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Production, Characterization and Immunodiagnostic Application of a Monoclonal Antibody to Shiga Toxin

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ABSTRACT

A mouse monoclonal antibody (MAb ICT7) that is specific for Shiga toxin was produced. The MAb neutralises the cytotoxic effects of both purified Shiga toxin and culture extracts of *Shigella dysenteriae* type 1 in HeLa cells. Using MAb ICT7 and polyclonal rabbit antiserum, a sandwich ELISA was developed. This test detects Shiga toxin in both *S. dysenteriae* type 1 bacterial extracts and in stools of patients with *S. dysenteriae* type 1 infection. The ELISA also detects toxin in enterohaemorrhagic *Escherichia coli* (EHEC) strains positive for Shiga-like toxin I. The test could detect a minimum of 100 pg of purified Shiga toxin. Furthermore, the ELISA did not detect toxin in non-*S. dysenteriae* type 1 *Shigella* species or Shiga-like toxin II produced by EHEC strains.

Key words: *Shigella; Escherichia coli,* Enterohaemorrhagic; Antibody, Monoclonal; Cytotoxins

INTRODUCTION

Shiga toxin (ShT) produced by strains *of Shigella dysenteriae* type 1 is a heat-labile protein having neurotoxic, cytotoxic and enterotoxic properties (1,2). It is a cell-associated toxin (3,4) and is released into the culture medium after cell death (5,6). The toxin has been detected in stools of patients with *S. dysenteriae* type 1 infection (7). These patients also develop serum neutralizing antibody to ShT (8). It has been suggested that *Shigella* species, other than S. *dysenteriae* type 1, also produce relatively small amounts of a toxin similar to ShT (9,10). However, a conclusive evidence whether this toxin is identical to ShT is not available, since these studies were only based on the cytotoxic effect on HeLa cells, neutralization of these activities with polyclonal rabbit antisera or serological responses in patients (6,9,10). Recently an iron-dependent toxin, termed *Shigella* enterotoxin (ShET1), has been detected in strains of *S. flexneri* 2a (11). The term ShT is generally used for indicating toxin produced by *S. dysenteriae* type 1 only, while Shiga-like toxin (SLT) is used for indicating related toxins produced by other *Shigella* species or by other bacteria, especially enterohaemorrhagic *Escherichia coli* (EHEC) (6,12). The toxin SLT-I produced by EHEC is neutralized by antibodies against ShT. The properties of ShT and SLT-I are very similar, and the sequences of the genes encoding the two toxins show more than 99% identity (13). Another toxin, Shiga-like toxin II (SLT-II) of EHEC, shares approximately 55% overall nucleotide and amino acid sequence homologies with the structural gene of SLT-I (6,13). SLT-II is not neutralized by anti-ShT/SLT-I antibodies. Interest has also been generated in EHEC strains which produce SLTs since they can cause diarrhoea with severe complications, such as haemorrhagic colitis and the haemolytic-uraemic syndrome. To detect *S. dysenteriae* type 1 producing ShT, and EHEC strains producing SLT-I and SLT-II, procedures using toxin-specific polyclonal and monoclonal antibodies (MAb) have been developed (7,12,14). We have produced a MAb (ICT7) to ShT. We report here its characterisation and use in immunodiagnostic assays to detect the toxin in strains of *Shigella* and EHEC as well as in diarrhoeal stools. In addition, we have attempted to assess whether MAb ICT7 could discriminate between the toxin produced by *S. dysenteriae* type 1 and the toxins of other *Shigella* species and serotypes, as well as other bacteria.

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MATERIALS AND METHODS

Bacterial strains and media

Strains of *Shigella* species studied were isolated from patients at the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka. Other bacteria tested were *S. dysenteriae* type 1 strain 60R, a ShT-positive rough mutant (15) and SC-501 (provided by Dr. P. Sansonetti) (16) – a non-toxigenic mutant of *S. dysenteriae* type 1. Fourteen EHEC strains positive for SLT-I, SLT-II or both SLT-I and SLT-II were provided by Dr. Peter Echeverria of the Armed Forces Institute of Medical Sciences, Bangkok, Thailand. *E. coli* K-12 (strain DH1) (17) was used as negative control for the experiments. Other bacteria tested were from the culture collection of ICDDR,B. Strains were stored at -70 °C in trypticase soy (TS) broth (Gibco Diagnostic, Madison, WI) containing 15% glycerol. They were subcultured either on MacConkey agar (Difco, Detroit, MI) or TS agar containing 0.6% yeast extract. Diarrhoeal stool samples tested were obtained from patients at the Clinical Research and Service Centre. Only single stool samples were obtained from patients immediately after admission to the hospital. For *S. dysenteriae* type 1 patients, this was approximately from 2 to 4 days after the onset of illness.

