A COMPETITIVE IMMUNOSORBENT ASSAY FOR THE DETECTION OF HEAT-STABLE ENTEROTOXIN OF *ESCHERICHIA COLI*

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SUMMARY. A competitive ELISA procedure for the detection of *Escherichia coli* heat stable enterotoxin (ST) with monoclonal antibody has been developed. This test is 10 times more sensitive than the suckling-mouse bioassay and it is specific, simple and cheap. A set of 882 strains of *E. coli* isolated from man were tested both by ST-ELISA and suckling-mouse bioassay, the latter serving as the reference method. Positive results in both tests were obtained with 152 strains. The remaining strains gave negative results in both tests, with the exception of two strains, known to be ST producers, that gave negative results in the suckling-mouse assay, but gave positive results by the ELISA method.

INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* implicated in diarrhoeal disease of man and domestic animals produce a heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST). Two subgroups of ST have been described (Burgess *et al.*, 1978; Olsson and Soderlind, 1980): STa or STI is methanol-soluble and has biological activity in suckling mice and piglets; and STb or STII, which is methanol-insoluble and has secretory activity only in piglets. The role of STb in pathogenesis has not been established. At least two genetically distinct STa (STI) enterotoxins have been described (Moseley *et al.*, 1980; So and McCarthy, 1980; Moseley *et al.*, 1983): STp is produced by porcine, bovine and human strains and STh is produced only by human strains. STp and STh are also labelled as STIa and STIb respectively. Both STp (STIa) and STh (STIb) have been purified and their amino-acid sequences are known (Aimoto *et al.*, 1982; Takao *et al.*, 1983).

In contrast to the experience with LT, many attempts in the past to raise antibodies against ST in animals by injection of a highly purified form or a crude extract were

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unsuccessful (Sack, 1975; Alderete and Robertson, 1978). Only recently, it has been shown that purified ST can be rendered weakly antigenic if coupled to bovine serum albumin (Frantz and Robertson, 1981), or bovine immunoglobulin G (Giannella *et al.*, 1981), or if it is polymerised by treatment with either glutaraldehyde (Van Wijnendaele *et al.*, 1982) or toluene-2,4-diisocyanate (Takeda *et al.*, 1981). The availability of ST antibodies enabled several authors to develop either radioimmunoassays (Frantz and Robertson, 1981; Giannella *et al.*, 1982) or enzyme-linked immunosorbent assays (ELISA) (Van Wijnendaele, personal communication; De Mol *et al.*, 1983). These developments encouraged us to produce anti-ST monoclonal antibodies (Hemelhof *et al.*, 1984). Recently, two other ELISA procedures were described and one of them (Thompson *et al.*, 1984) that made use of monoclonal antibodies was technically similar to the system that we reported previously (De Mol *et al.*, 1983). The other system (Klipstein *et al.*, 1984) is based on a double sandwich technique.

Presently available bioassays for ST, including the suckling-mouse bioassay, are cumbersome and time-consuming but they are the only tests for ST detection used routinely. A simple, rapid immunological assay for ST would facilitate diagnostic work and epidemiological investigations. In this paper we describe and evaluate the ST-ELISA system that we based on our development of a monoclonal anti-STh antibody.

MATERIAL AND METHODS

Escherichia coli strains. The 882 strains used in this study were from three different sources: (a) 200 strains used in a multilaboratory evaluation by the Diarrhoeal Diseases Control Programme of the World Health Organization were isolated at the Osaka Airport Quarantine Station from patients recently returned from South East Asia with traveller's diarrhoea; they were tested blindly in parallel by ST-ELISA and by the suckling-mouse assay; (b) 237 isolates were collected in Brussels during a routine survey of enterotoxigenic *E. coli* (ETEC) in liquid stools; (c) 445 isolates were isolated from diarrhoeic and non-diarrhoeic patients in Rwanda, Africa, during an epidemiological study of enteropathogenic agents. Strains were kept on Dorset agar slants at room temperature until used.

