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Recombinant Fusion Protein for Simple Detection of *Escherichia coli* Heat-Stable Enterotoxin by GM1 Enzyme-Linked Immunosorbent Assay

JOAQUIN SANCHEZ,[1]* JAN HOLMGREN,[2] AND ANN-MARI SVENNERHOLM[2]

Center for Research on Infectious Diseases, National Public Health Institute, Apdo Postal 222, Cuernavaca, Morelos, 62020 Mexico,[1] and Department of Medical Microbiology and Immunology, University of Göteborg, S-413 46 Göteborg, Sweden[2]

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A recombinant gene fusion protein composed of an *Escherichia coli* heat-stable enterotoxin (STa) peptide epitope fused to the amino end of the cholera toxin B subunit was used to detect STa produced by clinical isolates of enterotoxigenic *E. coli* (STa-ETEC) by a single monoclonal antibody-based inhibition GM1 enzyme-linked immunosorbent assay. In this test, 100% sensitivity and 100% specificity were observed for use of the recombinant protein in either its purified form or as crude *Vibrio cholerae* culture supernatants in detection of STa-ETEC.

Escherichia coli capable of producing enterotoxins is a major cause of diarrhea in children in developing countries and in visitors to those areas (1). Similar enterotoxigenic *E. coli* (ETEC) bacteria also cause diarrheal disease in young farm animals (12). Among the enterotoxins produced by ETEC are the so-called heat-labile (LT) and heat-stable (ST) enterotoxins (2). While useful in vitro detection methods for LT have been available for some time (15), detection of STa (an *E. coli* heat-stable enterotoxin) has predominantly been done by intragastric inoculation in infant mice (5). However, this technique has been limited to a few laboratories because it requires animal facilities and trained personnel. More recently, alternative procedures have been developed (4, 6, 8, 9), including a simple GM1 enzyme-linked immunosorbent assay (ELISA) based on inhibition of binding of anti-STa monoclonal antibodies to STa chemically coupled to the B subunits of cholera toxin (CTB) or LT (LTB) (16, 18). The limiting reagent in this latter assay has been the preparation of reproducible conjugates between STa (native or synthetic) and purified CTB or LTB. Therefore, to facilitate the use of the STa-GM1 inhibition ELISA, we here tried to substitute the STa-LTB with a recombinant protein carrying an STa-related decapeptide (Cys-Ala-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys) at the amino end of CTB (13, 14). This plasmid-encoded decapeptide-CTB protein has been previously shown to be able to oligomerize, to be excreted in high concentrations by *Vibrio cholerae*, to bind strongly to GM1, and to be recognized efficiently by anti-STa antibodies (14).

MATERIALS AND METHODS

Chemical conjugates between purified native STa to CTB (16) or synthetic STa to LTB were prepared as previously described (17). Recombinant decapeptide CTB-protein was prepared from *V. cholerae* JS1569 (13), a derivative of the nontoxigenic strain CVD103 (7), which carried plasmid pJS8 that encodes the decapeptide-CTB protein under the control of the inducible *tacP* promoter (14). The bacterial strain was grown in LB broth (10) with continuous shaking at 30°C in the presence of 100 μg of ampicillin per ml and 25 μg of rifampin per ml until the culture reached an optical density of 1.0 at 660 nm. At this point, the *tac* inducer isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 50 μg/ml and growth continued under the same conditions for an additional 4 h. Cells and culture supernatants were separated by centrifugation, and the supernatants were treated for purification of the decapeptide-CTB protein by affinity chromatography. Alternatively, samples were processed to give semicrude recombinant protein by selective hexametaphosphate precipitation as previously described (11).

For affinity chromatography purification of the decapeptide-CTB protein, culture supernatants from 2-liter cultures of strain JS1569(pJS8) were adjusted to pH 7.0, and NaCl and phosphate concentrations were brought to 50 and 10 mM, respectively. Adjusted samples were passed through a Spherosil-GM1 column (19) of 2-cm diameter and 2- to 3-cm bed height at a flow rate of 250 ml/h. Unbound material was removed by exhaustive washing with phosphate-buffered saline (PBS) (pH 7.0). Bound decapeptide-CTB protein was released with 50 mM citrate buffer (pH 2.8). Aliquots (5 ml) were collected and quickly neutralized with either 3 M K_2HPO_4 or 1 M Tris (pH 8.0). Aliquots whose A_{280} was greater than 0.1 were pooled and dialyzed overnight against PBS in the cold. Dialyzed samples were concentrated by ultrafiltration on Amicon (Danvers, Mass.) YM10 membranes to give a protein concentration between 1 and 5 mg/ml. Concentrated material was stored frozen at −20°C until used.

