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# Monoclonal Antibodies Against *Escherichia coli* Heat-Stable Toxin (STa) and Their Use in a Diagnostic ST Ganglioside GM1-Enzyme-Linked Immunosorbent Assay

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**Seven monoclonal antibodies (MAbs) against heat-stable enterotoxin (ST) from a human *Escherichia coli* isolate were prepared and evaluated for their usefulness in an ST immunodetection assay, the ST ganglioside GM1-enzyme-linked immunosorbent assay (ELISA). This assay is based on the ability of STa, as present in, for example, culture filtrates from ST-producing *E. coli*, to inhibit specific anti-ST antibody from binding to solid-phase-bound ST ganglioside (GM1-bound ST-cholera B subunit). Four of the MAbs were of immunoglobulin G1 (IgG1), one was of IgG2b, and two were of IgM isotype. All the IgG1 MAbs could be completely inhibited by addition of free ST; 0.2 to 0.4 ng of purified ST inhibited binding of these MAbs by 50%. The non-IgG1 MAbs were, in contrast, not inhibited by 200-fold-higher amounts of purified ST, probably because they were directed against linkage epitopes or were of low affinity or both. When the IgG1 MAbs were tested in the ST GM1-ELISA, ST could be detected in culture filtrates from stock human *E. coli* isolates with 100% sensitivity and specificity. ST in filtrates from fresh stool cultures was demonstrated with higher sensitivity with the MAb ST GM1-ELISA than with the conventional infant mouse test. Both subtypes of STa, STaI and STaII, could be detected by the ST GM1-ELISA by using either IgG1 MAb in the immunodetection step, whereas infant-mouse-active ST from *Yersinia enterocolitica* failed to react.**

Enterotoxin-producing *Escherichia coli* (ETEC) is an important cause of diarrheal disease both in humans and in animals (14, 15). The bacteria produce one or both of a heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST), which by their actions on small-intestine fluid transport processes are responsible for the diarrhea induced. LT is a complex, high-molecular-weight (~86,000) protein that is structurally and functionally closely related to cholera toxin (10); like cholera toxin, LT is also strongly immunogenic (10). In contrast, ST is a small polypeptide (molecular weight, <5,000) that is nonimmunogenic in its natural state; it can, however, be made immunogenic by coupling it to a carrier protein, e.g., serum albumin (6, 8). Two classes of ST which differ functionally and structurally were described. One is the methanol-soluble STa (STI) produced by human ETEC isolates and active in the suckling mouse assay (3, 7, 21). The other is the methanol-insoluble, infant-mouse-inactive STb (STII), which was originally found in and thought to be restricted to porcine ETEC strains but in exceptional cases has been found also in strains isolated from humans (3, 7, 27). DNA hybridization tests indicated that STa (STI) can be further subdivided into STaI (STIa or $ST_p$) and STaII (STIb or $ST_h$) that differ in their primary structure (16); human ETEC isolates usually produce STaII, although formation of STaI alone or in combination with STaII is also common (13, 16). Polyclonal antisera raised against STaII seem to react well with both STaII and STaI (26), but the possibility that subtype-specific epitopes exist cannot be excluded.

In many geographic areas, ETEC strains that produce ST either alone or with LT are common and may even predominate over strains that produce LT only (14). A number of useful immunoassays have been developed for detection of LT (for a review, see reference 12 and A.-M. Svennerholm, M. Wikström, L. Lindholm, C. Czerkinsky, and J. Holmgren, Proc. 20th U.S.-Japan Cholera Conf., 1984, in press). In contrast, only a few radioimmunoassays (6, 8) and some enzyme-linked immunosorbent assays (ELISAs) (11, 12, 20) for detection of ST have been described. Since the latter tests are based on access to highly specific polyclonal anti-ST sera, their use is still restricted to a few laboratories. There is a great need for immunodetection methods for ST (and LT) that could be widely used all over the world.