Bacterial cultivation. A loopful of stock culture was inoculated into a 100-ml Erlenmeyer flask containing 10 ml of 3% Trypticase Soy Broth (TSB, bio Mérieux, Marcy l'Etoile 69260, Charbonnières-les-Bains, France). The strains were grown aerobically for 18 h at 37°C while shaking in a Gallenkamp orbital incubator (200 rpm). After incubation, the cultures were centrifuged at 8000 *g* for 20 min, and the supernates were assayed that day or stored at -20°C for no longer than 1 week before testing.

Preparation of STh and monoclonal anti-STh antiserum. STh was purified by reverse-phase high-performance liquid chromatography (Aimoto *et al.*, 1982) and polymerised by glutaraldehyde (Van Wijnendaele *et al.*, 1982). Female Balb/c mice (6 weeks old) received a priming intraperitoneal dose of the polymerised STh (equivalent to 50 µg of pure STh) emulsified with complete Freund's adjuvant. After 8 weeks, each mouse received two further doses of 50-µg equivalent of polymerised STh intraperitoneally, 3 and 2 days before they were killed. Spleen cells were fused by a modification of the polyethyleneglycol method of Kohler and Milstein (1975) with Sp 2/0 myeloma cells. Anti-STh in culture fluids of fused cells was sought by enzyme-linked immunosorbent assay (ELISA). Specific antibody-producing hybrids were cloned by the limiting-dilution method. Large-scale production of anti-STh was done by intraperitoneal injection of 10⁵ hybridoma cells into female Balb/c mice (6 weeks old) after priming with 0.5 ml of Pristane (2,6,10,14-tetramethylpentadecane; Janssen Chimica, Beerse, Belgium). Ascitic fluid was taken 7–10 days after injection of the hybridoma cells. The properties of this antibody preparation will be described elsewhere. It neutralises both STh and STp in the

suckling-mouse bioassay with a potency about 10 times greater than that of conventionally prepared anti-STh and anti-STp antisera.

ST-ELISA procedure. ST was estimated by measuring the extent of inhibition of binding of monoclonal anti-ST antibodies to an excess of solid-phase, fixed, purified STh. The wells of a polystyrene 96-well microtitration plate (Nunc Immunoplate I B.P., 280, Kamstreep, DK-4000 Roskilde, Denmark) were coated overnight at 4°C with 100 µl of carbonate-bicarbonate buffer (pH 9.6) containing purified STh (2.5 µg/ml). The plates were then washed three times with 0.1 M phosphate-buffered saline containing 0.01 M sodium phosphate and 0.1 M NaCl (PBS; pH 7.2) and 0.05% v/v of polyoxyethylene sorbitan monolaurate (Tween 20, U.S. Biochem. Corp., P.O.B. 22400, Cleveland, OH, 44122, USA). Plates were either used immediately or stored at -20°C for up to 1 month. For the test, 50 µl of test supernate was added to each well (two wells for one test), and immediately thereafter 50 µl of monoclonal anti-STh (optimum dilution determined by checkerboard titration) was added. The plates were gently shaken to mix and then incubated for 1 h at 37°C. After another washing procedure, 100 µl of peroxidase-labelled rabbit anti-mouse conjugate (Miles Lab Ltd, POB 37, Stoke Poges, Slough, SL2 4LY) (optimum dilution determined by checkerboard titration) was added to each well and the plates were incubated for 1 h at 37°C. After a final washing procedure, 100 µl of a substrate solution containing 9 parts of 5-aminosalicylic acid (Sigma) 0.08% v/v in distilled water (pH 6.0) and 1 part of H₂O₂ 0.05% v/v was added to each of the wells; the plates were incubated at room temperature for 30 min and thereafter placed at 4°C for another 30 min. Optical densities (OD) from clear (positive test) to dark brown (negative test) were read visually by two different technicians and measured with a spectrophotometer (Virion Reader, Institute Virion Ltd, Weingartenstr 9, CH-8803 Ruschlikon, Switzerland) at 450 nm. The net OD was calculated by subtracting the OD of the background control wells from that of the corresponding test well. The results were expressed as a percentage of the reading for a negative control (percentage inhibition of the extinction value). Reactions showing an inhibition of 30% or more of the negative control were considered to be positive.

