Enzyme-Linked Immunosorbent Assay for Shigella Toxin

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An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of shigella toxin. For the assay, a mouse monoclonal antibody against the B subunit of the toxin and a rabbit polyclonal antibody against the holotoxin were employed. The monoclonal antibody was used to coat wells of a microtiter plate, and the polyclonal antibody preparation was used as the detecting antibody. The amount of bound polyclonal antibody was determined by using a goat anti-rabbit immunoglobulin G-alkaline phosphate conjugate and substrate. The ELISA was able to detect as little as 12 pg (0.06 ng/ml) of shigella toxin. The assay was specific for shigella toxin, not detecting a variety of other bacterial enterotoxins and lethal toxins. The ELISA values correlated well with cytotoxic activity during toxin purification. Shigella toxin was detected by ELISA and by immunoblot analysis in human fecal specimens from persons with S. dysenteriae infections, demonstrating that this toxin is produced in vivo.

Shigella toxin is a potent bacterial protein toxin consisting of six subunits: one A subunit (Mr = 32,000) and five B subunit monomers (Mr = 6,500 each) (3). The A subunit is responsible for the known biochemical effect of toxin, inhibition of eucaryotic ribosomal protein synthesis, apparently by irreversible and probably catalytic inactivation of the 60S ribosomal subunit (14). The B subunit mediates toxin binding to cell surface receptors, which is necessary for expression of cytotoxicity against susceptible mammalian epithelial cells (3). Because the same toxin also causes isotonic fluid secretion by rabbit small intestine (1, 4, 7, 10), shigella toxin is postulated to be an important virulence attribute of the organism (7).

Shigella toxin also causes death of susceptible animals when administered parenterally (5, 7, 10). Hence, assay of toxin now depends on measuring the 50% lethal dose (LD50) for mouse lethal activity, fluid secretion in rabbit intestine for enterotoxic activity (7), or the tissue culture LD50, which measures cytotoxic activity (12). Because there are drawbacks to each of these methods, we developed an enzyme-linked immunosorbent assay (ELISA) utilizing toxin-specific monoclonal mouse and polyclonal rabbit antitoxin antibodies that we have produced and previously characterized (3). This assay is specific for shigella toxin, is highly sensitive, detecting subnanogram quantities of toxin, and was employed in this study to demonstrate toxin in stools of patients with S. dysenteriae 1 infection.

MATERIALS AND METHODS

Bacterial toxins. Escherichia coli heat-stable and heat-labile enterotoxins were kindly provided by Cambridge Research Laboratories (Cambridge, Mass.). Cholera, diphtheria, and tetanus toxins were kindly provided by D. Michael Gill, Tufts University School of Medicine (Boston, Mass.).

Purification of shigella toxin. Shigella toxin was purified by a previously reported procedure (3). Briefly, washed S. dysenteriae 1 60R cells were lysed by sonication in 10 mM Tris hydrochloride (pH 7.4), and the resulting lysate was applied to a column containing Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N.J.). The bound material, which contains the toxin, was eluted with the same buffer containing 0.5 M NaCl, dialyzed against 25 mM Tris-acetate (pH 8.3), and applied to a column of Polybuffer Exchanger 94 (Pharmacia) which had been equilibrated with 25 mM Tris-acetate (pH 8.3). Bound material was then eluted with Polybuffer 96 (Pharmacia) diluted 1:13 in water and adjusted to pH 6.0 with acetic acid. The cytotoxic material which eluted at pH 7.0 to 7.1 was concentrated and applied to a column containing Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 20 mM NH4CO3. Purified cytotoxin was eluted with the same buffer, and the toxin-containing fractions were pooled and lyophilized.

Preparation of polyclonal rabbit antiserum to shigella toxin. Rabbit antiserum to shigella toxin was prepared by immunizing rabbits with Formalin-treated, purified shigella toxin. The production and characterization of this antiserum has been previously reported (3).

Monoclonal anti-B subunit antibody. Monoclonal antibodies were prepared by immunizing BALB/c mice with Formalin-treated toxin. Spleen cells were fused to P3X63Ag 8.653 myeloma cells, and hybridomas producing antibody to shigella toxin were screened by their ability to immunoprecipitate 125I-toxin. Ascites fluid was prepared from stable hybridoma cell lines by intraperitoneal injection of 5 × 106 cells into BALB/c mice that had been primed 2 weeks earlier by intraperitoneal injection of 0.5 ml of Pristane. The subunit specificity of the antitoxin antibodies was analyzed by Western blotting as previously described (3).

