Rapid Method To Detect Shiga Toxin and Shiga-Like Toxin I Based on Binding to Globotriosyl Ceramide (Gb₃), Their Natural Receptor

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Shiga toxin and the closely related Shiga-like toxins produced by Escherichia coli represent a group of very similar cytotoxins that may play an important role in diarrheal disease and hemolytic uremic syndrome. These toxins have the same biologic activities and according to recent studies also share the same binding receptor, globotriosyl ceramide (Gb₃). They are currently detected, on the basis of their ability to damage several cell lines, by using expensive and tedious assays that require facilities for and experience with tissue cultures and are therefore most suitable for research laboratories. We have developed a rapid method to detect Shiga toxin and Shiga-like toxin I based on specific binding to their Gb₃ natural receptor, which was coated onto microtiter plates. Bound toxin was then detected by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies. The sensitivity of the Gb₃ ELISA was 0.2 ng (2 ng/ml) of purified toxin. The assay was positive with sonic extracts of Shigella dysenteriae serotype 1 strain 60R (a Shiga toxin producer), E. coli serotype O26:H11 strain H30, and E. coli serotype O157:H7 (both Shiga-like toxin I producers). The assay was very specific in that no cross-reactivity was noted with purified cholera toxin, E. coli heat-labile and heat-stable enterotoxins, and Clostridium difficile cytotoxin, or sonic extracts of other cytotoxin-producing organisms, such as other shigellae, pathogenic and nonpathogenic E. coli, Salmonella spp., Campylobacter spp., and Aeromonas spp. These results were in complete agreement with a [³H]thymidine-labeled HeLa cell cytotoxicity assay and with detection of the structural genes by DNA hybridization studies with a Shiga-like toxin I probe. Quantitative analysis showed a high correlation between Gb₃ ELISA and HeLa cell assay when fractions obtained at various stages of toxin purification were examined by both methods (r = 0.99, P < 0.01). This rapid Gb₃ ELISA is sensitive and specific and may be diagnostically useful in cytotoxin-related infections.

Cytotoxin production by enteric organisms has been increasingly investigated in recent years. Information regarding the role of cytotoxins in the intestinal manifestations of Shigella spp. (26), Escherichia coli (6), and Clostridium difficile (3) is gradually accumulating. Hemorrhagic colitis syndrome has been recently defined and related to high-level cytotoxin-producing E. coli, such as serotypes O157:H7 and O26:H11, which have been called enterohemorrhagic E. coli (19, 24, 27, 29). In addition, cytotoxin production probably plays a major pathogen role in the development of hemolytic uremic syndrome (HUS) (5, 12). For example, E. coli O157:H7 has been observed in one-half of the patients with HUS during a prospective study in the northwestern United States (20), and the syndrome developed in 22% of patients involved in an outbreak of hemorrhagic colitis related to this organism (4). Evidence of infection with cytotoxin-producing E. coli has been demonstrated in 75% (12) to 88% (5) of patients with idiopathic HUS. It is currently thought that the cytotoxins damage vascular endothelial cells, thereby initiating the abnormalities of HUS (5).

The cytotoxins are defined by their ability to damage mammalian cells. Among the most important cytotoxins are Shiga toxin, produced by Shigella strains (mainly Shigella dysenteriae serotype 1) (2, 21), and the closely related Shiga-like toxins (SLTs), produced by E. coli (23). The latter are also referred to as verotoxins by those who assay cytotoxic activity in Vero cells (15). At least two immunologically distinct toxins, SLT-I and SLT-II, have been defined (19, 25, 29). SLT-I is neutralized by antibodies raised against Shiga toxin, to which it is virtually identical; there is only a three-nucleotide difference, resulting in a single-amino-acid difference, between the two toxins (11). Although immunologically distinct, SLT-II is very similar to Shiga toxin and SLT-I with regard to structure and biologic activities. These toxins act by inhibiting protein synthesis by catalytic inactivation of the 6OS ribosomal subunits. Recent studies have shown that these toxins also share the same glycosphingolipid receptor, globotriosyl ceramide (Gb₃) (9, 10, 16, 17, 31). Other less-defined variants of SLTs also exist (23).

