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Isolation and Characterization of Monoclonal Antibodies to Shiga-Like Toxin II of Enterohemorrhagic *Escherichia coli* and Use of the Monoclonal Antibodies in a Colony Enzyme-Linked Immunosorbent Assay

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The major obstacle in large-scale epidemiological investigations of the incidence of Shiga-like toxin (SLT)-producing *Escherichia coli* in diarrheal stools is the lack of a rapid, specific test to detect toxin. Enterohemorrhagic *E. coli* produces elevated levels of SLT-I, SLT-II, or both cytotoxins (also called Verotoxins). SLT-I but not SLT-II can be neutralized by antiserum to purified Shiga toxin and by monoclonal antibodies to the B subunit of SLT-I. In this study, monoclonal antibodies were generated against a crude preparation of SLT-II produced by an *E. coli* K-12 strain lysogenized with the 933W toxin-converting phage of enterohemorrhagic *E. coli* 933. Hybridoma culture supernatants were screened for anti-SLT-II antibodies by a cytotoxicity neutralization assay and by an enzyme-linked immunosorbent assay (ELISA). Of 53 ELISA-positive lines, 5 were capable of neutralizing the cytotoxicity of SLT-II but not of SLT-I, Shiga toxin, or a variant of SLT-II produced by *E. coli* that causes edema disease of swine. All five monoclonal antibodies immunoprecipitated the isolated A subunit of SLT-II but not the B subunit. Of these five neutralizing monoclonal antibodies, four were of the immunoglobulin M class and one belonged to the immunoglobulin G1 subclass. All five lines had κ light chains. These neutralizing monoclonal antibodies have been used as probes in a colony ELISA to detect SLT-II-positive bacterial colonies. The colony ELISA with these monoclonal antibodies is a specific, sensitive test with potential diagnostic value.

Enterohemorrhagic *Escherichia coli* (EHEC) strains which are primarily of serotypes O157:H7, O26:H11, and O111:H8 (15) have been incriminated as etiologic agents of hemorrhagic colitis and the hemolytic-uremic syndrome. Hemolytic-uremic syndrome is a generalized disease characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (10). Shiga-like toxins (SLTs), which are also called Verotoxins, have been implicated as possible virulence factors in the pathogenesis of these diseases as well as in edema disease of swine (20). There are several distinct *E. coli* SLTs (20). SLT-I and SLT-II are produced by EHEC and are enterotoxic for ligated rabbit ileal segments, paralytic and lethal for rabbits and mice, and cytotoxic for HeLa and Vero cells (21, 27). SLT-I and Shiga toxin share >99% deduced amino acid sequence homology (25), whereas SLT-I and SLT-II share about 60% deduced amino acid sequence homology (6). SLT-I and SLT-II are antigenically distinct (27). SLT-I can be neutralized by antiserum against purified Shiga toxin from *Shigella dysenteriae* 1 and monoclonal antibodies (MAbs) to the B subunit of SLT-I, whereas SLT-II cannot (27). Antisera to SLT-II do not neutralize Shiga toxin or SLT-I but do neutralize a variant of SLT-II (designated SLT-IIv) produced by strains of *E. coli* that cause edema disease (18). SLT-IIv is cytotoxic for Vero but not HeLa cells, distinguishing it from SLT-II (18).

The major obstacle in large-scale epidemiological investigations of the incidence of SLT-producing *E. coli* in diarrheal stools is the lack of a rapid, simple and specific test to detect toxin. In this paper, we describe the production and characterization of MAbs against SLT-II and their use in an enzyme-linked immunosorbent assay (ELISA) to detect SLT production by individual bacterial colonies.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* strains used in this study included clinical isolates from humans with diarrhea, hemorrhagic colitis, or hemolytic-uremic syndrome, calves with diarrhea, and pigs with edema disease (17, 18). The *E. coli* lysogen C600(933W) which produces SLT-II has been described previously (27).

Preparation of crude SLT-II. Crude SLT-II was prepared from toxin-converting phage plaque preparations. Confluent 933W coliphage plaque lawns were prepared by inoculation of Luria-Bertani agar plates with a mixture of an exponential culture of *E. coli* C600 and a stock filtrate that contained approximately 10^5 PFU of W phage per ml. The filtrate was prepared by inducing the W phage from *E. coli* C600(933W) with mitomycin C as described previously (18). The plaque lawns were then overlaid with SM buffer (16) and gently rocked on a platform shaker for 3 h at room temperature to elute phage and toxin from the agar. The phage were removed from the mixture by ultracentrifugation at 50,000 × *g* for 3 h. The clarified supernatant contained 10^5 to 10^6 HeLa cell 50% cytotoxic doses per ml (CD_{50}/ml) and was lethal for mice when inoculated intraperitoneally. Unlike SLT-I, SLT-II production is not suppressed in the presence of iron (27a).