In a recent study, we introduced the ganglioside GM1-ELISA principle, which was originally developed for the demonstration of cholera toxin or LT (10, 22, 24), for the immunodetection of ST (23). In this ST GM1-ELISA, the inhibition of binding of specific anti-ST antibody to solid-phase ST is assessed. The solid-phase ST is obtained by first coupling ST covalently to the purified B subunit of cholera toxin (CTB) and then binding this conjugate to GM1 ganglioside (i.e., the receptor for CTB [10]) absorbed to ELISA microtiter wells. The rationale for using a GM1-ELISA method also for the detection of ST is that binding of ST to a solid phase via GM1 seemed to result in a considerably more efficient presentation of the toxin to subsequently added antibodies than did direct coating of plastic surfaces with free ST (23). Furthermore, it is possible that essentially the same method could be used for parallel detection of LT and ST.

We here describe the production and characterization of monoclonal antibodies (MAbs) against STa and evaluate their usefulness for the demonstration of ST in the ST GM1-ELISA method. We show that both STaI and STaII can be detected with high sensitivity and specificity by using selected MAbs. We propose that this MAb ST GM1-ELISA is suitable for the demonstration of STa from clinical ETEC isolates.

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## MATERIALS AND METHODS

**Bacterial strains.** A total of 95 *E. coli* strains with different enterotoxin profiles representing LT- only, LT- and ST-, ST-only, or non-ST- and non-LT-producing strains (kindly provided by Y. Takeda, Osaka University, Osaka, Japan) isolated from humans with diarrhea were used. All of the ST-producing strains produced STa, which is methanol soluble and active in the infant mouse model (7). Some 27 of the ST-producing strains used were analyzed by M. Lung, Hong Kong, for production of STaI or STaII by using ST probes; 12 of these strains produced STaI, and 15 produced STaII (Y. Takeda, personal communication). All the strains were kept in deep agar at room temperature or frozen in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 15% (wt/vol) glycerol at −70°C until used. Before being tested, the bacteria were inoculated onto blood agar plates to ascertain growth and purity. In addition, *E. coli* colonies from 31 fresh stool cultures on MacConkey agar, from 31 Bangladeshi patients with mild to moderate watery diarrhea, were tested (courtesy of D. A. Sack, International Center for Diarrhoeal Disease Research, Dacca, Bangladesh). Eight *Yersinia enterocolitica* serotype O:3 strains isolated at the Department of Clinical Bacteriology, University of Göteborg, from stools of patients with diarrhea were also tested.

**Cultivation of bacteria.** Culture filtrates of the 95 stock *E. coli* strains and the 31 fresh stool cultures were prepared by inoculating the bacteria from agar plates into 100-ml flasks containing 30 ml of a Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract medium (5) and then incubating the cultures at 37°C for 16 to 18 h with shaking (200 rpm). The *Y. enterocolitica* strains were also cultured in flasks with Casamino Acids-yeast extract medium but at 25°C for 48 h as described previously (17). After centrifugation of the cultures at 3,000 × *g* for 10 min, the supernatants were heated at 65°C for 30 min and frozen in equal portions until used.

**Ganglioside.** Purified ganglioside GM1 was purchased from Supelco Inc., Bellefonte, Pa., or kindly provided by L. Svennerholm, University of Göteborg.

**ST and ST conjugates.** ST was purified from an *E. coli* strain (64111) producing ST only that was isolated from a patient with diarrhea in Bangladesh and kindly provided by M. Merson, World Health Organization, Geneva, Switzerland. Purification was performed as described by Alderete and Robertson (2) with some modifications described previously (23). Approximately 150 μg of purified ST was obtained from each liter of culture filtrate by this procedure (23). The purified ST was coupled to bovine serum albumin (BSA) by the carbodiimide method (6, 12) or by using glutaraldehyde as a cross-linking reagent, as recently described by Lockwood and Robertson (12). ST was also coupled to purified CTB (provided by the Institut Mérieux, Lyon, France), by mixing 0.5 mg of purified ST with 25 mg of purified CTB in the presence of carbodiimide. The ST conjugates were frozen in portions at −70°C until used.