The cytotoxins are currently determined by their damage to several cell lines, which is usually evidenced by morphologic changes. These assays are expensive and tedious. They require facilities for and experience with tissue cultures and are therefore not suitable for routine usage and less-well-equipped laboratories. In light of recent data indicating that the well-defined cytotoxins share the same binding receptor, we have developed a rapid-detection method for Shiga toxin and SLT-I based on their binding to Gb₃, the natural receptor, with detection of bound toxin by enzyme-linked immunosorbent assay (Gb₃ ELISA). Results were compared with cytotoxicity determined by a quantitative radiolabeled HeLa cell assay that we have described previously (2, 6, 26) and with the structural genes for toxin production detected by DNA hybridization assay (21, 22).

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TABLE 1. Procedure for Gb3 ELISA

<table>
<thead>
<tr>
<th>Step and compound</th>
<th>Concn or dilution</th>
<th>Diluent</th>
<th>Vol (μl/well)</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Coat plates with Gb3</td>
<td>10 μg/ml</td>
<td>Chloroform-methanol (2:1)</td>
<td>100</td>
<td>Room 4 h</td>
</tr>
<tr>
<td>2. Wash</td>
<td></td>
<td>Tween-PBS*</td>
<td>200</td>
<td>Room 1 min (5×)</td>
</tr>
<tr>
<td>3. Block with BSA</td>
<td>50 mg/ml</td>
<td>PBS</td>
<td>200</td>
<td>37°C 1 h</td>
</tr>
<tr>
<td>4. Wash</td>
<td></td>
<td>Tween-PBS</td>
<td>200</td>
<td>Room 1 min (3×)</td>
</tr>
<tr>
<td>5. Add test sample</td>
<td></td>
<td>0.1% BSA in Tween-PBS</td>
<td>100</td>
<td>37°C 1 h</td>
</tr>
<tr>
<td>6. Wash</td>
<td></td>
<td>Tween-PBS</td>
<td>200</td>
<td>Room 1 min (5×)</td>
</tr>
<tr>
<td>7. Add monoclonal antibodies to SLT-I</td>
<td>1:1,000</td>
<td>Tween-PBS</td>
<td>100</td>
<td>37°C 1 h</td>
</tr>
<tr>
<td>8. Wash</td>
<td></td>
<td>0.1% BSA in Tween-PBS</td>
<td>200</td>
<td>Room 1 min (5×)</td>
</tr>
<tr>
<td>9. Add peroxidase-conjugated rabbit anti-mouse IgG*</td>
<td>1:1,000</td>
<td>Tween-PBS</td>
<td>200</td>
<td>Room 1 min (5×)</td>
</tr>
<tr>
<td>10. Wash</td>
<td></td>
<td>Tween-PBS</td>
<td>200</td>
<td>Room 1 min (5×)</td>
</tr>
<tr>
<td>11. Add substrate (orthophenylene diamine)</td>
<td>0.4 mg/ml</td>
<td>Citrate-phosphate buffer</td>
<td>100</td>
<td>Room 15 min</td>
</tr>
<tr>
<td>12. Stop reaction with 2.5 N H2SO4</td>
<td></td>
<td></td>
<td>100</td>
<td>Room</td>
</tr>
</tbody>
</table>

* Tween-PBS, 0.05% Tween 80 in PBS (0.2 M sodium phosphate, 0.2 M potassium phosphate).

b IgG, Immunoglobulin G.