Preparation of toxoids. Crude SLT-II was converted to a toxoid by either formaldehyde or glutaraldehyde treatment. To prepare SLT-II toxoid by exposure to formaldehyde, samples of toxin containing 100 μg of protein were treated

for 3 days at 37°C with 0.1 M Na_2HPO_4 (pH 8.0) containing 1% formaldehyde, and the residual formaldehyde was removed by dialysis against phosphate-buffered saline (PBS) (3). The resultant toxoid contained no residual toxicity for HeLa cells and was not lethal for mice. To prepare SLT-II toxoid by treatment with glutaraldehyde, crude toxin samples containing 50 μg of protein were incubated at 37°C in 0.11% glutaraldehyde in 0.1 M Na_2HPO_4 (pH 8.0) for 30 min (1). The resultant toxoid had less than 1% of the original cytotoxicity and was not lethal for mice.

Production of MAbs to SLT-II. Groups of 4- to 8-week-old female BALB/c mice were immunized intraperitoneally with 25 μg of formalinized toxoid or 25 μg of gluteraldehyde-treated toxoid in complete Freund adjuvant. Mice were boosted intraperitoneally three times at 7-day intervals with the same dose of toxoid in incomplete Freund adjuvant. The mice were periodically bled from the retro-orbital capillary plexus to assess the titers of neutralizing antibodies. Four weeks later, the mice were boosted intravenously with the same dose of toxoid in PBS via the tail vein. Three days later, two mice from each group were sacrificed by cervical dislocation. Spleen cells were prepared and fused to Sp2/0-Ag 14 mouse myeloma cells at a ratio of 10 spleen cells to 1 myeloma cell, according to published procedures (5). Fused cells were distributed into microdilution plates. Each microdilution plate well contained spleen cells from nonimmunized BALB/c mice as a feeder layer. After 3 to 4 weeks in culture, hybrid culture supernatants were assayed for toxin-specific antibodies by ELISA and by a cytotoxin neutralization assay with crude SLT-II as previously described (17). Cultures positive for neutralizing antibodies were expanded and cloned twice by limiting dilution. Assays with the MAbs were performed with either unconcentrated or concentrated hybridoma culture supernatants. Supernatants were concentrated by precipitation with 50% saturated ammonium sulfate (pH 7.0) followed by dialysis against 0.1 M phosphate buffer (pH 7.4) Isotypes were determined by immunodiffusion with class-specific antisera.

ELISA. An ELISA was developed to screen for binding antibodies. Crude toxin (25 μg/ml) in carbonate buffer (pH 8.8) was dispensed in 200-μl volumes into 96-well microdilution plates (Nunc-Immuno Plate I type; Nunc, Roskilde, Denmark). The plates were incubated overnight at 4°C and then washed six times in a high-salt washing buffer that contained (wt/vol) 2.92% NaCl, 0.02% KH_2PO_4, 0.29% $Na_2HPO_4 \cdot 12H_2O$, 0.02% KCl, and 1% Tween 20 adjusted to pH 7.4. Undiluted hybridoma culture supernatant (200 μl) was added to each well, and the plates were incubated at 37°C for 2 h. The plates were then washed six times with high-salt washing buffer. Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) + IgM (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was diluted 1:1,000 in high-salt washing buffer, and 50 μl of the diluted conjugate was added to each well. The plates were incubated for 2 h at 37°C and washed vigorously six times as described above. Peroxidase activity was detected colorimetrically after adding 100 μl of freshly made substrate-dye solution containing 0.04% *ortho*-phenylenediamine and 0.45% H_2O_2 in phosphate-citrate buffer (pH 5.0). *E. coli* C600 culture supernatant was included as a control so that only antibodies specific for the crude SLT-II were selected.