Preparation of culture extracts of bacteria

Bacterial strains were cultured on heart infusion agar slants (Difco) overnight at 37 °C and then treated with polymyxin B sulphate to obtain culture extracts of bacteria (12).

Immunization of mice and production of hybridomas and rabbit polyclonal antibody to ShT

Purified Shiga toxin was a gift from Dr. A. Donohue-Rolfe of the New England Medical Center, Boston, MA, USA. Monoclonal antibodies were prepared by immunizing BALB/c mice with formalin-treated toxin (7) four times (5 µg/dose) at weekly intervals. The first dose was mixed with an equal volume of Freund's complete adjuvant and administered subcutaneously. The second and third doses were mixed with equal volumes of Freund's incomplete adjuvant and given intraperitoneally. The last dose was given intravenously. Four days after the last dose, spleen cells from two immunized BALB/c mice were fused with SP2/0 myeloma cells (18). Rabbit polyclonal serum against formalin-treated toxin was prepared as described previously (7). Hyperimmune rabbit antiserum was tested using purified ShT in an ELISA (18) and an end-point titre of $5x10^6$ was calculated. End-point titres were defined as the reciprocal of the dilution giving an absorbance of 0.2 above background.

Protein determination

Quantification of purified ShT and of ShT in bacterial extracts was carried out on the basis of protein measurement, using the Bio-Rad assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

Screening of hybridomas by ELISA and production of ascites fluid

Supernatant fluids harvested from hybridomas were tested by ELISA in microtitre plates coated with 100 µl each of purified ShT (1 µg/ml), culture extracts of strains of *S. dysenteriae* type 1 (25 µg/mL) or the lipopolysaccharide (LPS) of *S. dysenteriae* type 1 (10 µg/mL) (17). Culture extracts of the non-toxigenic *S. dysenteriae* type 1 strain SC-501 and *E. coli* K-12 were also tested. Ascites tumours were produced in pristane primed mice by injecting them with $5x10^6$ cloned cells. Ascites fluids were tested by ELISA for reactivity to ShT and other antigens mentioned above. To determine the isotype of MAbs, supernatants obtained from hybridoma serum-free medium (Gibco) were tested by ELISA (18).

Immunoblot

Shiga toxin (5 µg), cholera toxin (5 µg), and LPS of *S. dysenteriae* type 1 (5 µg) were separated on 13.5% sodium dodecyl sulphate polyacrylamide gels (19) and stained with Coomassie blue. Antigens were transferred to nitro–cellulose membranes (20), probed with MAb ICT7 and immunoblots developed as described previously (18). Pre-stained molecular weight markers (Bio-Rad) of range 106 to 18.5 kDa were used.

Stool extract

Single stool samples obtained from patients immediately after hospitalization were frozen at -20 °C and tested within 7 days of collection. Stools were cultured for detection of *Shigella* spp. and other enteric pathogens (21). Approximately 1 g of stools was suspended in 1 mL of phosphate buffered saline (PBS) and vortexed for about 2 minutes (12). After centrifugation at 6000X*g* for 15 minutes and filtration (0.45 µm filter, Sartorius, Göttingen, Germany), the supernatants were tested.

Cytotoxicity assay

The cytotoxic effect of ShT and culture extracts was studied, using the method of Gentry and Dalrymple (22). HeLa cells were seeded in microtitre plates ($2x10^4$ cells/well) and grown for 18-20 hours at 37 °C in a 5% CO_2 atmosphere to near confluency. Cells were then treated with varying amounts of toxin (1 µg to 1 pg/well) and incubated for an additional 18-20 hours. Culture extracts of bacteria (100 µL/well) were also tested.

Negative controls of culture extracts of non-toxigenic mutant of *S. dysenteriae* type 1 (SC-501) and *E. coli* K-12 (DH1), as well as wells containing cells not exposed to toxin, were included. Purified ShT and culture extracts of *S. dysenteriae* type 1 strain 60R were used as positive controls. All assays were carried out in duplicate.

