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Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*

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Three monoclonal antibodies, designated MAb 16E6, MAb 13C4, and MAb 19G8, were produced which recognize Shiga-like toxin (SLT) from *Escherichia coli*. All three monoclonal antibodies neutralized the cytotoxicity of *E. coli* SLT and were able to immunoprecipitate intact labeled toxin with *Staphylococcus aureus* protein A. The three antibodies were of the G1 heavy and kappa light chain classes. MAb 16E6 bound to the B subunit of SLT in Western blots and also neutralized the lethality of the toxin for mice and the enterotoxicity of the toxin in ligated rabbit ileal loops. The ability of MAb 16E6 to neutralize the cytotoxicity, lethality, and enterotoxicity of *E. coli* confirms the hypothesis that all three activities are associated with a single toxin. MAb 16E6 and MAb 13C4 also neutralized the cytotoxicity of purified Shiga toxin from *Shigella dysenteriae* type 1 and Shiga-like toxic activities in crude cell extracts from *Shigella flexneri*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Salmonella typhimurium*. Thus, Shiga toxin and the SLTs from *E. coli*, *Shigella flexneri*, *V. cholerae*, *V. parahaemolyticus*, and *Salmonella typhimurium* share a common B subunit epitope that is involved in neutralization. MAb 13C4 has been successfully used in a colony blot assay for the detection of bacterial colonies which produce high levels of SLT. Sixty-two different strains of bacteria were tested by both the cytotoxicity and colony blot assays for the presence of SLT. The colony blot assay detected all strains of bacteria which produce $\geq 10^5$ 50% cytotoxic doses of SLT per ml of sonic lysate. There were no false-positive results among the 62 samples tested.

In addition to its heat-stable and heat-labile enterotoxins, *Escherichia coli* has been reported to produce another toxin which has enterotoxic activity. This toxin, referred to either as Verotoxin or *Shigella dysenteriae* 1 (Shiga)-like toxin (SLT), was first described in 1977 by Konowalchuk et al. (11). Like Shiga toxin, the SLT of *E. coli* is enterotoxic for ligated rabbit ileal loops, paralytic and lethal for mice, and cytotoxic for selected cell lines in vitro (18). The cytotoxicity of SLT is also neutralized by polyclonal antiserum against Shiga toxin (20). SLT contains one A subunit and several copies of a B subunit (18). However, the function of each subunit of SLT has not been directly demonstrated. Studies with the prototype Shiga toxin (4, 23) indicate that its A subunit contains the biologically active portion of the toxin while its B subunit contains binding component(s). The SLT of *E. coli* has been implicated as a possible virulence factor in the pathogenesis of certain diarrheal diseases (3, 27, 28, 32), hemorrhagic colitis (12, 13, 21, 25), and hemolytic uremic syndrome (10, 29). However, collection of epidemiological data on the association of SLT production with disease has been hampered by the fact that the methods currently available for screening of *E. coli* for SLT are expensive, time consuming, and labor intensive. Moreover, attempts to develop immunochemical tests to detect toxin-producing bacteria in our laboratory have been hampered by the presence of cross-reacting antibacterial antibodies in polyclonal antitoxin and normal sera. The use of monoclonal antibodies rather than polyclonal antisera should improve the specificity of immunochemical tests for the detection of SLT.

Monoclonal antibodies have been produced against the heat-stable enterotoxin of *E. coli* (2), heat-labile toxin of *E. coli* (1), cholera toxin from *V. cholerae* (9, 15, 24, 26), and Shiga toxin from *Shigella dysenteriae* type 1 (4, 8). These monoclonal antibodies have been helpful in cross-neutralization studies, epitope-mapping experiments, and in the development of rapid diagnostic tests for the presence of various antigens in patient and food samples. In this paper, we describe the production and characterization of three monoclonal antibodies against SLT and the successful use of one of these antibodies in the development of an assay to detect high-SLT-producing bacteria growing on agar plates.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study were obtained from J. G. Wells, J. J. Farmer, and I. K. Wachsmuth of the Center for Disease Control, Atlanta, Ga.; M. A. Karmali of the Hospital for Sick Children, Toronto, Ontario, Canada; S. B. Formal of the Walter Reed Army Institute of Research, Washington, D.C.; H. W. Smith of the Houghton Poultry Research Station, Huntingdon, Cambridgeshire, United Kingdom; L. R. Trabulsi of the Escola Paulista de Medicina, Sao Paulo, Brazil; D. Sherwood of the Moredun Research Institute, Edinburgh, United Kingdom; M. Levine and J. Kaper of the Center for Vaccine Development, Baltimore, Md.; T. Mapes of the National Naval Medical Center, Bethesda, Md.; and lysogens constructed in this laboratory. The strains were stored frozen at −70°C and maintained in the laboratory by periodic transfer on tryptic soy agar slants.