ELISA for shigella toxin. An indirect ELISA was performed by using a modification of the procedure of Voller et al. (15). Ascites fluid from the hybridoma 4D3, which produces immunoglobulin G antibody specific for the B subunit of the toxin, was diluted to a protein concentration of 10 μg/ml in 50 mM Na2CO3-NaHCO3 (pH 9.6; carbonate buffer). Diluted ascites fluid (0.2 ml) was then added to each well of microtiter plates (Nunc-Immuon Plate I; Nunc, Kamstrup, Denmark) and incubated overnight at 4°C. At that time, wells were emptied and 0.2 ml of 1% bovine serum albumin in carbonate buffer was added to each well and incubated for 1 h at room temperature. After this, wells were washed five times with 0.2 ml of phosphate-buffered...
saline—0.05% Tween 20 (PBS-T). Test samples of antigen (0.2 ml) diluted in PBS-T were then added, and the plates were incubated for 16 h at 4°C. After five washes with PBS-T, 0.2 ml of a 1:5,000 dilution of rabbit polyclonal antiserum to shigella toxin in PBS-T was added and incubated at room temperature for 2 h. Wells were again washed five times with PBS-T and incubated with 0.2 ml of goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.), diluted 1:1,000 in PBS-T, for 1 h at room temperature. Wells were washed five times with PBS-T and then incubated for 1 h at room temperature with enzyme substrate (0.2 ml of a 1-mg/ml solution of p-nitrophenylphosphate [Sigma] in diethanolamine buffer (pH 9.8)). Finally, the absorbance in each well was measured with a microplate colorimeter (Dynatech Laboratories, Inc., Alexandria, Va.). Net absorbance was determined by subtracting the absorbance in wells treated with PBS-T in place of a test sample from the absorbance in wells with toxin-containing test samples.

Detection of shigella toxin in fecal specimens. Fecal samples were diluted 1:1 in PBS-T and clarified by centrifugation (5,000 x g for 5 min). The samples were stored at -70°C. The clarified fecal sample was used for the ELISA without further dilution.

To detect toxin in stool by immunoblot analysis, 2 ml of the fecal sample in PBS was first chromatographed over a 1 ml column of Protein A-Sepharose 4B A (Pharmacia). The eluate was collected, dialyzed against 10 mM Tris hydrochloride (pH 7.4), applied to a 2-ml column of Blue Sepharose, and washed with 10 ml of 10 mM Tris hydrochloride (pH 7.4) buffer. The bound material containing cytotoxin was eluted with 10 M Tris hydrochloride containing 0.5 M of NaCl, dialyzed against 20 mM NaHCO₃ buffer, and lyophilized. The lyophilized sample was suspended in sodium dodecyl sulfate (SDS) electrophoresis sample buffer and subjected to SDS-polyacrylamide gel electrophoresis in 15% acrylamide slab gels (2). Proteins in the SDS-gel were then electrophoretically transferred (2 h at 300 mA) to a Zeta-Probe membrane (Bio-Rad). The buffer used in the transfer was 15.6 mM Tris—120 mM glycine (pH 8.3). Following this, the membrane was incubated for 1 h at 50°C in 0.1 M Tris hydrochloride (pH 7.4)—0.9% NaCl (Tris-saline) containing 10% bovine serum albumin. The filter was then incubated overnight at 4°C with a 1:1,000 dilution of polyclonal rabbit antitoxin serum, and washed for three 10-min periods with Tris-saline. To visualize antibody bound to antigen, the filter was incubated for 2 h at room temperature in Tris-saline containing 5% bovine serum albumin and 5 x 10⁻⁶ cpm of [³²P]-labeled protein A per ml (100,000 cpm/ng). The filter was then washed extensively in Tris-saline, air dried, wrapped in plastic wrap, and developed by autoradiography.

Cytotoxin assay. Cytotoxin activity and the tissue culture LD₅₀ were determined by the method of Keusch et al. (12). HeLa cell monolayers were grown in microtiter plates in modified McCoy 5a medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum to near confluency. Cells were then treated with fresh McCoy medium or McCoy medium with toxin for 18 h and washed to remove dead or loosely adherent cells. Remaining attached cells were removed with 0.025% trypsin, suspended in PBS, and counted in a hemocytometer chamber. HeLa cell mortality was calculated by comparison of cell counts from control and toxin-treated monolayers.

