection by competitive erythrocyte assay.** GM1 ganglioside adsorbed on V-shaped wells had a high affinity for binding LTh (Fig. 1). The coated wells were filled successively with the test sample and LTh-coupled sheep erythrocytes. This assay was based on the principle that if the test sample has no toxin, the erythrocytes, which are linked to LTh, become attached to the GM1 which coats the solid surface, with the result that none of them are available to settle at the bottom of the well. Thus, a homogeneous layer of erythrocytes would be observed. If, on the other hand, the stool supernatant contains toxin, the binding of erythrocytes carrying LTh on their surfaces is inhibited, in direct proportion to the amount of toxin present in the sample. At a saturating concentration of toxin in the sample, the adsorption of sensitized erythrocytes to the solid surface would be completely prevented. The erythrocytes would then fall to the bottom of the well and would form a pellet. Sixteen positive cell-free supernatants were identified by this technique, by which as little as 0.8 ng of LTh per ml was detected. LTh was detected in 13 of the 17 (76%) stool specimen supernatants diluted with PMSF (4 stool specimen supernatants were positive at a 1/4 dilution and 9 were positive at a 1/5 dilution) and in 4 (23%) stool specimen supernatants diluted with PBS (1 stool specimen supernatant was positive at 1/4 and 3 were positive at 1/5) (Table 1).

**LTh detection by erythrocyte assay with chimera antibody.** If the stool sample to be tested is positive for LTh, the chimera antibody would be retained on the solid GM1-coated surface and the sheep erythrocytes would also be retained, leading to the formation of a homogeneous layer. If the stool sample is devoid of toxin, the erythrocytes would not be retained and would form a pellet. Seventeen positive cell-free supernatants were identified by this method, by which as little as 0.2 ng of LTh per ml was detected (Fig. 2). LTh was detected in 16 of the 17 (94%) stool specimen supernatants diluted with PMSF (5 stool specimen supernatants were positive at a 1/4 dilution and 11 were positive at a 1/5 dilution) and in 4 (23%) stool specimen supernatants diluted with PBS (titer, 3) (Table 1).

By both methods treatment of diarrheal stool specimens with 2,000 IU of polymyxin per ml, before or after freezing, did not modify LTh titers in stool specimen supernatants.

Concentrations of ethanol up to 20% (vol/vol) had no effect on the detection of five LTh-positive supernatants by both techniques. Heating of the supernatants changed all positive samples (stools and cell-free supernatants) to negative by both tests. The test was also positive (titer, 2) with one stool specimen supernatant in which no etiological agent was identified. In blocking tests, all the positive reactions, including the discordant positive reactions, became negative.

**DISCUSSION**

The goal of this study was to find a rapid, simple, and economical method to detect the LTh produced by enterotoxin-positive stool samples.
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genic *E. coli* directly in stool specimens. Erythrocytes have been used as markers in various qualitative and quantita
tive immunological techniques (13–15, 25). In the present study we compared two assays; in one, the LTh of the test sample competed with the same antigen coupled to sheep erythrocytes for binding to the GM1 ganglioside coated on the solid surface; and in the other, a chimera antibody, which was prepared by coupling polyclonal antibodies spe
cific for LTh to monoclonal antibodies for sheep erythrocytes, was used. Both techniques were convenient, insofar as a large number of samples could be tested and the amount of enterotoxin in feces or cultures could be determined. The assays were run on 96-well microdilution plates, which offers a great advantage for laboratories that receive a large num
er of specimens, but the dilution of stool specimens and centrifugation of these specimens is not a simple matter; filtra
tion through a cellulose acetate filter could avoid these problems. The coupling procedure was very simple and rapid, and the chimera antibody preparation was used without further purification. For both techniques the results (either a clear pellet or a homogeneous carpet) were easy to interpret. As described previously (16, 17, 33), GM1 is highly specific for LTh and has a high affinity for binding the enterotoxin.