Toxin purification. Shiga toxin from *Shigella dysenteriae* type 1 strain 60R and SLT from *E. coli* H30 were prepared and purified by published techniques (18, 19).

Production and purification of monoclonal antibodies. BALB/c mice (female, 6 to 8 weeks old) were immunized with purified, biologically active SLT from *E. coli* H30. The mice were periodically bled from the orbital plexus to assess the titers of neutralizing antibody. Four mice were immunized with 0.4 to 1.6 μg of purified toxin on days 1, 37, 54, 83, and 101. The toxin was administered intraperitoneally in

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Freund complete adjuvant (1:1; Difco Laboratories, Detroit, Mich.) (1:1) on day 1, subcutaneously in Freund incomplete adjuvant (1:1; Difco) on day 37, intraperitoneally in phosphate-buffered saline (PBS) on day 54, and intravenously in PBS on days 83 and 101. On day 105, two mice were sacrificed by cervical dislocation for use in fusion 1. To the remaining mice, toxin (1.0 μg in PBS) was administered intraperitoneally on days 174, 175, 176, and 177. On day 178, toxin (1.0 μg in PBS) was administered intravenously, and on day 179, the mice were sacrificed for fusion 2.

Cell fusion with splenocytes and SP2/0-Ag 14 myeloma cells, selection, and growth of hybrids were performed as described previously (16). Cell culture supernatants were screened for toxin-specific antibodies by a cytotoxin neutralization assay with purified *E. coli* H30 toxin as previously described (6). Positive hybrids were subcloned twice by limiting dilution at 1 and 0.1 cells per well and retested for antibody production by neutralization. Isotypes were determined by immunodiffusion with class-specific antisera (Litton Bionetics, Kensington, Md.).

Assays with monoclonal antibodies were performed with either unconcentrated or concentrated culture supernatants. Concentrated culture supernatants were prepared by precipitation with 50% saturated ammonium sulfate (pH 7.0) followed by dialysis against 0.1 M phosphate buffer (pH 7.4).

Immunoprecipitation of SLT by monoclonal antibodies. Immunoprecipitation of toxin-monoclonal antibody complexes with *Staphylococcus aureus* was performed as described previously (30). Radioiodination of purified SLT from *E. coli* H30 was performed by the chloramine T method by published procedures (18). Proteins immunoprecipitated by the monoclonal antibodies were resolved by electrophoresis on sodium dodecyl sulfate–15% polyacrylamide gels (14) and identified by fluorography in the presence of X-Omatic regular intensifying screens (Eastman Kodak Company, Rochester, N.Y.).

Immunoblot analysis of monoclonal antibodies. Purified SLT from *E. coli* H30 was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 15% acrylamide gels (approximately 50 μg of toxin per gel) (14). When electrophoresis was completed, the gels were removed, and the separated toxin subunits were electrophoretically transferred to nitrocellulose paper (pore size, 0.45 μm; BA 85; Schleicher & Schuell, Inc., Keene, N.H.) BA 85, 0.45 μm) essentially as described by Towbin et al. (31). The nitrocellulose paper-gel sandwich was electrophoresed overnight under a constant current of 150 mA at 10°C. After electrophoresis, the nitrocellulose paper was stained immunochemically or with amido black (0.1% [wt/vol]). All manipulations of the nitrocellulose paper were done at room temperature. Immunochemical staining was performed by first blocking the paper with 3% (wt/vol) gelatin in Tris-buffered saline (TBS; 20 mM Tris hydrochloride, 500 mM NaCl; pH 7.5) for 30 min and then incubating it with monoclonal antibodies (undiluted culture supernatants). The nitrocellulose paper was then washed with TBS (three 15-min washes with 200 ml of buffer per wash) and subsequently stained for 3 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (whole molecule) (Sigma Chemical Co., St. Louis, Mo.) diluted 1:2,000 in TBS–1% (wt/vol) gelatin. The nitrocellulose paper was washed as described above and developed in TBS containing 0.05% (wt/vol) 4-chloro-1-napthol and 0.015% (vol/vol) hydrogen peroxide for 30 min.

