GM1 Ganglioside Enzyme-Linked Immunosorbent Assay for Detection of Heat-Labile Enterotoxin Produced by Human and Porcine Escherichia coli Strains

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Human and porcine enterotoxigenic strains of Escherichia coli were cultivated in tryptone-yeast extract medium or brain heart infusion broth and tested for production of heat-labile enterotoxin by the GM1 ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) and the Y1 adrenal cell assay. When testing for enterotoxigenicity by the GM1-ELISA technique, homologous antisera for human and porcine heat-labile enterotoxins had to be used to detect enterotoxigenic strains of human and porcine origin, respectively. This observation indicates a serological difference between the heat-labile enterotoxins produced by human and porcine strains. Furthermore, brain heart infusion broth was found to have an inhibitory effect on detection of enterotoxin both in the GM1-ELISA and in a toxin-binding modification of the Y1 adrenal cell test, but not in the conventional adrenal cell assay.

For the detection of the heat-labile enterotoxin of Escherichia coli, several techniques are available, such as the rabbit ileal loop test (3), the rabbit skin test (7), the infant rabbit test (8), and various in vitro assays with different cell types, e.g., Y1 adrenal cells (4), Chinese hamster ovary cells (9), or Vero cells (15). The in vitro assays are preferable in the routine screening of enterotoxigenic E. coli isolates, since they are inexpensive and easy to perform and permit the study of a large number of strains simultaneously. In particular, the Y1 adrenal cell assay has been widely used for this purpose. However, cell tests may be hampered by bacterial growth in the cell medium or by cytotoxic substances produced by the bacteria. These drawbacks were avoided by the development of a toxin-binding modification of the adrenal cell assay (1, 12).

The second generation of in vitro assays consists of immunological techniques, such as the passive immune hemolysis assay (6) and the enzyme-linked immunosorbent assay (ELISA) (5, 17). A refinement of the latter test uses the toxin receptor, ganglioside GM1, as the sorbent for the active toxin and is designated the GM1-ELISA (2, 16).

Upon routine testing of strains cultivated in a tryptone-yeast extract (TY) medium (10) or brain heart infusion (BHI) broth, some incongruous results were obtained. The aim of this study was to clarify the primarily negative results obtained with the GM1-ELISA. These discrepancies were shown to depend on the occurrence of inhibitory substances in the BHI broth and an immunological difference between enterotoxins of human and porcine origins.

(These results were presented in part at the International Pig Veterinary Society Congress, Copenhagen, 30 June to 3 July 1980.)

MATERIALS AND METHODS

Bacterial strains and cultivation. A total of 10 enterotoxigenic strains of E. coli were isolated from children with diarrhea (1), and 10 enterotoxigenic strains were isolated from piglets with neonatal diarrhea (13). The human strains carried the O antigens 6, 8, 25, 26, 78, 88, and 140. Three were nontypable. The porcine strains carried the O antigens 147 and 149. The strains were cultivated in a modified TY medium (10) containing 5 g of glucose per liter and in BHI broth (Difco Laboratories, Detroit, Mich.) in test tubes with 5 ml of medium at 37°C on a rotary shaker overnight. For higher yields of enterotoxin, some strains were cultivated in TY medium in 100-ml shake flasks overnight at 37°C.

Antiserum. Antiserum against heat-labile enterotoxin produced by a human strain (H-serum) was prepared by immunizing rabbits intramuscularly with a crude enterotoxin preparation from the human strain E. coli 411-5 carrying the O antigen 78 (2). Antiserum against a porcine strain (P-serum; kindly provided by O. Söderlind, National Veterinary Institute, Uppsala) was prepared by immunizing rabbits subcutaneously with a whole-cell lysate of the porcine strain E. coli Bd 853/67 carrying the O antigen 149 (14). Both H-serum and P-serum showed equal neutralization capacities when tested against homologous or heterologous en-
Enterotoxin in the adrenal cell assay.