After incubation, detached cells, medium and toxin were removed. The remaining cells were fixed with a 2% solution of formalin for one minute. The fixative was removed, and the plates were stained with 0.13% crystal violet in PBS (containing 50% ethanol and 2% formalin) for 20 minutes. For quantification, the stain in the monolayer was dissolved with absolute ethanol. Absorbance was determined at 595 nm in a Titertek Multiscan spectrophotometer (Flow Laboratories, Oxfordshive, UK). The percentage of cell detachment (CD) resulting from incubation of different amounts of toxin was used for constructing a standard curve. The amount of toxin resulting in 50% cell detachment, i.e. 50% dye uptake, was denoted as the CD_{50} value.

Neutralization of cytotoxicity by MAb ICT7

Neutralization of cytotoxicity was carried out after mixing equal volumes of test samples and MAb ICT7. Serial dilutions of culture extracts and pure ShT were incubated at 37 °C for two hours with an equal volume of MAb ICT7 (1:10 dilution). Then, 0.1 mL of the toxin-antibody mixture was incubated with the HeLa cell monolayer in duplicate wells for 18 hours. Neutralization of cytotoxicity was ascertained by the crystal violet assay (22). HeLa cells treated with only toxin (at appropriate dilutions) or only MAb ICT7 (1:20 dilution) were also used as controls.

MAb ICT7-based sandwich ELISA

An indirect sandwich ELISA (7,12) was set up by using the MAb ICT7 and rabbit polyclonal antibody to ShT. Briefly, MAb ICT7 (as ascites fluid) was diluted 1:1000 in 0.05 M carbonate buffer, pH 9.6 and 100 µL was added to each well of a microtitre plate (Nunc, Roskilde, Denmark). After overnight incubation at 4 °C in a moist chamber, the plate was washed three times with wash-diluent buffer (1% fetal bovine serum in PBS with 0.05% Tween-20). The remaining binding sites were blocked with 0.5% skim milk in PBS (100 µL/well) for 30 minutes at room temperature. After washing, purified ShT (2 ng to 20 pg), culture extracts of bacteria or stool extracts (both 100 µL/well) were added to the wells. After incubation for one hour at 37 °C in a moist chamber, the plate was washed three times and rabbit polyclonal antibody to ShT diluted 1:500 in buffer was added (100 µL/well) and incubated for one hour at 37 °C in a moist chamber. The plate was washed three times and peroxidase conjugated swine anti-rabbit immunoglobulins (Dako, Denmark) diluted 1:500 in buffer was added (100 µL/well) and incubated for one hour at 37 °C. After washing the plate, substrate solution [3,3',5,5'-tetramethyl-benzidine (TMB)] (1 mg/mL) was added. The reaction was stopped after 10 minutes by adding 50 µL of 1 M H_2SO_4 and absorbance was measured at 450 nm. Purified ShT (1 µg/mL) and culture extract of *S. dysenteriae* type 1 (strain 60R) were used as the positive controls in all assays. Culture extracts from non-toxigenic mutant of *S. dysenteriae* type 1 and *E. coli* K-12 were used as negative controls. In addition, wells containing PBS instead of toxin were also used as negative controls. All assays were carried out in duplicate. A cut–off point of 0.2 was used for determining the presence or absence of toxin. This was based on the OD values plus 2 standard deviations of less than 0.200, obtained with the non-toxigenic mutant of *S. dysenteriae* type 1 strain SC-501. Absorbance values plotted against the amounts of ShT tested were used for determining the sensitivity and detection range for the assay. Linear regression analysis was carried out to determine the amounts of toxin present in samples.

Inhibition ELISA

The MAb ICT7 was also tested in sandwich ELISA inhibition studies. These studies were carried out with purified ShT (1 to 20 µg/mL) and culture extracts of *S. dysenteriae* type 1 strain 60R (7.5 to 750 µg/mL) which were mixed with MAb ICT7 and incubated overnight at 4 °C. The toxin preparation alone and the toxin-MAb ICT7 mixture were added to a microtitre plate that had previously been coated with 100 µL/well of MAb ICT7 diluted 1:1000 in 0.05 M carbonate buffer (pH 9.6) and blocked with 0.5% skim milk in PBS (100 µL/well). The plate was developed as described for the MAb ICT7-based ELISA.