Biological assays. (i) Rabbit ileal loop assay. Ammonium sulfate-concentrated culture supernatants of monoclonal antibody 16E6 (MAb 16E6) and MAb 32D3 (negative control, IgG1/kappa anti-cholera toxin; for additional description see reference 9) were tested for their ability to neutralize the enterotoxic effects of purified SLT from *E. coli* H30. Three milliliters of antibody solution and 3 ml of toxin (0.66 μg of toxin per ml diluted in PBS containing 0.1% [wt/vol] gelatin) were mixed and incubated 1 h at 37°C and overnight at 4°C. The antibody-toxin solution (1 ml per loop) was tested for enterotoxicity in the rabbit ileal loop assay by published procedures (5).

(ii) Cytotoxicity assay for SLT. Sonic lysates at various dilutions or culture supernatants at a 1:100 dilution were tested for cytotoxicity on HeLa cells as previously described (6). Neutralization of Shiga toxin and SLT was performed by published procedures (6). Neutralization of the cytotonic effect of heat-labile enterotoxin (LT), LT-like toxin, and cholera toxin for Y-1 adrenal cells was also performed by published procedures (7, 9).

(iii) Mouse lethality. Ammonium sulfate-concentrated culture supernatants of MAb 16E6 and MAb 32D3 were tested for their ability to neutralize the lethality for mice of purified SLT from *E. coli* H30. The samples were prepared by using the same antibody and toxin stock solutions used for the enterotoxicity neutralization study. One microgram of toxin was mixed with the undiluted monoclonal antibody solution and incubated as described for the enterotoxicity assay. The neutralized samples were tested for lethality in CD-1 mice (female, 30 to 35 g; Charles River, Kingston, N.Y.) as previously described (22).

Colony blot assay for the detection of SLT. Syncase agar plates were prepared by mixing modified syncase broth (20) with Noble agar (final concentration, 1.5% [wt/vol]; Difco) and Chelex 100 (final concentration, 2% [wt/vol]; Bio-Rad Laboratories, Richmond, Calif.) for 2 h at room temperature with stirring. After Chelex treatment, the whole mixture (agar and Chelex) was autoclaved at a pressure of 15 lb/in^2 and a temperature of 121°C for 30 min. Immediately before the plates were poured, trimethoprim and sulfamethoxazole (Septra; intravenous infusion, 16 mg of trimethoprim per ml and 80 mg of sulfamethoxazole per ml; Burroughs-Wellcome Co., Research Triangle Park, N.C.) was added to give a final concentration of 0.021 μg of trimethoprim per ml and 0.104 μg of sulfamethoxazole per ml in the agar.

Bacterial colonies were inoculated on Chelex-treated syncase agar containing trimethoprim and sulfamethoxazole and incubated 18 to 24 h at 37°C. Dry nitrocellulose membranes (diameter, 82 mm; pore size, 0.45 μm; Millipore Corp., Bedford, Massachusetts, or Schleicher & Schuell) were then placed on top of the colonies and allowed to absorb moisture from the plates. Bubbles trapped between the nitrocellulose membranes were expressed manually, and 2 ml of polymyxin B solution (2 mg of polymyxin B sulfate [Sigma] per ml in PBS) were layered over the membranes and allowed to permeate the membranes for 45 min at 37°C to release toxin from the bacteria. The liberated toxin was bound by the membrane, and the bacterial debris adhering to the membranes was washed off with TBS (pH 7.5). Identical membranes were prepared for staining with positive and negative monoclonal antibodies. Precautions for infectious material were taken during the blotting and staining procedures since viable organisms had been observed to remain on the plates after the polymyxin B treatment.