MATERIALS AND METHODS

**Bacterial strains.** Bacteria which characteristically produce Shiga toxin and SLT-I were initially used for development of the assay. They included *S. dysenteriae* serotype 1 strain 60R (a Shiga toxin producer); *E. coli* serotype O26: H11 strain H30 (a SLT-I producer), originally described by J. Konowalchuk (15); and *E. coli* serotype O157:H7 (a SLT-I producer), provided by John Mathewson, who originally obtained the strain from Richard Wilson at the *E. coli* Reference Center, Pennsylvania State University. For specificity studies, other bacterial strains were used: *E. coli* K-12 strain C600 (a non-cytotoxin-producing strain) and lysogenic *E. coli* K-12 strain C600 (933w) (a SLT-II producer), provided by A. D. O’Brien, Uniformed Services University of the Health Sciences, Bethesda, Md. Other bacterial isolates examined were *Shigella* spp. (2), pathogenic and nonpathogenic *E. coli* strains (6), *Salmonella* spp. (1), *Aeromonas* spp. (13), *Campylobacter* spp., and *Crotobacter* spp.

**Preparation of bacterial sonicate.** Bacteria were grown in iron-depleted synace broth for 48 h with shaking at 200 rpm, harvested, lysed by sonication, and filter sterilized, as previously described (2, 6). Protein concentration was determined by protein assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin (BSA) as a standard.

**Preparation of purified Shiga toxin.** Shiga toxin was purified from a sonicate of *S. dysenteriae* serotype 1 strain 60R by Affi-Gel Blue column, chromatofocusing, and hydroxyapatite column, as we have described elsewhere (26). The final cytotoxnic fraction was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel (15%) under denaturing conditions (β-mercaptoethanol), and purity was confirmed with silver staining. The purified toxin had a specific activity of 10^7.3 50% cytotoxic doses (CD50) per mg of protein.

**Preparation of anti-Shiga toxin serum.** Rabbits anti-Shiga toxin serum, which also neutralized SLT-I, was obtained by repeated injections of formaldehyde-treated purified Shiga toxin (100 μg per dose) in complete Freund adjuvant as previously described (2).

**Gb ELISA.** The detailed procedure of the Gb ELISA is described in Table 1. Gb (Supelco, Bellefonte, Pa.) was obtained and diluted for use in chloroform-methanol (2:1). After inoculation, the polyvinyl chloride microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) were left uncovered, so that the diluent evaporated and Gb, attached to the plates. Monoclonal antibodies (13C4, mouse ascitic fluid) to SLT-I (B subunit) were obtained from N. A. Strockbine, Centers for Disease Control, Atlanta, Ga. (28), and used at a 1:1,000 dilution. Peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Bionetics Laboratory Products, Charleston, S.C.) was then added at a dilution of 1:1,000. After being washed, the substrate, orthophenylene diamine (0.4 mg/ml)-0.1% H2O2 in citrate-phosphate buffer (25 mM citrate, 50 mM phosphate [pH 5]), was added. The reaction was stopped with 2.5 N H2SO4, and then samples were transferred to new plates and the A_{490} was measured (Dynatech microELISA plate reader MR580). Control wells, to which 0.1% BSA in 0.05% Tween 80 phosphate-buffered saline (PBS) (sample diluted) instead of the sample was added and in which all steps were performed, gave readings of near zero (0.000 to 0.003). Positivity was defined as absorbance of 0.1 or more.

Reproducibility of the assay was determined by duplicate testing. Sensitivity was determined by titration curve with twofold dilutions of a known amount of purified Shiga toxin and sonic extracts of SLT-I-producing bacteria. Specificity was determined by examining cross-reactivity with (i) other purified toxins (including cholera toxin and *E. coli* heat-stable enterotoxin [Sigma Chemical Co., St. Louis, Mo.]; *E. coli* heat-labile enterotoxin [B subunit], provided by John Mathewson, who originally obtained the toxin from J. Clements, Tulane Medical School, New Orleans, La.; and *C. difficile* cytotoxin [Barrels Immunodiagnostic Supplies, Bellevue, Wash.]), (ii) sonic extracts of well-characterized bacterial strains that produce SLT-II (*E. coli* C600 [933w]) or are non-cytotoxin producing (*E. coli* C600), and (iii) sonic extracts of a variety of other cytotoxin-producing bacterial isolates (Table 2).