**Immunization of animals.** Inbred 8- to 14-week-old BALB/c mice of both sexes were given 3 to 4 injections with ST-BSA conjugate in doses corresponding to an initial immunization of 2 μg of purified ST, and the following injections corresponded to 1 μg. The initial immunization was given intraperitoneally with Freund complete adjuvant, and the following injections were given intravenously without adjuvant. The interval between the first and second immunizations was 6 weeks, and that between the remaining immunizations was 2 to 6 weeks. At 4 days after the last immunization, the animals were killed, the spleens were excised, and the spleen cells were prepared for production of MAbs.

**Preparation of MAbs.** The technique by De St. Groth and Scheidegger was followed (4). F 0 myeloma cells in Iscove medium containing 10% (vol/vol) fetal calf serum were propagated, and exponentially growing myeloma cells were fused with spleen cells from the immunized mice by using polyethylene glycol. Fused cells were distributed into microtiter plates, and stable hybrid cell lines were selected by growth in Iscove medium–10% fetal calf serum containing hypoxanthine-aminopterine-thymidine. At 10 days after fusion, culture fluids were tested for anti-ST antibodies by means of an ST GM1-ELISA (see below). All anti-ST-producing hybrids were expanded by cultivation in 2-ml-well plates, and stable antitoxin-producing hybrids were cloned and expanded by cultivation in 100 ml of Iscove medium–10% fetal calf serum in tissue culture bottles. Culture fluid from established antitoxin-secreting hybridomas was harvested and frozen in portions at −30°C until use. Some of the stable anti-ST-producing hybridomas were also injected into pristane-treated mice (~$5 \times 10^5$ hybrid cells per animal) for production of ascitic fluid (9); 3 to 5 ml of ascitic fluid was obtained from each animal.

**GM1-ELISA for detection of anti-ST antibodies.** The screening of culture fluid from cell hybrids for anti-ST was done by an ST GM1-ELISA (23). Polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va., or Nunc A/S, Copenhagen, Denmark) were coated with GM1 by incubating the plates with purified GM1 (1 μg/ml) diluted in phosphate-buffered saline (PBS) at room temperature overnight (0.1 ml per well). After the plates were washed twice in PBS, the remaining binding sites were blocked by incubation with a 0.1% BSA-PBS solution (0.2 ml per well) at 37°C for 30 min followed by repeated washing in PBS. All subsequent incubations were performed at room temperature with 0.1 ml per well and were followed by three washings in PBS containing 0.05% Tween. ST-CTB conjugate (approximately 500 ng of ST per ml) diluted in PBS was then added, and after incubation for 1 h, culture fluids were diluted 1:2 in PBS-Tween containing 0.1% BSA and allowed to react for 1 h. After incubation for 2 h with anti-mouse immunoglobulin-horseradish peroxidase conjugate (Dakopatts, Copenhagen, Denmark) diluted 1:500 in PBS-Tween-BSA, the reactions were visualized by the addition of orthophenylenediamine-$H_2O_2$ (prepared by dissolving 10 mg of orthophenylenediamine in 10 ml of 0.1 M sodium citrate buffer [pH 4.5], to which was added 4 μl of 30% $H_2O_2$); after 20 min, the plates were read by eye or spectrophotometrically at 450 nm. An $A_{450}$ of ≥0.1 above the background level was defined as positive; an $A_{450}$ of 0.1 always exceeded three standard deviations of the mean for the background. The titration of MAbs in culture fluids was done with the same method by testing fivefold serial dilutions of the sample; the anti-ST titer was determined as the interpolated dilution giving an $A_{450}$ of 0.2 above the background.

**GM1-ELISA for detection of ST.** ST in culture filtrates or in clinical specimens was determined by its ability to inhibit binding of anti-ST antibody to the GM1-bound ST-CTB conjugates (see above). The specimens or dilutions thereof were added in 50-μl volumes to the ST-CTB-coated plates, followed by the addition of anti-ST MAb diluted in PBS-BSA to a concentration corresponding to ~20 times the anti-ST ELISA titer (50 μl per well). In each plate, culture filtrates from an ST-positive and an ST-negative control strain as well as fivefold dilutions of an ST standard (1 μg of ST per

ml) were similarly incubated with anti-ST MAb. After incubation at room temperature for 1 h, binding of anti-ST antibody was assayed as described for determination of anti-ST antibody. The concentration of ST was determined by comparing the dilution of specimens with that of the known ST standard which inhibited antibody binding to solid-phase-bound ST-CTB by 50%. Culture filtrates of *E. coli* strains giving ≥50% inhibition of the binding of diluted anti-ST MAb to GM1-bound ST-CTB were regarded as positive for ST.