After the toxin was released from the bacteria, the blots were stained immunochemically for the presence of SLT. Immunochemical staining was performed as follows: (i) the blots were blocked with 3% (wt/vol) gelatin in TBS for 30

TABLE 1. Characterization of monoclonal antibodies

Monoclonal antibody	Characteristic[a]			
	Holotoxin immunoprecipitation	Neutralization of the following SLT activity		
		Cytotoxicity	Enterotoxicity	Mouse lethality
MAb 16E6	+	+	+	+
MAb 13C4	+	+	NT	+
MAb 19G8	+	+	NT	NT

[a] All three monoclonal antibodies are of the IgG1/kappa isotype. MAb 16E6 and MAb 13C4 exhibited binding specificity for B subunit in Western blots. MAb 19G8 was not reactive. NT, Not tested.

min at room temperature with gentle agitation, and (ii) the blots were incubated for 2 to 3 h at room temperature or overnight at 4°C with either monoclonal antibodies (positive or negative) or rabbit sera (immune or normal). A 1:10 dilution (in TBS–1% [wt/vol] gelatin) of culture supernatants containing monoclonal antibodies or a 1:2,500 dilution of rabbit serum was used in the first incubation.

Unbound antibody was removed by three 15-min washes with TBS at room temperature. The blots were then incubated with an enzyme-linked second antibody (1:3,000 dilution of goat anti-mouse IgG [heavy and light chain]-horseradish peroxidase conjugate in TBS–1% [wt/vol] gelatin; Bio-Rad Laboratories) for 1.5 to 2 h at room temperature with gentle agitation. Unbound second antibody was removed as described above. The blots were developed with 0.05% (wt/vol) 4-chloro-1-napthol and 0.015% (vol/vol) hydrogen peroxide in TBS at room temperature with gentle agitation for 30 min. After development, the membranes were rinsed with deionized water and photographed while wet.

RESULTS AND DISCUSSION

Isolation and characterization of monoclonal antibodies. Three hybrid cell lines, designated 16E6, 13C4, and 19G8, were recovered and cloned from two fusions involving the SP2/0-Ag 14 myeloma cell line and spleen cells from BALB/c mice immunized against purified, biologically active SLT from *E. coli* H30. Approximately 2,000 hybrids were screened from the two fusions. Hybrid cell line 16E6 was isolated from fusion 1, and lines 13C4 and 19G8 were derived from fusion 2.

The limiting factor in the development of monoclonal antibodies against SLT was the availability of purified toxin for immunizations, screening assays, and characterization studies. The purification of SLT routinely took 3 weeks, and the yield of pure toxin from an 8-liter culture was only approximately 200 μg. Large amounts of toxin were expended in an attempt to develop an enzyme-linked immunosorbant assay for the detection of nonneutralizing antibodies. These efforts were, in practical terms, not successful, due to the need for quantities of toxin that were not feasible for routine screening. The only sensitive and specific assay available in our laboratory for identifying antitoxin antibodies was the neutralization assay which was used as the screening assay for both fusions. Genetic studies are in progress to obtain larger quantities of holotoxin as well as A and B subunits alone. Once available, these reagents should facilitate the production of additional monoclonal antibodies for epitope-mapping studies.

Table 1 summarizes the results of the antibody characterization studies. All three antibodies were of the IgG1 heavy chain and kappa light chain class. All three antibodies immunoprecipitated the holotoxin (Fig. 1) and neutralized the cytotoxicity of the toxin (Table 1). Western blot analysis showed that MAb 16E6 and MAb 13C4 recognized the B subunit of the toxin (Table 1; data not shown). MAb 16E6 was also shown to neutralize both the lethality of the toxin for mice and the enterotoxicity of the toxin in ligated rabbit ileal loops. The neutralization of the three biological activities of purified SLT by MAb 16E6 supports the hypothesis that the three activities are associated with a single toxin molecule and that neutralization of these three activities by this monoclonal antibody involves a determinant on the B subunit.