The ELISA procedure was also performed with conventional antiserum produced in guinea pigs by immunisation with polymerised STh.

Suckling-mouse assay. The suckling-mouse assay was done as previously described (Dean *et al.*, 1972). Each supernate was tested in three mice. A ratio of gut weight to remaining body weight (G/B) of <0.080 was considered to be a negative result; ratios in the range 0.080-0.095 were considered to be questionably positive results and performed again; and ratios >0.095 were considered to be positive.

RESULTS AND DISCUSSION

The titration plot for purified *E. coli* STh is presented in fig. 1, which compares the results obtained with the ST-ELISA performed with monoclonal antibody and with guinea-pig antibodies classically produced by immunisation with polymerised STh. The ST-ELISA procedure with monoclonal antibody is more sensitive and the titration shows a very steep slope, indicating good specificity, and a more clear-cut difference between negative and positive tests. Our ST-ELISA procedure with monoclonal antibody detects *c.* 300 pg of STh/well (6 ng/ml in the test sample) when the cut-off point is fixed at 30% inhibition. Comparatively, in our experience, the suckling-mouse assay becomes positive with a dose of 5 ng of STh in 0.1 ml (50 ng/ml) and is therefore ten-fold less sensitive than ST-ELISA. We did not encounter loss of ability to bind antibodies to unmodified STh bound to the solid phase, as described by Thompson *et al.* (1984). Furthermore, in our experience, a blocking step after coating did not increase the sensitivity of the assay. These differences may be related to variations in the quality of the microtitration plates or of the antibody preparations. Reproducibility of the tests is fairly good; the variations in the percentages of

FIG. 1.—Titration curve of purified ST in ST-ELISA tests with two different antibody preparations. (Δ = monoclonal antibody; $\circ$ = conventional guinea-pig antibodies)

inhibition by the same supernate from one day to another was $< 10\%$. One of the 237 Belgian strains and 53 of the 445 Rwanda strains were found to be ST-positive by both techniques. Results of testing reference strains are presented in fig. 2. A discrepancy was observed between the assays for two ST-producing reference strains which gave positive results in our ST-ELISA system and negative results by the suckling-mouse assay ($G/B \leq 0.075$). Two further strains, listed as positive, gave negative results by both tests and may, possibly, have lost the relevant *Ent* plasmid during storage. Linear regression analysis of the results shows a regression coefficient of 0.91.

The ST-ELISA procedure is very easy to perform and does not require expensive apparatus or materials, with the exception of the need for preparations of purified ST and antibody which are not commercially available; the assay is rapid and can be interpreted in 2.5 h but, in common with the suckling-mouse and most of the other tests, it requires supernates of agitated *E. coli* cultures that have to be incubated overnight after primary isolation. Large numbers of supernates (184) can be processed on the same day by one technician who does not need special training. The sensitivity and reproducibility of the ST-ELISA system are very good. Visual differences between a negative and a positive test are usually clear. As the monoclonal antibody also effectively neutralises STh and STp, our ELISA procedure would probably detect both toxins and may be of value in the veterinary field. In comparison with bioassay

FIG. 2.—Comparisons of results obtained with ST-ELISA (with monoclonal antibodies) and suckling-mouse bioassay in parallel tests with 200 different strains. (Δ = positive ST; $\bullet$ = negative ST)

procedures, the ST-ELISA offers an easier alternative with greater sensitivity for screening large numbers of strains. It does not require isotopes, as are needed for radioimmunoassay work, and it can be performed in relatively unsophisticated laboratories in field conditions that might be encountered in large epidemiological surveys. We find that it can be performed routinely without problems in our laboratory where it now replaces the cumbersome suckling-mouse assay.

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