**Infant mouse test.** The infant mouse test was performed as previously described (7). A gut/body ratio of ≥0.085 was considered a positive result.

**Determination of MAb isotype and concentration.** The isotypes of the MAbs were determined by single radial immunodiffusion or double diffusion-in-gel analyses (18) with mouse immunoglobulin-specific antisera (immunoglobulin G1 [IgG1], IgG2b, IgG3, IgA, and IgM; Meloy Laboratories, Inc., Springfield, Va.). The immunoglobulin concentration was determined by single radial immunodiffusion by assaying appropriate dilutions of the MAb preparations in isotype-specific anti-mouse immunoglobulin using preparations of the respective immunoglobulin isotype (Meloy) with known immunoglobulin concentrations as standards.

## RESULTS

In three different cell fusion experiments, 30 hybridomas which produced significant levels of anti-ST antibodies were identified. Seven of these hybridomas, which after expansion and cloning continued to produce high quantities of anti-ST antibodies, were used for large-scale production of MAbs in tissue culture or ascites form or both. The isotypes and concentrations of the different MAbs were determined by double diffusion-in-gel and single radial immunodiffusion tests. One of the clones, ST-1:3, gave rise to more than 20 mg of IgG1 per ml in ascitic fluid (Table 1). The specific anti-ST titer in culture fluid or ascitic fluid, as determined by the ST GM1-ELISA, in general corresponded well to the total immunoglobulin concentration of the respective MAb (Table 1).

The possibility of inhibiting the binding of different anti-ST MAbs to GM1-bound ST-CTB by free ST was studied. It was found that all four IgG1 MAbs could be inhibited by free ST in the ST GM1-ELISA (Fig. 1). When the MAbs were diluted to 20 times the ST GM1-ELISA titer, the inhibitory concentration was 4 to 7 ng/ml, or 0.2 to 0.4 ng of purified ST. (Fig. 1). The MICs of ST were considerably lower if the anti-ST MAbs tested were more dilute or if less inhibition, e.g., 25%, of the antibody to the solid phase was determined. Purified ST or ST in culture filtrates in concentrations up to 1 μg of ST per ml had no inhibitory effect on the two IgM or the IgG2b anti-ST MAbs. Carbodiimide-coupled ST-BSA conjugate, on the other hand, effectively inhibited binding of ST-2:8 to the solid-phase-bound ST, whereas neither carbodiimide- nor glutaraldehyde-coupled ST-BSA inhibited binding of ST-4:4.

TABLE 1. Presentation of anti-ST MAbs used

| MAb[a] | Isotype | Production and detection in: | | | |
|---|---|---|---|---|---|
| | | Culture fluid | | Ascitic fluid | |
| | | Immunoglobulin concn (mg/liter) | ELISA titer | Immunoglobulin concn (mg/liter) | ELISA titer |
| ST-1:3 | IgG1 | 68 | 1/4,000 | 22,500 | 1/(2 × 10⁶) |
| ST-2:8 | IgG2 | 6 | 1/125 | 610 | 1/25,000 |
| ST-3:5 | IgG1 | <3 | 1/125 | 2,500 | 1/50,000 |
| ST-4:4 | IgM | 34 | 1/500 | ND[b] | |
| ST-7:1 | IgM | 30 | 1/500 | ND | |
| ST-26:5 | IgG1 | 55 | 1/8,000 | 5,500 | 1/(2 × 10⁶) |
| ST-27:3 | IgG1 | 120 | 1/16,000 | 11,400 | 1/(4 × 10⁶) |

[a] ST-1 to ST-4 were obtained after immunization with carbodiimide-coupled ST-BSA (6), and the remaining MAbs were obtained after immunization with glutaraldehyde-coupled ST-BSA (12).