Neutralization studies with MAb 16E6 and MAb 13C4 were performed to evaluate the antigenic relatedness of several enterotoxins. MAb 16E6 and MAb 13C4 neutralized the cytotoxicity of purified Shiga toxin from *Shigella dysenteriae* type 1 strain 60R; purified SLT from *E. coli* H30 (026); and crude SLT from *E. coli* 933 (O157:H7), *E. coli* H19, *E. coli* (K-12) C600, *Shigella flexneri* M4243, *V. parahaemolyticus* FC1011, *V. cholerae* JBK70, and *Salmonella typhimurium* W118. In Y-1 adrenal cells, MAb 16E6

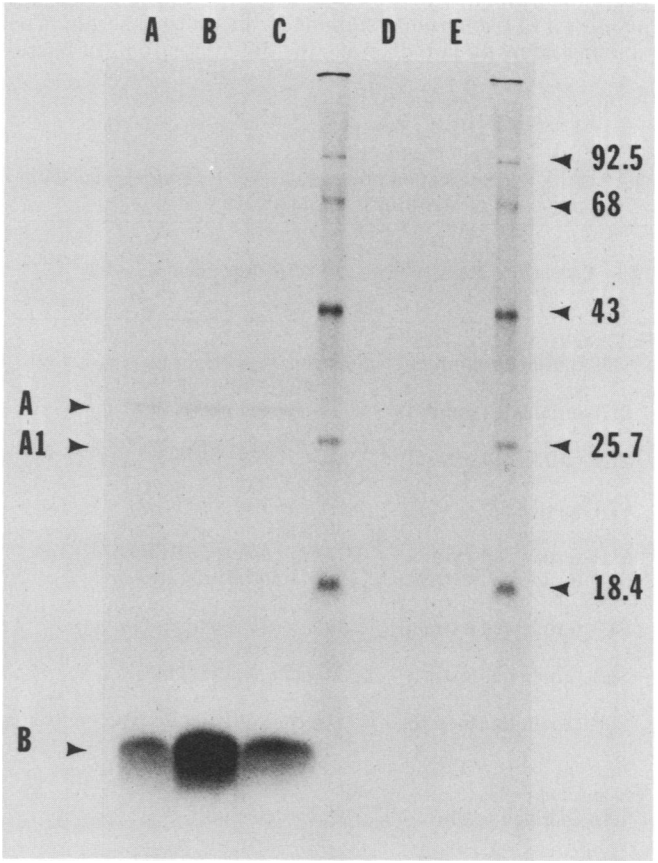

FIG. 1. *Staphylococcus aureus* protein A immunoprecipitation of ^{125}I-labeled SLT by monoclonal antibodies. Lanes A, B, and C contain the immune precipitates obtained with anti-SLT MAb 16E6, MAb 13C4, and MAb 19G8, respectively. Lanes D and E contain the precipitates obtained with anti-cholera toxin MAb 32D3 and MAb 34B6, respectively. ^{14}C-labeled molecular weight standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) are included in the center and side lanes.

and MAb 13C4 did not neutralize the cytotonic activity of purified cholera toxin, purified LT of porcine or human origin, or LT-like toxin from *E. coli*. These neutralization studies confirm earlier observations (20) that Shiga toxin and SLT are antigenically related and that *Shigella flexneri*, *V. parahaemolyticus*, *V. cholerae*, and *Salmonella typhimurium* produce SLT (17).

Rapid epidemiologic screening for *E. coli* strains that produce high levels of SLT. To facilitate the detection of bacteria producing high levels of SLT, a colony blot assay was developed. Two events contributed to the successful development of the colony blot assay. These were (i) the production of SLT-specific monoclonal antibodies and (ii) the observation that subinhibitory concentrations of the antibiotics trimethoprim and sulfamethoxazole increased the production of toxin for *E. coli* 933 (H. Karch, A. D. O'Brien, P. Goroncy, and W. Opferkuch, Abstr. Annu. Meet. Am. Soc. Microbiol., 1985. Blll, p. 36).

Figure 2 compares the results of colony blot assays performed with monoclonal antibodies and with rabbit polyclonal antisera. SLT in 33 of the 62 different strains tested was detected by MAb 13C4. There was no background staining with MAb 32D3, the negative control antibody (IgG1/kappa) directed against the B subunit of cholera toxin (9). In contrast, SLT in bacterial colonies stained with the polyclonal rabbit sera was not readily discernible due to a high level of background staining by the normal serum. The staining intensity noted among the different strains of bacteria tested by using the polyclonal antisera did not correlate with the presence of SLT.