Protein determinations. For protein determinations we used the Bio-Rad assay kit II with bovine serum albumin as the standard.

RESULTS

ELISA for shigella toxin. To capture the toxin molecule, microtiter plates were coated with ascites fluid containing monoclonal antibody against the B subunit of the toxin. To detect bound toxin, we first added polyclonal rabbit antiserum containing antibody to both A and B chains of shigella toxin. Next, bound rabbit antitoxin antibodies were measured by sequentially adding goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and substrate. The response of shigella toxin in this ELISA was dose dependent (Fig. 1). The assay was capable of detecting as little as 12 pg of toxin and was linear between 0.012 and 0.2 ng. Specificity was demonstrated by the negative results obtained with cholera toxin (1 μg/ml), E. coli heat-labile enterotoxin (1 μg/ml), E. coli heat-stable enterotoxin (1 μg/ml), diphtheria toxin (1 μg/ml), and tetanus toxin (10 μg/ml) (data not shown). When toxin was incubated at 90°C for 30 min, a treatment known to inactivate biological activity of shigella toxin, the ELISA sensitivity to toxin was decreased by 90%.

We also tested the reverse assay, that is, by coating the microtiter wells first with the rabbit antitoxin serum and using the mouse monoclonal antibody as the detecting antibody. Bound monoclonal antibodies were determined by sequentially adding rabbit anti-mouse immunoglobulin G-alkaline phosphatase conjugate and substrate. This procedure was approximately 10-fold less sensitive.

Correlation between ELISA values and cytotoxin titers. To correlate ELISA values and cytotoxin activity, we performed both assays on samples obtained at various stages of toxin purification. The correlation was excellent (Table 1), and antigenicity and bioactivity during purification were closely related.

Detection of toxin in fecal samples from patients. We prepared a series of twofold dilutions of toxin added to a fecal specimen from a healthy infant and then immediately examined these for the presence of toxin by the ELISA method. The toxin-feces mixture gave a dose response curve similar to that obtained with toxin diluted in PBS-T (Fig. 1). Thus, the presence of feces in the assay sample did not interfere with toxin detection by the ELISA methodology. We then tested nine fecal samples from patients in Bangladesh who were culture positive for S. dysenteriae. All nine produced high ELISA readings (Table 2). Based on a
standard curve generated by diluting the purified toxin in PBS-T, these values corresponded to fecal toxin concentrations ranging from 0.5 to 12 ng/ml.

To confirm that the positive ELISA values in stools of patients were indeed due to the presence of bona fide shigella toxin, we employed immunoblot analysis on the fecal specimen from patient no. 6. It was necessary to pass the clarified fecal specimen over a protein A-Sepharose column to remove protein A-binding proteins to permit immunoblot detection with radioactive protein A. Figure 2 shows the autoradiograph of toxin, purified as described previously, subjected to SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to Zeta-Probe membranes, and reacted with antitoxin antibody followed by 125I-labeled protein A. The fecal specimen (lane 2) contained two major bands which exactly corresponded to the positions of the A and B subunits of purified shigella toxin (lane 1). The relatively weak reaction to the B subunit from the fecal specimen remains unexplained at this time but could conceivably reflect preferential proteolytic sensitivity of this subunit.

**DISCUSSION**

Using polyclonal and monoclonal antibody preparations, we developed a rapid and sensitive ELISA to detect shigella toxin. In the assay, absorbance readings were directly related to toxin concentrations from 12 to 200 pg per well. The assay was highly specific in that no reaction was obtained with three other bacterial enterotoxins (cholera toxin and heat-stable and -labile E. coli enterotoxins) or with lethal toxins (diphtheria or tetanus toxin). The ELISA readings also correlated well with shigella toxin bioactivity during toxin purification.

At the present time there are no nonbiological assays for this toxin. Biological assays which measure neurotoxicity, enterotoxicity, and cytotoxicity employ mouse lethality, rabbit intestinal fluid responses, and HeLa (or Vero) cell lethality, respectively. The most simple, reproducible, and sensitive of the three assays is HeLa cell cytotoxicity. However, even this assay takes approximately 24 h to perform from the time cell monolayers are available. Moreover, cytotoxicity present in crude toxin preparations should be confirmed by neutralization experiments using toxin-specific antibody before conclusions are drawn about its specific relationship to the toxin content of the sample.