Morgan et al. (24) and Merson et al. (23) examined the possibility of detecting LTh directly in stool in countries in which ET
EC is a major cause of diarrhea by the Y1 adrenal cell assay, ELISA, and counterimmunoelectrophoresis. They compared these methods and showed that ELISA may soon replace the Y1 adrenal cell assay and counterimmunoelectrophoresis, since it is a rapid and sensitive assay that is easy to perform and interpret (24). In a field setting, how
ever, especially in tropical countries in which the environ
mental temperatures and humidity are high and refrigeration is not available universally. ELISAs face difficulties because of the instability of the biocatalyst (8, 15). Furthermore, conjugates and substrates are more expensive than erythrocytes. On the other hand, sheep erythrocytes are available even in remote areas, and techniques for their handling and stabilization are well established in developing countries and have been adapted to minimally equipped laboratories (14, 15, 25).

Both methods are reproducible and almost as sensitive as the ELISAs (8, 23, 24, 27, 33). The sensitivity of the GM1 erythrocyte immunoassay with chimera antibodies is higher than that of the GM1 competitive erythrocyte assay, but the choice between these two methods depends on their appli

cations. Both are serviceable and give valid information. In routine diagnosis work on ETEC, the qualitative determina
tion of enterotoxin production is of primary interest and the quantitative determination of enterotoxin in stool specimens is less important. For qualitative purposes, the competitive procedure is quicker than the sandwich type erythrocyte immunoassay; the latter technique, nevertheless, offers sev
eral advantages. It seems to be more sensitive, and erythro
cytes are used in their native form. The advantage of the competitive technique is that it can give valuable information rapidly and thus aid in the control of infection.

The in vitro results suggest that the stool specimens contain a factor that interferes with the detection of the LTh present in feces. This activity was observed in practically all positive stool specimens tested. Similar activity has been widely studied by Hovi et al. (20) and Prevot et al. (26), as it interferes with the detection of antigens in stool specimens by solid-phase immunoassays. Our results document the protease activity in stool samples which may cause false-negative results. This problem is avoided in most assay procedures by the addition of PMSF, a potent broad-spec
trum protease inhibitor. We expect that the proteases present in stool specimens might digest part or all of the enterotoxin in the sample. Discrepancies between microbiolo
gical identification of ETEC LTh+ and direct detection in stool specimens diluted in PBS have been described previ
ously (24). Our results correlate well with those of Merson et al. (23), who used fetal calf serum and horse serum as diluents, which are known to contain several types of protease inhibitors (23). They reported that much of the LTh activity can be destroyed by exposure of the stool specimens to room temperature for 45 to 60 min (23), in contrast to the results reported by Morgan et al. (24). The destruction of LTh is probably caused by the enzymatic activity of the protease that is present in the stool specimen (20).

It is not surprising that both erythrocyte assays did not identify some of the known LTh-positive samples, even when PMSF was used. A possible enterotoxin-binding effect of free gangliosides in stool specimens has been found by several investigators (16).

One sample gave a positive reaction by both techniques, and this reaction was confirmed by the blocking test, but no ETEC LTh+ strain or other etiological agent was identified. Perhaps an LTh-producing ETEC was present in the sample, but in such a small quantity that it was not detected by the isolation procedure used. Furthermore, the patient had clinical signs of cholera-like watery diarrhea but without significant dehydration (i.e., the loss of <3% body weight). Merson et al. (23) have suggested that direct LTh is diag
osed more easily in patients with significant dehydration who would presumably be more heavily colonized with ETEC in their small intestine and perhaps have more toxin in their stool specimens.

At last, except for two cultures, the use of the polymyxin release technique is not necessary to increase LTh titers, either in culture supernatants or in stool specimens. In conclusion, in this study we demonstrated that both the competitive erythrocyte assay and the erythrocyte assay with chimera antibodies are suitable to detect LTh either in cell-free supernatants or in nonsterile stool supernatants diluted with PMSF, especially in countries in which refrigeration facilities are not readily available and laboratories are poorly equipped.
E. COLI ENTEROTOXIN DETECTION

LITERATURE CITED