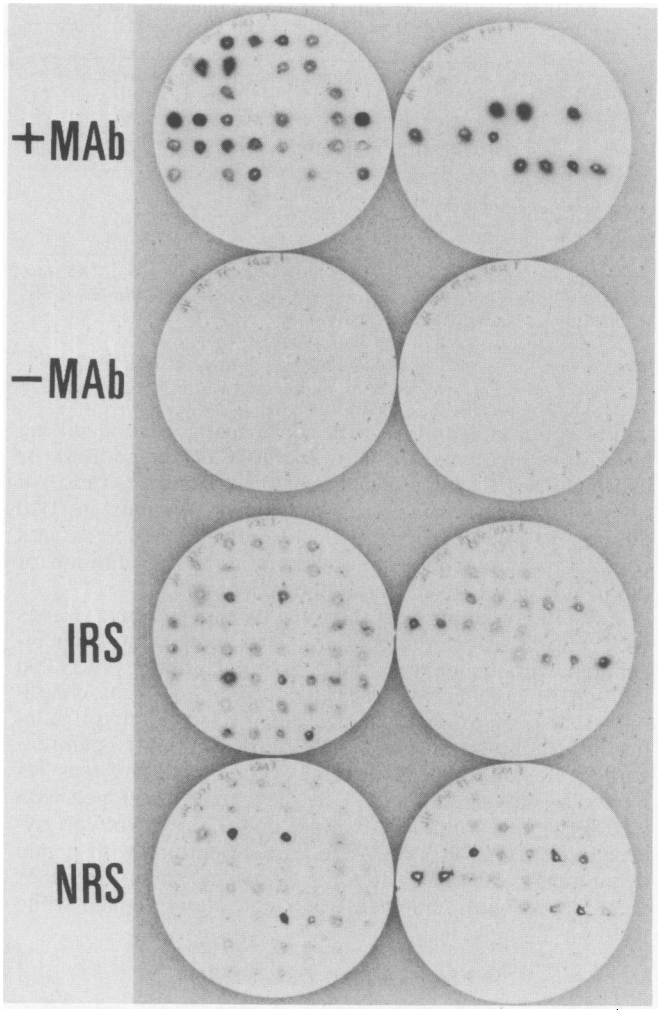

FIG. 2. Colony immunoblot assay for the detection of SLT in bacteria on agar plates. Nitrocellulose membranes containing polymyxin B-released toxin were stained with monoclonal antibodies against SLT (13C4, +MAb), monoclonal antibody against cholera toxin (32D3, −MAb), anti-Shiga toxin immune rabbit serum (IRS), or normal rabbit serum (NRS). The monoclonal antibodies were used at a 1:10 dilution of the culture supernatants, and the rabbit sera were used at a 1:2,500 dilution.

TABLE 2. Neutralization of several bacterial enterotoxins with MAb 16E6 and MAb 13C4

Test culture, toxin[a]	Source strain	Neutralization of activity[b]
HeLa cells		
SLT (purified toxin)	*Escherichia coli* H30 (O26)	+
SLT (crude cell extract)	*Escherichia coli* H19 (O26)	+
SLT (crude cell extract)	*Escherichia coli* 933 (O157:H7)	+
SLT (crude cell extract)	*Escherichia coli* (K-12) C600	+
SLT (crude cell extract)	*Vibrio cholerae* JBK70	+
SLT (crude cell extract)	*Vibrio parahaemolyticus* FC1011	+
SLT (crude cell extract)	*Salmonella typhimurium* W118	+
SLT (crude cell extract)	*Shigella flexneri* M4243 2A	+
Shiga toxin (purified toxin)	*Shigella dysenteriae* type 1 strain 60R	+
Y-1 adrenal cells		
LTh (purified toxin)	*Escherichia coli* HE22pTD2	−
LTp (purified toxin)	*Escherichia coli* HE12	−
LT-like toxin (partially purified toxin)	*Escherichia coli* SA-53	−
CT (purified toxin)	*Vibrio cholerae* 569B	−

[a] Abbreviations: SLT, Shiga-like toxin; LTh, LT purified from a human isolate; LTp, LT purified from a porcine isolate; CT, cholera toxin.