Correlation with a quantitative HeLa cell assay was determined by simultaneous examination of purified toxin and sonic extracts by both methods. In addition, we examined samples obtained during various stages of Shiga toxin purification for cytotoxicity (CD50 per milligram of bacterial protein) and by Gb ELISA (micrograms of toxin) (blind format). The fractions were a crude sonic extract of *S. dysenteriae* serotype 1 strain 60R, a cytotoxin fraction eluted from an Affi-Gel Blue column, a cytotoxin fraction eluted by chromatofocusing, and purified Shiga toxin.
TABLE 2. Detection of Shiga toxin and SLT-I in sonic extracts of cytotoxin-producing bacteria by Gb3 ELISA and comparison with HeLa cell assay and DNA hybridization

<table>
<thead>
<tr>
<th>Bacterium (toxin)</th>
<th>Gb3 ELISA</th>
<th>HeLa cell cytotoxin assay</th>
<th>DNA probe for SLT-I</th>
</tr>
</thead>
</table>

**Reference strains**
- *S. dysenteriae* serotype 1 strain 60R (Shiga toxin) + + +
- *E. coli* O157:H7 (SLT-I) + + +
- *E. coli* O26:H11 strain H30 (SLT-I) + + +
- *E. coli* C600 (933w) (SLT-II) - - -

**Other bacteria (no. of strains)**
- *S. dysenteriae* serotype 1 (10) + + ND
- *Shigella sonnei* (13) - - -
- *Shigella flexneri* (13) - - -
- *Shigella boydii* (4) - - -
- Nonpathogenic *E. coli* (11) - - ND

**Pathogenic *E. coli***
- EAEC (11) - - ND
- EIEC (30) - - ND
- EPEC (6) - - ND
- ETEC (10) - - ND
- *E. coli* O26:H11 (SLT-I) (3) + + ND
- *E. coli* O157:H7 (SLT-I) (4) + + ND
- *E. coli* O157:H7 (SLT-II) (4) - - ND
- *Salmonella enteritidis* (30) - - -
- *Salmonella typhi* (12) - - -
- *Salmonella choleraesuis* (15) - - -
- *Aeromonas hydrophila* (30) - - -
- *Campylobacter jejuni* (25) - - ND
- *Citrobacter spp.* (5) - - ND

* All sonic extracts were examined with a protein concentration of 1 mg/ml. Positivity with Gb3 ELISA was defined as an A₅₆₀ of 0.1 or more. Positivity with the cytotoxicity assay was determined when neutralization with anti-Shiga toxin serum was demonstrated. DNA hybridization was performed with a probe for SLT-I (22), and positivity was determined by comparison with results for control strains.

* ND, Not determined.

* EAEC, Enteroadherent *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.

**RESULTS**

Gb3 ELISA. We found that the Gb3 concentration for coating the plates which gave highest sensitivity and reproducibility was 10 to 30 μg/ml; 10 μg/ml was then used in all further assays and for evaluation of the method. Coating was achieved after 4 h, but coated plates were kept for as long as 2 weeks without affecting the results. Because positive assays (optical density at 490 nm of 0.1 or more) had an easily detectable yellowish color, while the background was clear with a reading near zero, visual detection of positive results was possible. The sensitivity of the assay was determined by titration curve with different concentrations of purified Shiga toxin (Fig. 1). The assay was capable of detecting as little as 0.2 ng of toxin (100 μl of 2-ng/ml purified toxin). The assay was positive with sonic extracts of *S. dysenteriae* serotype 1 strain 60R (Shiga toxin), *E. coli* O26:H11 strain H30 (SLT-I), and *E. coli* O157:H7 (SLT-I) until a dilution of at least 1:256 (initial concentration, 1 mg of bacterial protein per ml) was achieved. In addition, culture supernatants were also examined and gave positive results, although optic densities suggest that the amount of toxin detected was about fivefold lower than that in sonic extracts.