Colony ELISA for detection of SLTs. The colony ELISA was a modification of that described by Strockbine et al. (26). Bacterial colonies were inoculated onto modified syncase agar plates containing 1.5% Noble agar and 0.125 μg of trimethoprim-sulfamethoxazole per ml. After 20 to 24 h of growth at 37°C, the agar surface was overlaid with a dry nitrocellulose membrane (pore size, 0.45 μm). Care was taken to avoid trapping of any air bubbles. A 2-ml portion of polymyxin B sulfate solution (2 mg/ml of PBS) was then added, and the nitrocellulose membranes were incubated at 37°C for 1 h to allow the polymyxin B solution to permeate and release the toxin from the bacteria. The membrane was gently peeled off from the agar surface and washed with PBS to remove adherent bacterial debris. The membrane was then transferred into a glass petri dish containing 20 ml of Tris-buffered saline (pH 7.5) plus 3% gelatin. The membrane was kept in this gelatin solution for 1 h at room temperature with gentle agitation to block proteins from nonspecifically binding to the membrane. Next, the membrane was incubated for 18 to 20 h at room temperature with 20 ml of hybridoma culture supernatant diluted 1:10 in Tris-buffered saline–1% gelatin. Unbound antibody was removed by washing the membrane with PBS. The membrane was then incubated with 20 ml of peroxidase-conjugated goat anti-mouse IgG + IgM diluted 1:1,000 in Tris-buffered saline with 1% gelatin for 2 h at room temperature with gentle agitation. Unbound second antibody was removed as described for the primary antibody. Finally, the membrane was developed with 0.05% (wt/vol) 4-chloronaphthol and 0.015% (vol/vol) H_2O_2 in Tris-buffered saline at room temperature for 30 min.

Immunoprecipitation. Immunoprecipitation of toxin-MAb complexes with *Staphylococcus aureus* was performed according to published procedures (12, 13). Radioiodination of crude SLT-II was performed by the chloramine T method as described previously (21). Immunoprecipated complexes were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 or 15% polyacrylamide gels essentially as described by Laemmli (14). The gels were dried and exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens.

Western blot (immunoblot) analysis. The subunit specificities of the antitoxin MAbs were analyzed by using Western blotting (2). Crude SLT-II (50 μg) was subjected to SDS-PAGE in 15% polyacrylamide slab gels. The dissociated protein subunits were electrophoretically transferred (2 h at 200 mA) to nitrocellulose paper, which was then incubated at 4°C overnight in 0.1 M Tris hydrochloride (pH 7.4)–0.9% NaCl containing 10% bovine serum albumin. Next, the nitrocellulose sheet was incubated for 2 h at room temperature with undiluted hybridoma culture supernatant and then washed three times by rocking for 10 min in 0.1 M Tris hydrochloride–0.9% NaCl containing 0.05% Tween 20. The sheet was then incubated at room temperature with a 1:1,500 dilution of peroxidase-conjugated goat anti-mouse IgG + IgM serum and washed three times as described above. Peroxidase activity was detected colorimetrically by adding chromogen solution (0.1 M Tris hydrochloride–0.9% NaCl containing 0.05% [wt/vol] 4-chloronaphthol and 0.015% [vol/vol] H_2O_2) for 30 min.

Separation of A and B subunits. An ^{125}I-labeled crude toxin sample (200 μg of protein per ml) was treated with 10 M urea at 37°C for 30 min (23) followed by filtration with a 10,000-molecular-weight-cutoff microconcentrator (Centricon 10; Amicon Division, W. R. Grace and Co., Danvers, Mass.). The retentate and filtrate were collected separately and dialyzed against PBS extensively with 3,000-molecular-weight-cutoff dialysis tubing.

Toxin neutralization assay. The cytotoxin-neutralizing abilities of the MAbs were assayed on HeLa cells or Vero cells as previously described (17).

TABLE 1. Characterization of MAbs

MAb line[a]	Isotype	Cytotoxin-neutralizing activity against[b]:			
		Shiga	SLT-I	SLT-II	SLT-IIv
11F11	IgM(κ)	−	−	+	−
11G10	IgM(κ)	−	−	+	−
2E1	IgM(κ)	−	−	+	−
10E10	IgM(κ)	−	−	+	−
11E10	IgG1(κ)	−	−	+/−	+/−

[a] MAbs all specific for subunit A.
[b] Ability of undiluted hybridoma culture supernatants to neutralize 10 CD_{50} of cytotoxin. +, Full protection from cytotoxic activity; −, no protection from cytotoxic activity; +/−, partial protection from cytotoxic activity.