[b] Shiga toxin and SLT kill HeLa cells but do not effect Y-1 adrenal cells. LT, LT-like toxin, and CT do not effect HeLa cells but cause rounding (cytotonicity) in Y-1 adrenal cells.

The strains studied in the colony blot assay had previously been tested for the levels of SLT in the sonic lysates using the cytotoxicity assay (L. R. M. Marques, M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien, manuscript in preparation). The bacteria in that study were classified as high, low to moderate, or trace toxin producers by the following criteria: high toxin producers made $\geq 10^5$ 50% cytotoxic doses (CD_{50})/ml in the sonic lysate; low to moderate toxin producers made 6×10^2 to 10^4 CD_{50}/ml in the sonic lysate; and trace producers made $< 6 \times 10^2$ CD_{50} per ml in the sonic lysate. When analyzed with respect to the level of toxin produced, the colony blot assay appeared to be sensitive for the identification of high-SLT-producing bacteria (Fig. 3). All strains classified as high toxin producers were positive in the colony blot assay, while all strains that were classified as low to moderate or trace toxin producers were negative in the blot assay.

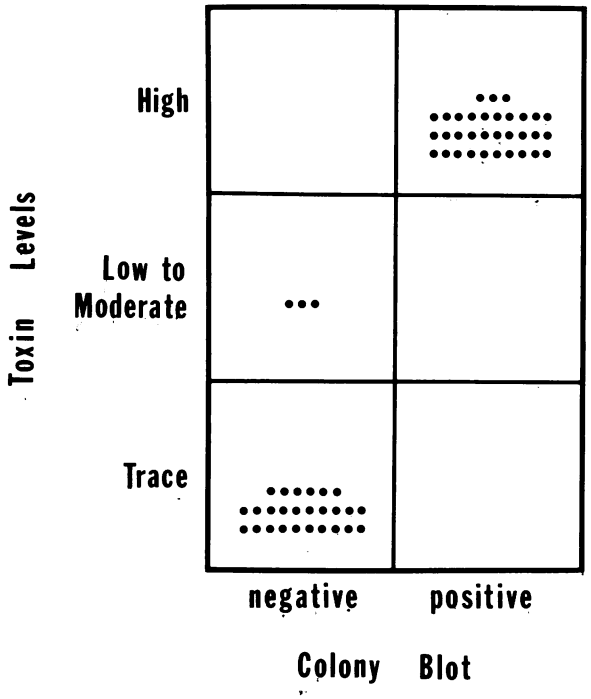

FIG. 3. Comparison of colony blot staining and level of toxin production as assessed by the cytotoxicity assay. The results of the colony blot assay are presented on the abscissa as positive (any detectable staining above background) or negative. The toxin level of a particular organism is shown on the ordinate. Each point represents one culture. Bacteria classified as high toxin producers made $\geq 10^5$ CD_{50}/ml in the sonic lysate while low to moderate toxin producers made 6×10^2 to 10^4 CD_{50}/ml, and trace toxin producers made $< 6 \times 10^2$ CD_{50}/ml in the sonic lysate. Strains for which serotype information was available were distributed as follows among the high, low-to-moderate, or trace categories: *E. coli* serotypes O157:H7, O26:H11, O111:H−, O111:H?, O146:H?, and O103:H2 were high toxin producers and were positive in the colony blot assay; *E. coli* serotype O9:H4 and *Shigella flexneri* serotype 2A were low to moderate toxin producers and were negative in the colony blot assay; and *E. coli* serotypes O55:H6, O145:−H4, and O145:−H were trace toxin producers and were negative in the colony blot assay. The remaining serotypes were not determined.

Twelve of the high toxin producers tested in this study were *E. coli* of the serotype O157:H7. All samples of this serotype were categorized as high toxin producers in the cytotoxicity assay, and all of these were positive in the colony blot assay. Since *E. coli* O157:H7 cells have been implicated as etiologic agents in several cases of food-borne hemorrhagic colitis (3, 27, 28, 32) as well as patients with hemolytic uremic syndrome (10, 29), the detection of these bacteria in patient and food samples is desirable. Where tissue culture facilities are not available or extensive bacteriologic workups are not practical, the colony blot assay may be a useful screening test for the identification of bacteria which make high levels of SLT.

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