For comparisons, hexametaphosphate or affinity-purified decapeptide-CTB recombinant protein were also stored in lyophilized form at 4°C or at room temperature for periods equal to or longer than 6 months. Culture supernatants without any purification were also stored frozen at −20°C.

For GM1 ELISA inhibition tests, microtiter plates were coated with GM1 and washed as previously described (19). Decapeptide-CTB recombinant protein (or STa-CTB or STa-LTB conjugate) were added in PBS, and the plates were incubated at room temperature for 1 h. Plates were subsequently washed, and a 0.1% (wt/vol) solution of bovine serum albumin in PBS was added to block unreacted sites. Culture supernatants and anti-STa monoclonal antibody

* Corresponding author.

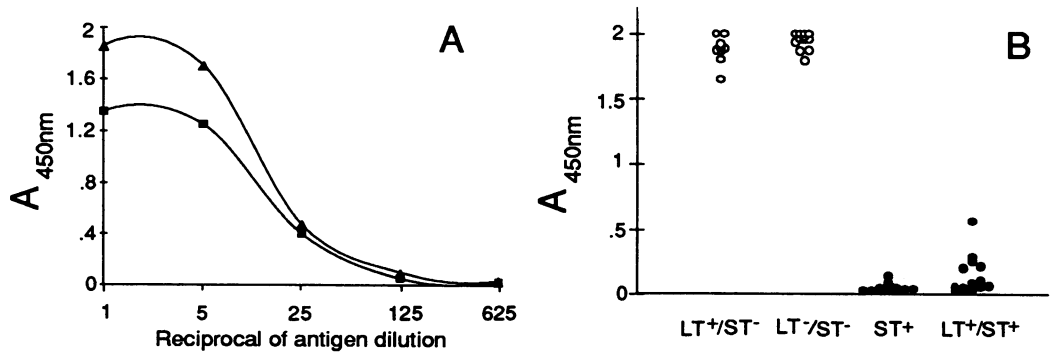

FIG. 1. (A) Titration of affinity chromatography-purified decapeptide-CTB recombinant protein (▲) and chemically coupled native STa-CTB protein (■). The proteins were bound to GM1 plates as described in the text. Initial concentrations of decapeptide-CTB and STa-CTB were 26 and 7 μg/ml, respectively; the ST 1:3 monoclonal antibody was diluted 1:800. Absorbance values were obtained with an ELISA reader. (B) Test for specificity of the ELISA on culture supernatants of clinical ETEC isolates using purified recombinant decapeptide-CTB protein. In this assay, GM1 plates were coated with the recombinant protein to be reacted with anti-STa monoclonal antibody 1:3 (as in panel A) in the presence of the bacterial cultures to be tested. Cultures having STa inhibited binding of 1:3 monoclonal antibody to the decapeptide-CTB, producing a decrease in absorbance. Thus samples giving low absorbances are considered to contain STa (●), while those giving high absorbance values are deemed to lack STa (○). ETEC profiles are shown on the *x* axis.

were then combined on the plates and reacted for 1 h at room temperature, followed by washing and addition of goat anti-mouse immunoglobulin G-peroxidase conjugate (Jackson Biological Laboratory, West Grove, Pa.). Plates were finally developed with H_2O_2-*ortho*-phenylene diamine substrate and read at 450 nm in an ELISA reader. Samples resulting in at least 50% inhibition of the absorbance values in comparison with a negative control (a culture filtrate from a known STa-negative *E. coli*) were regarded as STa positive. Optimal coating concentrations of decapeptide-CTB were determined by using the affinity chromatography-purified protein and constant concentrations of anti-STa monoclonal antibody (MAb) (Fig. 1A). Upon titration, a coating concentration of 1.5 to 2 μg of the purified decapeptide-CTB protein per ml in combination with a 1:300 dilution of culture medium of monoclonal antibody 1:3 (18) was deemed optimal.