[b] ND, Not done.

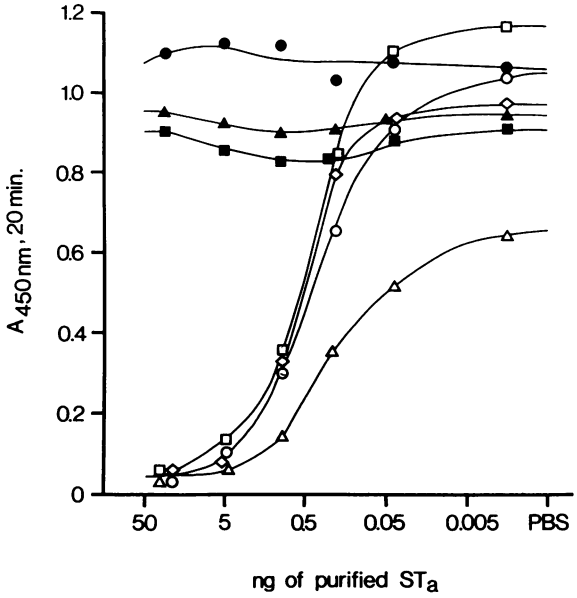


FIG. 1. Inhibition of binding of the different anti-ST MAbs to GM1-bound ST-CTB by graded doses of purified ST. Symbols: ◇, ST-1:3; △, ST-3:5; ○, ST-26:5; □, ST-27:3; ●, ST-2:8; ▲, ST-4:4; ■, ST-7:1.

The possibility of using some of these MAbs in the ST GM1-ELISA for the demonstration of ST production from clinical isolates was evaluated. In an initial experiment, 62 stock *E. coli* strains originally isolated from human feces, 31 of which were previously shown to produce ST, were tested for ST production by using different MAb preparations at final concentrations corresponding to 20 times the GM1-ELISA titer against ST. Culture filtrates of these stock strains were tested in a coded fashion. All filtrates of *E. coli* strains previously shown to produce ST were positive in the ST GM1-ELISA, i.e., they inhibited antibody binding by ≥50% when either tissue-cultured ST-1:3, ST-1:3 cultured in ascitic fluid, or tissue-cultured ST-3:5 MAb was used (Fig. 2). In no instance did filtrates from strains previously reported to produce LT only or to be both non-LT and non-ST producing give a positive result with any of the three MAb preparations tested (Fig. 2).

In continued experiments, culture filtrates of fresh *E. coli* isolates were tested in parallel by the ST GM1-ELISA and the infant mouse test. Of 31 *E. coli* strains isolated from the stools of 31 Bangladeshi patients with watery diarrhea, 19 strains were positive in the ST GM1-ELISA, and 16 of these were also positive in infant mice. The three strains that were positive in the ST GM1-ELISA but negative in the mice gave positive results in the latter test after the culture filtrates were concentrated two- to threefold. Similarly, concentrated culture filtrates of ST-negative *E.coli* control strains did not give positive results either in the ST GM1-ELISA or in the