DNA hybridization assay

Colony blot hybridization of the *Shigella* strains (Table I) and *E. coli* K-12 (strain DH1) was performed for SLT-I and SLT-II as described previously (23,24). DNA probes were prepared from recombinant plasmids containing the probe DNA as inserts. Plasmids were prepared, purified and digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD) and the appropriate restriction fragments were purified as described by Moseley *et al.* (25). The SLT-I probe was a 1.1-kb *Bam*HI fragment of pJN37-19 and the SLT-II probe was a *Sma*I-*Pst*I 850-bp digestion fragment of pNN110-18 (24). DNA fragments were labelled by random priming (26), using [^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol; Amersham International PLC, Aylesbury, UK) and a random primer DNA labelling kit (BRL). Colony blots were prepared, processed and hybridized under stringent conditions as

described earlier. The EHEC strains were tested for SLT-I and SLT-II at the Armed Forces Institute of Medical Sciences, Bangkok, Thailand.

RESULTS

Hybridoma secreting MAb ICT7

Antibody secreted by one clone obtained from the fusion of spleen cells of the immunized mice recognized ShT in ELISA. It also recognized culture extracts of *S. dysenteriae* type 1 strains. but not the non-toxigenic mutant of *S. dysenteriae* type 1 SC-501 or *E. coli* K-12. Non-specific reaction with LPS of *S. dysenteriae* type 1 or cholera toxin was not observed. This MAb was designated as ICT7 and was of IgG1 isotype. Ascites fluid raised against the ICT7 hybridoma recognized the toxin up to an endpoint titre of $5x10^6$.

Immunoblots

On immunoblotting (Fig.), MAb ICT7 recognized a small molecular weight polypeptide (Lane b) in the B subunit region of the purified toxin (<12 kDa in size) and did not recognize any band in the region of the A subunit. This suggested that MAb ICT7 specifically recognized the B subunit of the toxin. It did not recognize cholera toxin (Lane c). or LPS of *S. dysenteriae* type 1 (Lane d).

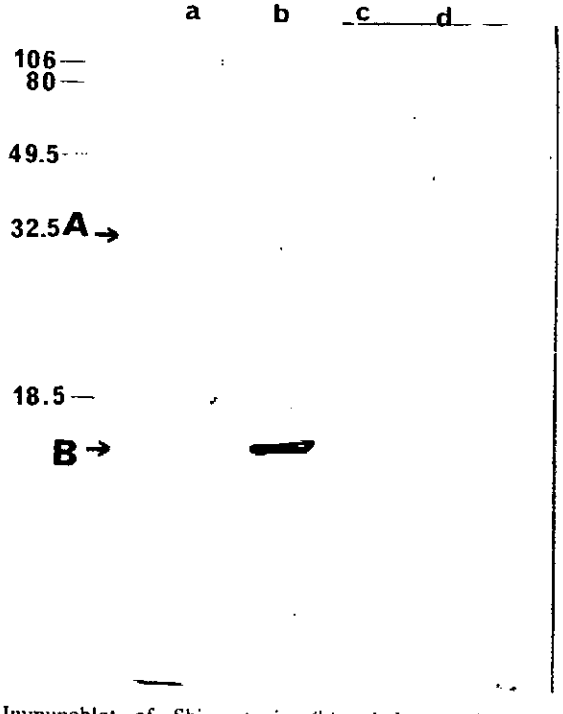

Fig. Immunoblot of Shiga toxin (b), cholera toxin (c), and lipopolysaccharide of *S. dysenteriae* type 1 (d) with MAb ICT7 (used at a 1:500 dilution). Pre-stained molecular weight markers (in KDa) are also shown (a). Arrow indicates the region of migration of the A and B subunits of Shiga toxin

Neutralization of cytotoxicity by MAb ICT7

Purified ShT and culture extracts of *S. dysenteriae* type 1 showed cytotoxic effects on HeLa cells. In this assay, one CD_{50} value of the toxin equalled 50 pg of the purified material and $2x10^4$ CD_{50} values were present per µg protein of pure ShT. At a 1:20 dilution of the ascites fluid, the MAb could neutralize 1000 CD_{50} doses of purified ShT. The cytotoxicity of undiluted culture extracts of 9 strains of *S. dysenteriae* type 1 was completely neutralized by MAb ICT7. These strains were also positive for toxin gene by colony blot hybridization with DNA probe for SLT-1.