![Graph showing purification of Shiga toxin](http://jcm.asm.org/ on October 28, 2017 by guest)
TABLE 3. Gb3 ELISA readings for purified toxins

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Conc (µg/ml)</th>
<th>A₄₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiga toxin</td>
<td>0.5</td>
<td>1.394</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>1</td>
<td>0.041</td>
</tr>
<tr>
<td><em>E. coli</em> heat-labile enterotoxin</td>
<td>1</td>
<td>0.040</td>
</tr>
<tr>
<td><em>E. coli</em> heat-stable enterotoxin</td>
<td>1</td>
<td>0.013</td>
</tr>
<tr>
<td><em>C. difficile</em> cytotoxin</td>
<td>1</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Specificity of the assay was determined by several experiments. Examination of other purified toxins (at a concentration of 1 µg/ml) showed no cross-reactivity with cholera toxin, *E. coli* heat-labile and heat-stable enterotoxins, and *C. difficile* cytotoxin (Table 3). Examination of other bacterial sonic extracts (1 mg of bacterial protein per ml) showed no cross-reactivity with sonic extracts of *E. coli* C600 (933w) (which produces SLT-II) or with the parent strain *E. coli* C600 (non-cytotoxin producing) (Table 2). In addition, we examined sonic extracts (at a concentration of 1 mg of bacterial protein per ml) of a variety of other cytotoxin-producing bacteria, including other shigellae, pathogenic and nonpathogenic *E. coli*, and strains of *Salmonella*, *Aeromonas*, *Campylobacter*, and *Citrobacter*. None of these sonic extracts showed positive results with the Gb3 ELISA.

Correlation of Gb3 ELISA with HeLa cell cytotoxicity assay and with DNA hybridization studies. Quantitative correlation with the cytotoxicity assay was determined by examination of fractions obtained at various stages of Shiga toxin purification with the two methods simultaneously. Increased specific activity (CD₅₀ per milligram of bacterial protein) during purification of the toxin resulted in simultaneous increased readings in the Gb3 ELISA and higher amounts of toxin detected (Fig. 2). Regression analysis indicated a high correlation between the two methods (r = 0.99, P < 0.01). The effect of heat treatment (100°C, 30 min) on purified Shiga toxin and sonic extracts of *E. coli* O26:H11 strain H30 (SLT-I) was examined. Heating caused inactivation of most cytotoxic activity in the HeLa cell assay (Fig. 3B) and at the same time reduced the absorbance in the Gb3 ELISA method to near zero (Fig. 3A). In addition, when various bacterial sonic extracts were examined, all extracts positive for Shiga toxin or SLT-I in the cytotoxicity assay (neutralized by rabbit antiserum to Shiga toxin) or hybridized with DNA probe for SLT-I were also positive in the Gb3 ELISA. Bacterial sonic extracts negative in the cytotoxicity assay (no neutralization with an anti-Shiga toxin serum) and in DNA hybridization assays were always negative in the Gb3 ELISA (Table 2).

**DISCUSSION**

Shiga toxin and SLTs are currently diagnosed by biologic assays. Three biologic activities have been ascribed to these cytotoxins: lethality in mice, fluid accumulation in ligated rabbit ileal loops, and toxic activity in several cell lines (8). Of these, the assays most frequently used to detect specific activity are based on cytotoxicity in HeLa or Vero cells. These assays are complicated, cumbersome, and time-consuming, and they require facilities for and experience with tissue cultures. They are usually based on cytotoxin-related morphologic changes of the cells or on radiolabeled assays. Identification of the specific cytotoxin also requires neutralization studies with the appropriate antiserum. In addition, the sensitivities of various cell lines to cytotoxic effects vary significantly (23). ELISA methods that use monoclonal antibodies to detect these cytotoxins have been described elsewhere (7, 14).