RESULTS

Production of MAbs to SLT-II. Twenty microdilution plates were seeded with mouse myeloma-spleen cell fusion mixtures, and more than 900 wells showed growth after 3 to 4 weeks of incubation. Culture supernatants from these hybrids were tested in an ELISA as well as in a toxin neutralization assay on HeLa cells to detect both binding and neutralizing antibodies. Fifty-three hybrid culture supernatants were positive by ELISA for binding antibodies against crude SLT-II; ten of these were positive by the toxin neutralization assay. From these 10 hybrids, 5 stable clones were selected for further characterization (Table 1). All five of these hybrids were obtained from spleens of mice immunized with the formalinized SLT-II toxoid. Four of the five hybridoma lines, designated 11f11, 11G10, 2E1 and 10E10, were of the IgM class, while the fifth, 11E10, belonged to the IgG1 subclass. All five lines had κ light chains.

Characterization of MAbs. The anti-SLT-II MAbs were tested for their capacity to immunoprecipitate ^{125}I-labeled crude SLT-II. Four of the SLT-II-neutralizing MAbs precipitated two peptides with approximate molecular weights of 33,000 and 8,000 (Fig. 1). The fifth cytotoxin-neutralizing MAb (11E10) was not included on the gel shown in Fig. 1 but did immunoprecipitate two peptides of the same molecular weights on a separate gel (not shown). The sizes of the immunoprecipitated peptides are consistent with the reported molecular weights of the A and B subunits of SLT-II (6, 28). These data reveal that the five SLT-II-neutralizing MAbs can immunoprecipitate the SLT-II holotoxin. To define the subunit specificity of the MAbs, Western blot analyses were attempted with all five of the MAbs. None of the MAbs were able to detect the denatured toxin proteins transferred to nitrocellulose membranes after SDS-PAGE. By contrast, polyclonal antiserum to crude SLT-II was able to detect the denatured toxin (data not shown). These findings suggest that the MAbs recognize conformational rather than sequence-determined epitopes. Next, we attempted to immunoprecipitate urea-treated fractions of crude SLT-II. Such treatment has been shown to separate the A and B subunits of Shiga toxin (23). In the higher-molecular-mass urea-treated fraction (over 10,000 daltons), all five of the MAbs precipitated a protein of 33,000 daltons (Fig. 2). Immunoprecipitation performed on the lower-molecular-mass fraction (less than 10,000 daltons) did not precipitate any protein (Fig. 2). These results indicate that the

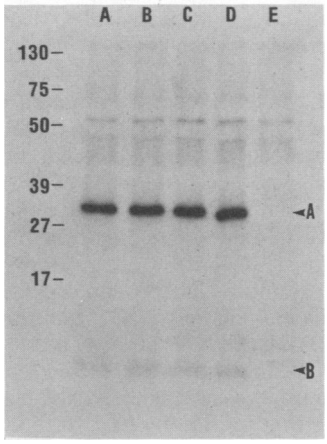

FIG. 1. Immunoprecipitation of ^{125}I-labeled crude SLT-II by MAbs. The immunoprecipitants were resolved by SDS-PAGE on a 10% polyacrylamide gel. Lanes A, B, C, and D contain the immunoprecipitants obtained with anti-SLT-II MAbs 11F11, 11G10, 2E1, and 10E10, respectively. Lane E contains the precipitate obtained with anti-cholera toxin MAb 32D3. The numbers on the left indicate molecular masses (in kilodaltons) of the polypeptide standards. A and B to the right of lane E indicate positions of A and B subunits.