RESULTS AND DISCUSSION

Detection of STa production by clinical isolates of *E. coli* using the decapeptide-CTB as a solid-phase antigen was possible either in culture supernatants of strains grown in flasks (Fig. 1B) or directly in GM1-coated ELISA plates for concomitant LT immunoassay detection (data not shown). This was examined by using ETEC clinical isolates with a previously determined enterotoxin profile (kindly provided by Y. Takeda, Tokyo, Japan) which had been grown in Casamino Acids yeast extract broth (3) at 37°C overnight with continuous shaking. ELISA detection of STa in clinical isolates correlated completely with the assigned phenotypes. The results show that the recombinant decapeptide-CTB protein acted as an excellent substitute for chemically prepared STa-LTB or STa-CTB proteins in the GM1 ELISA. Strains producing STa caused marked inhibition of binding of the STa-specific antibody to the coating decapeptide-CTB (as crude *V. cholerae* culture supernatant) in contrast to STa-negative strains (Fig. 1B). Moreover, results obtained using the chemically coupled STa-LTB (Fig. 2A) or STa-CTB (Fig. 2B) are virtually identical to those with the lyophilized *V. cholerae* crude culture supernatants (Fig. 2C). The use of culture supernatants as the source of the coating decapeptide-CTB may be particularly useful, especially in places where technical facilities are limited. Each liter of culture contains 5 to 6 mg of decapeptide-CTB (14), thus providing with material for coating approximately 250 to 300 ELISA plates. For convenience, crude supernatants may be sterilized, by filtration through membranes with a low protein-binding capacity, before being stored frozen or lyophilized until needed, without affecting the reactivity of the decapeptide-CTB protein.

The creation of CTB-derived recombinant fusion proteins for developing diagnostic tests as described in this study has several characteristics that may help make these tests more generally applicable. As with construction of other fusion proteins using oligonucleotide sequences, cloning provides the desired antigenic sequences attached to a carrier protein. Of distinctive importance is the fact that the recombinant protein preserves the ability inherent to CTB to recognize the GM1 receptor. Thus, reaction of the fusion protein in crude extracts or culture supernatants with GM1-coated

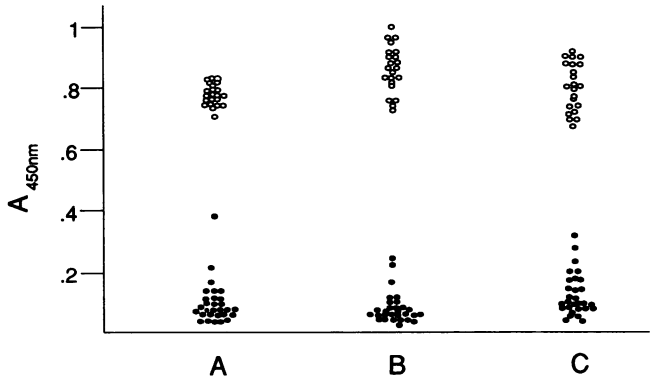

FIG. 2. STa detection by GM1 inhibition ELISA, as shown in Fig. 1B, in 30 STa-positive and 22 STa-negative ETEC clinical isolates. For the inhibition assay, GM1 plates were coated with either chemically coupled synthetic STa-LTB (A), chemically coupled native STa-CTB (B), or with a *V. cholerae* culture supernatant with the decapeptide-CTB which had been subjected to lyophilization and storage (C). Absorbance values were obtained by using an ELISA reader. Samples were either identified as STa positive (●) or STa negative (○).

plates will provide the CTB-derived protein essentially as a pure coating antigen for ELISA. In this regard, it is a notable advantage that expression of the CTB-derived proteins in *V. cholerae* hosts leads to their active secretion into the cell culture supernatant (14). This is most helpful when purification of the fusion proteins which can be carried out either by affinity chromatography, as described in this study, or by selective hexametaphosphate salting out (11) is desired.

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