**GM1-ELISA.** The test was performed as described previously (2, 16). Disposable polystyrene tubes (Heger Plastics, Stallarholmen, Sweden) were coated overnight with 0.2 ml of 1.5 μM ganglioside GM1 (Supelco, Bellefonte, Pa.) dissolved in 10 mM phosphate-buffered saline, pH 7.3. Remaining binding sites were blocked by incubation with 0.5 ml of 1% bovine serum albumin in phosphate-buffered saline for 30 min at 37°C. Bacterial supernatant (0.5 ml) was added to the coated tubes and incubated for 2 h at 22°C. Binding of *E. coli* heat-labile enterotoxin to ganglioside GM1 was visualized by the addition of 0.2 ml of H-serum (diluted 1:1000) or P-serum (diluted 1:200) in phosphate-buffered saline with 1% bovine serum albumin for 4 h, followed by the addition of 0.5 ml of enzyme-conjugated antibodies against rabbit gamma globulin and enzyme substrate as for an ordinary ELISA reaction (5). Alkaline phosphatase-conjugated antibodies (a gift from A. Lindberg, National Bacteriological Laboratory, Stockholm, Sweden) were diluted 1:500 before use in the assay. As the substrate, 1 ml of p-nitrophenylphosphate in 50 mM carbonate buffer, pH 9.6, was used. After 100 min of incubation at 37°C, the optical density of 400 nm was measured in a Hitachi 101 spectrophotometer. An optical density value of 0.2 above background was considered a positive result. As a negative control, tubes were incubated with phosphate-buffered saline.

**Adrenal cell assay.** (i) **Conventional assay.** Y1 adrenal cells, grown in microtiter trays, were incubated with 20-μl volumes of bacterial supernatant and 200 μl of cell medium at 37°C, 3% CO2, and 80% humidity for 24 h. A rounding up of 50% of the cells was considered a positive result (13).

(ii) **Toxin-binding assay.** After removal of the adrenal cell medium, 50-μl portions of bacterial supernatants were added to the wells. The cells were exposed to the toxin for 5 min after which the toxin preparations were poured off. The cells were incubated with fresh adrenal cell medium and read as for the conventional assay (1).

## RESULTS

**Enterotoxin titers by the GM1-ELISA.** Totals of 10 human and 10 porcine strains were cultivated in TY medium and tested in both assay systems. There was a clear-cut difference in the results obtained in the GM1-ELISA with the respective antisera (Fig. 1). When H-serum was used, all of the strains of human origin were positive, whereas all but one of the strains of porcine origin were negative. The opposite was the case when P-serum was used. All strains were positive in the adrenal cell test.

The relative titers of human and porcine enterotoxin preparations in the adrenal cell assay and the GM1-ELISA were determined for five human (O groups 8, 25, and 78) and five porcine (O group 149) strains cultivated in shake flasks overnight. The conventional and the toxin-binding assays in the adrenal cell test gave similar results for human and porcine *E. coli* enterotoxins (Table 1). The human strains yielded GM1-ELISA titers similar to adrenal cell titers only when H-serum was used. In contrast, the porcine strains yielded titers when P-serum, but not H-serum, was used in the GM1-ELISA, the enterotoxin of porcine origin yielding a median titer one to two dilution steps below adrenal cell titers (Table 1).

The GM1-ELISA technique was also used to test culture supernatants of all 20 strains cultivated in BHI broth. The strains were all negative in this assay when tested with either serum.

**Enterotoxin titers by the Y1 adrenal cell assay.** Strains cultivated in TY medium and BHI broth were also assayed for enterotoxin activity in the two adrenal cell assays. There was little difference in enterotoxin titers between assays with

![FIG. 1. Relative GM1-ELISA absorbance (A) values of culture supernatants of *E. coli* strains of human and porcine origin. The strains were cultivated in TY medium. H-serum, serum raised against a heat-labile enterotoxin preparation from a strain of human origin; P-serum, serum raised against a heat-labile enterotoxin preparation of porcine origin.](http://jcm.asm.org/)

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culture supernatants of both human and porcine strains grown in TY medium (Fig. 2A), but four human strains were negative in BHI broth. However, in the modified toxin-binding assay, several strains were negative when cultivated in BHI broth (Fig. 2B).