Recent data have shown that Shiga toxin, SLT-I, and SLT-II share the same binding receptor, Gb₃ (10, 16, 31). Because these well-defined toxins are emerging as the most important and clinically relevant cytotoxins produced by members of the family Enterobacteriaceae, we have developed a rapid diagnostic assay based on specific binding to this receptor; bound Shiga toxin or SLT-I was then detected immunologically. Gb₃ was obtained from the manufacturer in chloroform-methanol (2:1) solution. The Gb₃ was difficult to get into aqueous solution. Organic solvents were therefore used, and a special method for coating onto microdilution plates was necessary. A similar principle has been used to detect heat-labile enterotoxins by using their specific binding to the GM₁ receptor (30). However, since GM₁ is water soluble, it has been diluted in PBS, and a standard procedure has been used for coating.

The present study shows that the Gb₃ ELISA is sensitive and specific, detecting nanogram quantities of purified Shiga toxin. Sonic extracts of SLT-I-producing organisms (*E. coli* O26:H11 and *E. coli* O157:H7) were also positive in this assay. Production of SLT-I by these organisms was verified both by neutralization studies with specific antiserum and by DNA hybridization. The specificity of the assay was high in that no cross-reactivity was found with SLT-II or cytotoxins produced by a variety of other organisms, including other *Shigella* and *E. coli* strains and *Salmonella*, *Campylobacter*, and *Aeromonas* strains. Lack of SLT-I production was demonstrated in all of these strains by neutralization studies with specific antiserum in a HeLa cell assay, and in most of

**FIG. 2.** Correlation between specific activity and Gb3 ELISA readings of fractions obtained at various stages of Shiga toxin purification. Specific activity was determined with radiolabeled HeLa cell assay and expressed as CD₅₀ per milligram of protein. Readings of Gb₃ ELISA (A₄₉₀) were expressed as micrograms of purified Shiga toxin, obtained from the titration curve of purified toxin. The fractions examined were (dots from left to right) crude sonic extract of *S. dysenteriae* 1 strain 60R, cytotoxic fraction eluted from Affi-Gel Blue column, cytotoxic fraction eluted by chromatofocusing, and purified Shiga toxin. A high correlation was found between specific activity and Gb₃ ELISA readings (r = 0.99, P < 0.01).
them, SLT-I production was also demonstrated by DNA hybridization studies. Quantitative correlation with the radiolabeled HeLa cell assay was demonstrated by examination of fractions at the various stages of toxin purification by both methods. Because Gb3 is also the binding receptor of SLT-II (31), the latter toxin might also be detected by the same method by appropriate monoclonal antibodies to SLT-II. It would be very helpful if plates precoated with Gb3 could be used to detect the main cytotoxins, with specific identification of bound toxins by use of distinct antibodies.

The present Gb3 ELISA has obvious advantages over the HeLa or Vero cell assays: (i) it is inexpensive, simple, and rapid, can be performed in one working day, and can be used by relatively inexperienced personnel; (ii) there is no need for experience with or facilities for tissue cultures; (iii) Gb3, the monoclonal antibodies (American Type Culture Collection, cell line 13C4), and the second antibodies (rabbit anti-mouse immunoglobulin G) are commercially available; and (iv) objective results are obtained (optical density at 490 nm) rather than subjective interpretation of morphologic changes in cell lines.

The Gb3 ELISA seems comparable to the ELISA (7, 14). The methods described used anti-Shiga toxin monoclonal antibodies (7) or antibodies purified by immunoaffinity column chromatography (14) and then detection by enzyme immunoassay. The sensitivities of the assays were 0.06 ng/ml (7) and about 10 ng/ml (14), and the assays were specific when tested with other toxins. However, the entire Gb3 ELISA can be performed in one working day, the assay is based on commercially available materials, and it has the potential advantage of detecting additional cytotoxins that bind to Gb3 as their natural receptor.

Any rapid and simple method to detect this group of cytotoxins is of prime importance, as it may be also used by less-well-equipped laboratories, for example, in developing countries. This may significantly simplify epidemiologic studies of cytotoxin-related infections and increase our understanding of the role of these toxins in diarrheal disease and HUS.

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LITERATURE CITED