The present data demonstrate that in the laboratory setting the reproducibility, sensitivity, and linear dose response of the described ELISA allows quantitative estimation of toxin activity. This is useful for monitoring toxin production, for example, to determine optimum culture conditions to increase toxin yield as well as in monitoring purification.

The shigella toxin ELISA also gave positive results with fecal samples from patients infected with *S. dysenteriae* 1. Western blot analysis of one such stool showed that both toxin subunits were present, indicating that a positive stool ELISA does indeed indicate the presence of toxin. To our knowledge, this report represents the first documentation that shigella species produce toxin in vivo and is consistent with the previous finding of antitoxin antibody in sera of convalescent patients with *S. dysenteriae*, *S. flexneri*, and *S. sonnei* infections (9, 11, 13). Since all species of shigella have now been shown to produce toxin in vitro, this suggests that toxin is produced in vivo in all instances.

We must caution that, before this toxin ELISA can be used as a reliable general assay for toxin levels in stool, much further work is needed. The presence of stool specimens of interfering factors such as proteases (6) and antifibrinogen antibody (16) could affect the performance of the assay. The frequency of false-negative and -positive results in the assay system should be assessed by testing a

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**TABLE 1. Correlation between ELISA values and cytotoxic activity**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Tissue culture $LD_{50}$ (mg/ml)</th>
<th>ELISA (LD$_{50}$/μg)</th>
<th>Tissue culture $LD_{50}$/μg of toxin (ELISA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>$2 \times 10^4$</td>
<td>29</td>
<td>$6.9 \times 10^3$</td>
</tr>
<tr>
<td>Blue Sepharose eluate</td>
<td>$5.4 \times 10^4$</td>
<td>67</td>
<td>$8.1 \times 10^3$</td>
</tr>
<tr>
<td>Purified toxin</td>
<td>$1.8 \times 10^4$</td>
<td>240</td>
<td>$7.5 \times 10^3$</td>
</tr>
</tbody>
</table>

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**TABLE 2. ELISA of fecal samples from patients with *S. dysenteriae* infections**

<table>
<thead>
<tr>
<th>Fecal sample from patient no.</th>
<th>$A_{405}^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.229</td>
</tr>
<tr>
<td>2</td>
<td>0.560</td>
</tr>
<tr>
<td>3</td>
<td>0.548</td>
</tr>
<tr>
<td>4</td>
<td>0.683</td>
</tr>
<tr>
<td>5</td>
<td>1.204</td>
</tr>
<tr>
<td>6</td>
<td>&gt;1.5</td>
</tr>
<tr>
<td>7</td>
<td>1.09</td>
</tr>
<tr>
<td>8</td>
<td>1.202</td>
</tr>
<tr>
<td>9</td>
<td>0.313</td>
</tr>
</tbody>
</table>

*The $A_{405}$ of PBS blank was 0.1. Absorbance readings represent the averages of duplicate wells.*

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**FIG. 2. Immunological detection of shigella toxin in a fecal specimen from a person with *S. dysenteriae* infection.** Proteins were separated on 15% SDS-polyacrylamide gel electrophoresis and blotted to Zeta-Probe membranes. Rabbit anti-shigella antitoxin at a 1:1,000 dilution was used in the immunoblot analysis. To visualize antibody bound to antigen, the membrane was treated with 125I-labeled protein A and subjected to autoradiography. Lanes: 1, purified shigella toxin (50 ng); 2, 500 μg of protein from a fecal specimen.
larger number of stools from individuals with and without shigella infections.

The role of shigella toxin in pathogenesis remains somewhat controversial. Its ability to evoke an intestinal fluid response in rabbits (7, 14) suggests that toxin may be responsible for the diarrheal phase of shigellosis. In addition, its ability to cause inflammatory damage of rabbit gut (8) and to kill tissue culture cells by inhibiting protein synthesis (1) suggests that the toxin could also play a role in the dysentery phase of the disease. Although this hypothesis of a critical role for shigella toxin in pathogenesis is unconfirmed, all species and strains of the genus are toxigenic so that the virulence of nontoxigenic strains cannot be tested. The present paper adds one more necessary piece of evidence to the puzzle of pathogenesis: the proof that toxin is produced in vivo and is present in the gut lumen during infection with S. dysenteriae 1.

ACKNOWLEDGMENTS

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LITERATURE CITED