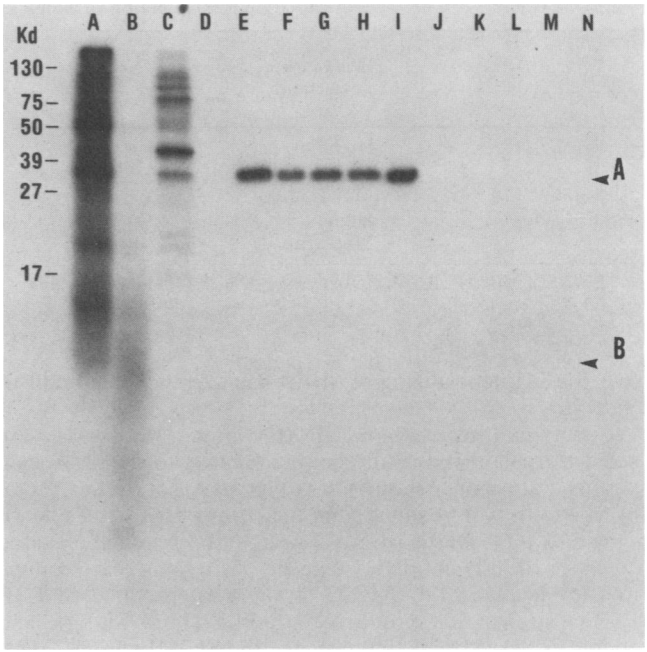

FIG. 2. Immunoprecipitation of ^{125}I-labeled crude SLT-II by MAbs after urea treatment and fractionation. The immunoprecipitants were resolved by SDS-PAGE on 15% polyacrylamide gels. Lane A, Crude SLT-II immunoprecipitated with polyclonal rabbit anti-SLT-II raised against crude SLT-II. Lane B, Lower-molecular-mass fraction obtained after treatment of crude SLT-II with urea. Lane C, Higher-molecular-mass fraction obtained after treatment of crude SLT-II with urea. Lane D, Crude SLT-II (not urea treated or fractionated) immunoprecipitated with anti-cholera toxin MAb 32D3. Lanes E, F, G, H, and I, Immunoprecipitants obtained when anti-SLT-II MAbs 11F11, 11G10, 2E1, 10E10, and 11E10, respectively, were used to immunoprecipitate the higher-molecular-mass fraction obtained after treatment of crude SLT-II with urea. Lanes J, K, L, M, and N, Immunoprecipitants obtained when anti-SLT-II MAbs 11F11, 11G10, 2E1, 10E10, and 11E10, respectively, were used to immunoprecipitate the lower-molecular-mass fraction obtained after treatment of crude SLT-II with urea. The numbers on the left indicate molecular masses (in kilodaltons) of the polypeptide standards. A and B to the right of lane N indicate positions of A and B subunits.

TABLE 2. Detection of Shiga-like toxins by colony ELISA with MAbs

Type of Shiga-like toxin	Level of toxin production[a]	No. of strains tested	No. of *E. coli* strains positive with MAb to:			
			SLT-I (13C4)	SLT-II (11E10)	SLT-II (2E1)	CT[b] (32D3)
I	High	21	21	0	0	0
I	Low	10	0	0	0	0
II	Moderate	10	0	7	6	0
I + II	High	18	18	17	18	0
IIv	Moderate	9	0	0	0	0

[a] Levels of cell-associated cytotoxin were defined as follows: low, $<2 \times 10^1$ to 6×10^2 CD_{50}/ml; moderate, 10^3 to 10^4 CD_{50}/ml; high, $>10^5$ to 10^8 CD_{50}/ml.
[b] CT, Cholera toxin.

five SLT-II-neutralizing MAbs recognize the A subunit of SLT-II.

Colony ELISA. MAbs 11E10 (IgG1) and 2E1 (IgM) were selected to be used in the colony ELISA to detect *E. coli* strains that produce moderate or high levels of SLTs. A total of 68 strains were evaluated in the colony ELISA (Table 2). These strains included EHEC strains that produce elevated levels of SLT-I, nonpathogenic *E. coli* strains that produce low levels of SLT-I, EHEC strains that produce SLT-II, EHEC strains that produce both SLT-I and SLT-II, and edema disease strains that produce the SLT-II variant. MAb 13C4 against the B subunit of SLT-I (26) and MAb 32D3 against the B subunit of cholera toxin (4) (kindly provided by R. Holmes, Uniformed Services University of the Health Sciences) were included as controls in the assay. Previous results by Strockbine et al. (26) showed that MAb 13C4 could detect EHEC strains that produce high levels of Shiga-like toxins (including strains that make SLT-I only or both SLT-I and SLT-II) but not *E. coli* strains that produce low levels of SLT-I. These findings were confirmed in this study (Table 2). As expected, MAb 32D3 to the B subunit of cholera toxin did not recognize any of the *E. coli* strains tested. As shown in Table 2, MAb 11E10 to SLT-II detected 7 of 10 *E. coli* strains that produced only SLT-II, whereas MAb 2E1 detected 6 of these strains. The same three SLT-II-producing *E. coli* strains that were not detected by MAb 11E10 were also not detected by MAb 2E1 (Table 2) or the three other neutralizing MAbs (data not shown). MAb 11E10 detected 17 of 18 strains that made SLT-I and SLT-II, whereas MAb 2E1 detected all 18 strains. Neither of the MAbs to SLT-II was able to detect strains which produced only SLT-I or only SLT-IIv.