Three strains each of *S. flexneri* type 1a, 1b, 2a, and 2b, *S. dysenteriae* type 13, *S. sonnei* Form 1, and *S. boydii* type 1-6, 7-11 and 12-15 produced cytotoxic effects on HeLa cells which could not be neutralized by MAb ICT7. The strains were also negative for toxin genes by colony blot hybridization with DNA probes for SLT-I, as well as SLT-II. Culture extracts of both SLT-I and SLT-II-producing EHEC (14 strains) showed cytotoxic effect on HeLa cells and were recognized by DNA probes. Neutralization of the cytotoxic effects of the EHEC strains by ICT7 was not done.

Sandwich ELISA with MAb ICT7

The ELISA procedure could detect a minimum of 100 pg of purified ShT. Pre–incubation with MAb ICT7 could inhibit the binding of a maximum of 1 µg of purified ShT. In crude extracts of *S. dysenteriae* type 1, toxin could be detected in as little as 70-90 µg of protein. The culture extracts of all 9 strains of *S. dysenteriae* type 1 prepared as described above were strongly positive in the MAb ICT7-based ELISA (≥1.5 OD at 450 nm). This could be related to the presence of about 0.85 µg of toxin/mL bacterial extract. The SLT-I-producing EHEC strains (11 strains) were also strongly positive in the ELISA, showing the presence of approximately 0.615 µg toxin/mL bacterial extract. The non-toxigenic strain of *S. dysenteriae* type 1 SC-501, other *Shigella* strains and serotypes, SLT-II-producing EHEC and the *E. coli* K-12 strain were negative in the ELISA.

Relatively high amounts of toxin (2-20 ng/mL) were detected in stools from patients with *S. dysenteriae* type 1-associated dysentery (Table). Shiga-toxin could not be detected in stools of patients with *S. flexneri* or *S. sonnei* infection. When stools from *Vibrio cholerae* O1 and *V. cholerae* O139 patients were tested in the ELISA, no reaction was observed. showing the absence of non–specific binding.

DISCUSSION

Our results show that MAb ICT7 is useful for the neutralization of cytotoxicity of purified ShT and crude

extracts of *S. dysenteriae* type 1 strains on HeLa cells. Furthermore, the cytotoxic effect of other species and serotypes of shigellae was not neutralized by this MAb. These results agree with those obtained by gene probe assay and by the MAb ICT7-based sandwich ELISA. Using the gene probe assay, except for *S. dysenteriae* type 1, all other shigellae were found to be negative for SLT-I and SLT-II. Similarly, in the sandwich ELISA, the bacterial extracts from these strains tested negative. Thus, there is a complete agreement between the three methods that we have used for detecting ShT in these bacteria. These results, thus, suggest that although these non-*S. dysenteriae* type 1 strains have a cytotoxic effect on HeLa cells, it must be due to a factor different from ShT or SLTs. It supports the findings by Bartlett *et al.* (27) and Johnson and Lior (28).

Table I. Detection of toxin in stools of diarrhoeal patients

Stool samples from patients infected with	Toxin in stool extract (ng/mL)
S. dysenteriae type 1 (15 patients)	8.1 (5.7)*
S. flexneri (2a, Y) (6 patients)	0
S. sonnei Form I (2 patients)	0
S. boydii (1-6) (2 patients)	0
V. cholerae O1 (6 patients)	0
V. cholerae O139 (6 patients)	0

* Value is mean (1 standard deviation of the mean)

The HeLa cell cytotoxicity assay has a higher sensitivity for detection of toxin (20 pg) than the sandwich ELISA that detects only a minimum of 100 pg. Similar results have been obtained earlier for SLT-I (11). However, the specificity of the cytotoxic effect on HeLa cells of the bacterial cultures had to be confirmed by neutralization with specific antibodies. Our results show that the ELISA is indeed specific for detecting ShT/SLT-I and could detect the toxin in bacterial as well as stool extracts. Either SLT-I or SLT-I and SLT-II-containing EHEC strains could also be detected by this ELISA. This is not surprising since the immunological and biochemical properties of the ShT and SLT-I are virtually identical (13,29). We show here that both *S. dysenteriae* type 1 and SLT-I-producing strains produce levels of toxins that can be easily detected in bacterial extracts.

In conclusion, we feel that ICT7 is a useful monoclonal antibody that can be used for immunodiagnostic assays for detection of toxin of bacterial strains and in diarrhoeal stools. The results presented here further support previous findings that Shiga toxin appears in the gut during the course of bacterial dysentery (7).

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