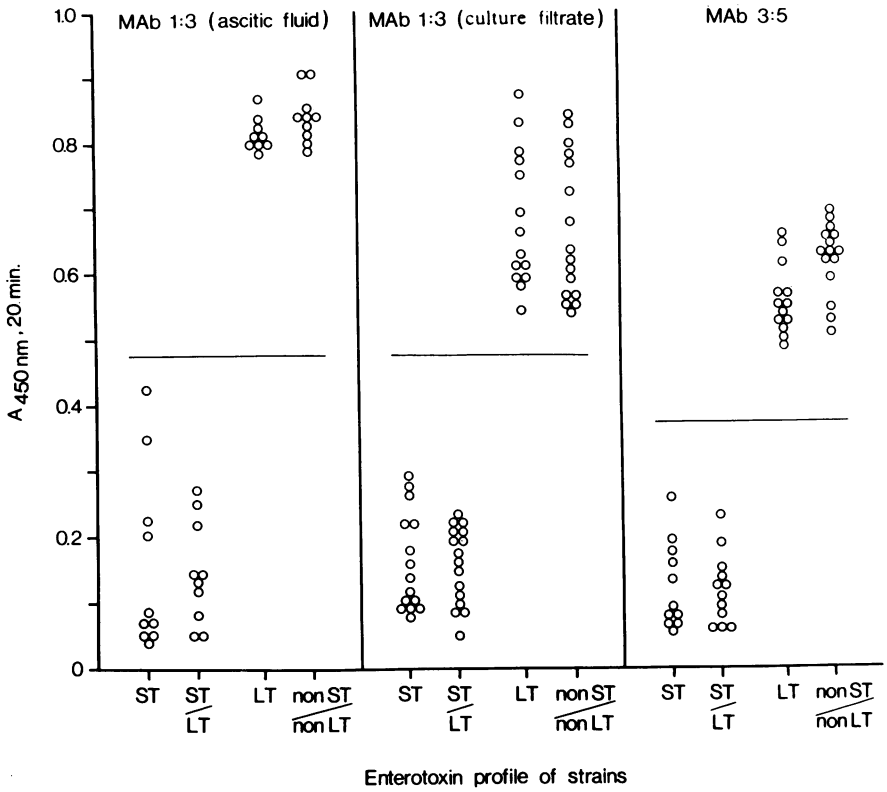


FIG. 2. Inhibition of binding of ST-1:3 ascitic fluid (diluted 1/50,000), ST-1:3 culture fluid (1/100), and ST-3:5 culture fluid (1/5) to solid-phase-bound ST by culture filtrates of 62 human fecal *E. coli* isolates with different enterotoxin profiles. Horizontal lines indicate the $A_{450}$ corresponding to 50% inhibition of antibody binding.

infant mouse test. None of the culture filtrates tested gave a positive result only in the infant mouse test.

To evaluate whether the ST GM1-ELISA could detect the two subtypes of STa when MAbs were used in the immunodetection step, 27 *E. coli* strains previously shown to produce STaI or STaII were evaluated with a number of positive and negative control strains in the ST GM1-ELISA. All except one STaI-producing strain gave positive results in the ST GM1-ELISA when either ST-1:3 or ST-3:5 was used (Table 2). The strain previously reported to produce STaI that was negative in the GM1-ELISA was also negative in the infant mouse test even when concentrated culture filtrate was tested.

Because *Y. enterocolitica* was found to produce an infant-mouse-active ST with structural, functional, and possibly also immunological similarities to *E. coli* STa (17, 25), culture filtrates of eight *Y. enterocolitica* strains were tested in parallel by using the infant mouse assay and the ST GM1-ELISA. Whereas five of the strains gave positive results for ST in infant mice, none of the eight culture filtrates gave any detectable inhibition of the binding of MAbs ST-1:3, ST-3:5, or ST-27:3 to the solid-phase ST. Neither were rabbit or mouse polyclonal antisera raised against the same ST-BSA conjugates used for production of the anti-ST MAbs (23) inhibited by any of the *Y. enterocolitica* culture filtrates in the ST GM1-ELISA.

## DISCUSSION

In this report, we characterized different anti-ST MAbs that may be useful in immunodiagnostic tests, especially the ST GM1-ELISA method (23). Development of ST immunodetection methods has previously been hampered by difficulties in producing homogeneous, high-titer anti-ST antibody preparations. Recently, the possibility of producing polyclonal antisera as well as MAbs against STa after immunization with different ST-protein conjugates was reported (6, 8). The advantage of using MAbs as immunoreagents is that they are homogeneous and may be produced in practically unlimited quantities. However, the high specificity of MAbs may result in the failure to bind to STs which differ in amino acid composition from the ST used for immunization. Furthermore, MAbs often have lower affinity than polyclonal antisera.

The MAb-based ST GM1-ELISA should be relatively easy to introduce in diagnostic laboratories, since the technology and most of the reagents are similar to those used for the demonstration of LT (A.-M. Svennerholm et al., in press). The coupling of ST to a solid phase via CTB and GM1 seems to result in particularly efficient presentation of ST for reacting with anti-ST antibodies (23). In contrast, our attempts to coat plastic surfaces directly with native ST for subsequent reaction with antibody failed, as also reported by Lockwood and Robertson (12), probably due to poor exposure of the small and very hydrophobic ST molecule on the plastic surface.