DISCUSSION

All the neutralizing MAbs generated in the present study recognized the A subunit of SLT-II, which suggests that the A subunit of the SLT-II toxoid was more immunodominant than the B subunit. In previous studies involving the production of MAbs to Shiga and Shiga-like toxins (3, 26), most of the MAbs were directed against the B subunit of the toxin. One explanation for these differences in immunodominant subunits among the Shiga-like toxin family is that the tertiary structures of Shiga toxin and SLT-I may differ from that of SLT-II. Alternatively, the process of inactivating SLT-II with formaldehyde or glutaraldehyde could have altered the conformation of the native SLT-II molecule such that either the A subunit epitopes which are normally masked became exposed or, conversely, the B subunit epitopes which are normally exposed became masked. Another difference between MAbs generated against SLT-II and those previously generated against SLT-I and Shiga toxin is that the SLT-II MAbs were primarily of the IgM class, whereas the SLT-I and Shiga toxin MAbs were of the IgG class. This may reflect the relatively short immunization schedule followed in immunizing the mice with SLT-II toxoid.

Immunoprecipitation of the SLT-II holotoxin revealed a more heavily iodinated A subunit than B subunit, in contrast to previous reports on Shiga toxin (22) and *E. coli* SLT-I (21, 26) in which the B subunit was more heavily iodinated than the A subunit. A differential iodination of Shiga toxin, SLT-I, and SLT-II B subunits due to differences in the number of tyrosine residues is not tenable based on the recently published nucleotide and deduced amino acid sequences for SLT-I (7) and SLT-II (6); the SLT-I B subunit and the SLT-II B subunit have two tyrosine residues each. However, differences in the number of tyrosine residues in the A subunit between Shiga toxin–SLT-I and SLT-II could contribute to enhanced iodination of the SLT-II A subunit, because the SLT-II A subunit contains 11 tyrosine residues, whereas the Shiga toxin and SLT-I A subunits contain only 7 tyrosine residues.

Epidemiological evidence strongly suggests that the SLTs of *E. coli* play a role in disease in both humans and animals (8, 10, 11, 17–19, and 24), although no direct proof for the involvement of SLTs in pathogenesis has yet been demonstrated. Because of this close association between SLTs and disease, an efficient and sensitive assay for detecting such toxin-producing *E. coli* strains in stools would be of value both clinically and epidemiologically. Karmali et al. (9) described a method for detecting Verotoxin (Shiga-like toxin)-producing *E. coli* strains in mixed stool cultures by a combination of colony sweeps (loopfuls of confluent bacterial growth) and polymyxin extraction of cell pellets. This is a sensitive assay capable of detecting both SLT-I- and SLT-II-producing *E. coli* strains. However, tissue culture facilities may not be available in many clinical laboratories, especially those in underdeveloped and developing countries. We previously described (26) a colony ELISA that can detect *E. coli* strains which produce high levels of SLTs, including strains making only SLT-I and those making both SLT-I and SLT-II. EHEC strains producing SLT-II at moderate levels could not be detected with the MAb to SLT-I used in that assay. The MAbs developed in the present study permit the colony ELISA to be upgraded so as to detect most strains of *E. coli* which produce only SLT-II as well.

Three observations from the colony ELISA should be noted. First, only *E. coli* producing moderate to high levels of Shiga-like cytotoxins can be detected (Table 2) (26). The fact that the assay does not detect low-level SLT producers is not a disadvantage because *E. coli* which produces low levels of SLT-I, unlike *E. coli* which produces moderate and

high levels of SLT-I, is not implicated in disease (17). Second, of the SLT-II-producing *E. coli* strains tested, all high-level cytotoxin producers were detected by at least one of the MAbs to SLT-II. However, it should be emphasized that SLT-II-producing strains in the high-level category ($>10^5$ to 10^8 CD_{50}/ml of cytotoxin in bacterial sonic lysate) are actually producing both SLT-I and SLT-II (17). The high level of cytotoxin made by these dual producers is a result of SLT-I synthesis; their SLT-II production is comparable with that of all other SLT-II-producing *E. coli* strains in the moderate-level category (10^3 to 10^4 CD_{50}/ml). Third, three of the strains that produced only SLT-II did not react in the colony ELISA with any of the neutralizing MAbs to SLT-II. We are examining the toxins of these three strains for possible antigenic differences with the prototype SLT-II of *E. coli* C600(933W). These three strains may produce SLTs more like the SLT-IIv of pig edema disease strains. In support of this possibility is the observation that none of the nine SLT-IIv-producing edema disease strains were detected by the MAbs to SLT-II in the colony ELISA. The existence of an antigenic variant among the SLT-II subfamily will necessitate the generation of MAbs to such a variant (or variants) to fulfill the goal of detecting all high- to moderate-level SLT-producing *E. coli*.

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