**Medium inhibitors.** To test whether inhibitory substance(s) was present in BHI broth, the supernatants of an overnight culture of a human strain *(E. coli* 411–5) and of a porcine strain *(E. coli* Bd 157/78) cultivated in shake flasks in TY medium were each mixed with equal volumes of freshly made BHI broth and TY medium. In the conventional assay there was no inhibition of either human or porcine *E. coli* enterotoxin (Fig. 3A). In contrast, when the toxin-binding assay was used, the addition of BHI broth to pre-

![Figure 2](http://jcm.asm.org/)

**FIG. 2.** Endpoint titers of culture supernatants of *E. coli* strains of human and porcine origin in the adrenal cell assay. Endpoint titers were defined as the highest dilution in a twofold serial dilution still giving a 50% rounding up of the adrenal cells. (A) Conventional assay. (B) Toxin-binding assay.

![Figure 3](http://jcm.asm.org/)

**FIG. 3.** Inhibition of preformed enterotoxin by BHI broth. Bacterial supernatants were preincubated for 15 min in adrenal cell medium (control [CONTR.]), TY medium, and BHI broth and measured in the adrenal cell assay. Human strains are represented by open bars, and porcine strains are represented by cross-hatched bars. (A) Conventional assay. (B) Toxin-binding assay.

**TABLE 1.** Median enterotoxin titers of five human and five porcine strains cultivated in shake flasks

<table>
<thead>
<tr>
<th>Enterotoxin assay</th>
<th>Median titers* of enterotoxin produced by:</th>
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<tbody>
<tr>
<td></td>
<td>Human strains</td>
</tr>
<tr>
<td>Y1—conventional assay</td>
<td>8 (4–32)b</td>
</tr>
<tr>
<td>Y1—toxin-binding assay</td>
<td>8 (2–32)</td>
</tr>
<tr>
<td>GM1-ELISA, H-serum</td>
<td>8 (&lt;1–32)</td>
</tr>
<tr>
<td>GM1-ELISA, P-serum</td>
<td>1 (&lt;1–16)</td>
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* Titers obtained by twofold serial dilution of bacterial supernatant.

* Parentheses indicate range.

**DISCUSSION**

Heat-labile enterotoxins of *E. coli*, whether the strains were isolated from humans or piglets, have until now been considered identical, although differences in immunological reactivity have been reported (11). There are minor differences of one or two amino acids in the primary structures of the enterotoxins of human and porcine origin (W. Dallas, California, personal communication). However, this difference appears to be of minor biological importance since the effect on adrenal cells is identical. Neverthe-
less, these differences might cause a conformational change in the tertiary structure of the protein, resulting in the observed immunological difference as indicated by the differences in GM1-ELISA reactivities. This also explains the negative results reported from other laboratories when the GM1-ELISA technique is used in combination with antiserum prepared against *E. coli* strains of human origin to detect enterotoxins of porcine origin (A. Lund, Oslo, and J. Holmgren, Gothenburg, personal communication).

When porcine strains are cultivated in shake flasks overnight, higher titers of enterotoxin are obtained than if they are cultivated in test tubes. When these enterotoxin preparations were tested in the GM1-ELISA with heterologous antiserum, positive results were obtained for some strains, indicating cross-reactivity (Table 1). However, when cultivated in test tubes for diagnostic purposes, it is important that homologous antiserum is used to avoid false negative results (Fig. 1).

Furthermore, it is important when screening for enterotoxigenicity to select a suitable nutrient medium. BHI broth was shown to inhibit heat-labile enterotoxin when tested in the GM1-ELISA as well as in the toxin-binding assay with adrenal cells. This phenomenon is probably due to the presence of gangliosides in BHI broth. The ganglioside GM1 is considered to be the receptor molecule for enterotoxin on the surface of the adrenal cell. If the binding between the ganglioside and toxin is weak, the toxin will be neutralized by free gangliosides in the medium when the toxin-binding assay or the GM1-ELISA is used. In the conventional adrenal cell assay with an incubation period of 20 h, an equilibrium is reached. In this equilibrium most toxin molecules become bound to the cells, since the cell surface exhibits a number of bound receptor molecules, each one anchoring one subunit of the toxin.

In conclusion, the present study indicates that negative results obtained in the GM1-ELISA may be due to inhibitory substances in the growth medium or to differences in immunological reactivity between enterotoxins of various sources.

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LITERATURE CITED