Of practical importance for the potential use of a particular MAb in competitive ST detection methods such as the ST GM1-ELISA is that it can be effectively inhibited by free ST from binding to solid-phase-bound ST. All of the IgG1 anti-ST MAbs had this property, although the MICs of ST were higher than those for the corresponding polyclonal

TABLE 2. Demonstration of STaI and STaII by different MAbs in the ST GM1-ELISA

| Reported enterotoxin production | Frequency of positive strains with MAb: | |
|---|---|---|
| | ST-1:3 | ST-3:5 |
| STaII | 6/7[a] | 6/7 |
| STaII and LT | 5/5 | 5/5 |
| STaI | 8/8 | 8/8 |
| STaI and LT | 7/7 | 7/7 |
| Non-ST | 0/6 | 0/6 |

[a] The one negative strain also failed to produce ST as tested by the infant mouse assay.

antisera, i.e., immune sera from the same animals as those used for the production of MAbs (23). This difference probably reflects a generally lower affinity of MAbs than of polyclonal antisera for binding to a specific antigen (9). Nonetheless, the IgG1-MAb-based ST GM1-ELISA identified ST in all except one culture filtrate of strains previously found to be ST producing. In a few instances, the MAb ST GM1-ELISA even detected ST production from fresh *E. coli* isolates that gave negative results in the infant mouse test. This difference was not due to false-positive reactions with the ST GM1-ELISA but reflected the higher sensitivity of this method. Thus, the infant-mouse-negative but ELISA-positive strains also gave positive results in mouse intestine after a two- to threefold concentration of the culture filtrates.

Although differences in amino acid composition have been reported between STaI and STaII (1, 16), both subtypes of STa could be detected by the MAbs used in the diagnostic ST GM1-ELISA test. In general, culture filtrates of STaII-producing strains were more effective in inhibiting the binding of the MAbs to solid-phase-bound ST than were filtrates of STaII-producing strains. We cannot tell, however, whether this difference is due to the structural differences between the two STa subtypes or to a generally higher ST production during in vitro culture by the STaII than by the STaI strains. Although the primary structures of STs produced by *Y. enterocolitica* and ETEC were reported to be very similar, at least in the C-terminal sequences of the two toxins (25), infant-mouse-positive culture filtrates of *Y. enterocolitica* strains failed to give positive results in the ST GM1-ELISA with either of the anti-ST MAbs. One possibility for this failure of *Y. enterocolitica* ST to inhibit the binding of MAbs raised against *E. coli* STa is that these MAbs were directed against epitopes located further to the $NH_2$-terminal sequence of the molecule. Alternatively, the anti-ST MAbs may have been directed against conformational determinants that are specific for *E. coli* ST.

In conclusion, MAbs reacting with both subtypes of STa were produced. When these MAbs were used in a competitive ST GM1-ELISA, highly specific and sensitive immunodetection of ST in culture filtrates of clinical isolates was achieved. The reagents needed for the MAb ST GM1-ELISA could be made easily available except for the ST-CTB conjugate. At present, ST-CTB is produced by coupling purified ST to the isolated B subunit of cholera toxin by the aid of carbodiimide, which has proved to be a simple and reproducible coupling procedure. Alternative approaches being tested are the use of synthetic ST or shorter synthetic peptides and chimeric ST-LT-B proteins achieved by gene fusions.

Our previously described LT GM1-ELISA was recently adapted for culturing bacteria directly in the GM1-coated plates and releasing the periplasmically located LT (19; A.-M. Svennerholm et al., in press). By transferring the overnight cultures from the GM1-coated wells to a new plate containing GM1-bound ST-CTB, parallel detection of ST and LT from the same *E. coli* colony could be achieved within 18 h after obtaining the clinical isolate. This combined LT-ST GM1-ELISA method, which is currently under clinical evaluation, promises to be a practical approach to the demonstration of enterotoxigenic *E. coli* isolates both in more advanced diagnostic units and in field laboratories.

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