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Affinity Purification and Characterization of Shiga-Like Toxin II and Production of Toxin-Specific Monoclonal Antibodies

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Shiga-like toxin II (SLT-II) was purified to apparent homogeneity from *Escherichia coli* K-12 strain NM522 containing the cloned toxin genes on recombinant plasmid pEB1. Purification was accomplished by a series of column chromatography techniques: anion-exchange, chromatofocusing, cation-exchange, and monoclonal antibody affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the pure toxin showed that SLT-II consisted of A and B subunits with apparent molecular weights of 32,000 and 10,200 ± 800, respectively. A band of molecular weight 25,000 was also observed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and identified as the A_1 subunit by Western immunoblot analysis with toxin-specific monoclonal antibodies (MAbs). The pI of the purified toxin was 5.2. Approximately 1 pg of pure SLT-II was a 50% cytotoxic dose for HeLa cells. The toxin was neutralized by polyclonal antibodies and MAbs to SLT-II, but was not neutralized by polyclonal antibodies or MAbs to SLT-I. Five hybridomas against SLT-II were produced (BC5 BB12, DC1 EH5, EA5 BA3, ED5 DF3, and GB6 BA4). Culture supernatant fluids containing MAbs from these hybridomas did not neutralize the cytotoxicity of SLT-I or Shiga toxin. Western blot analysis showed that two MAbs (MAb DC1 EH5 and MAb GB6 BA4) recognized the A and A_1 subunits of SLT-II and three MAbs (MAb BC5 BB12, MAb EA5 BA3, and MAb ED5 DF3) recognized the B subunit of SLT-II. MAb BC5 BB12 was used to prepare an affinity column for toxin purification.

In 1977 Konowalchuck et al. (12) reported that certain *Escherichia coli* strains associated with diarrheal disease produced a toxin cytotoxic to Vero cell monolayers. However, it was not until 1983 that *E. coli* O157:H7, the causative agent of hemorrhagic colitis (5, 27, 34), was shown to produce Vero toxin (VT) (W.M. Johnson, H. Lior, and G. S. Bezanson, Letter, Lancet i:76, 1983). A previously described *E. coli* Shiga-like toxin (SLT) (23) and VT were subsequently determined to be the same toxin (A. D. O'Brien, T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet i:702–703, 1983). In addition to hemorrhagic colitis, *E. coli* strains producing SLT (VT) have been associated with hemolytic uremic syndrome (HUS) (9; M. A. Karmali, M. Petric, C. Lim, P. C. Fleming, and B. T. Steele, Letter, Lancet ii:1299–1300, 1983), the leading cause of acute renal failure in children. Most recently, *E. coli* O157:H7 has been associated with an even more severe renal disease complicated with neurological symptoms, thrombotic thrombocytopenic purpura (6).

E. coli O157:H7 strains have been shown to produce two antigenically distinct toxins, designated VT1 and VT2 (S. M. Scotland, H. R. Smith, and B. Rowe, Letter, Lancet i:885–886, 1985) or SLT-I and SLT-II (33). Both toxins are bacteriophage encoded (24; S. M. Scotland, H. R. Smith, G. A. Willshaw, and B. Rowe, Letter, Lancet ii:216, 1983). SLT-I is structurally similar to Shiga toxin from *Shigella dysenteriae* type 1 and is neutralized by anti-Shiga toxin antibodies (19, 21–23). Both SLT-I and Shiga toxin are cytotoxic to Vero and HeLa cells, enterotoxic for ligated rabbit ileal segments, and paralytic-lethal for various laboratory animals (for a review, see reference 20). Based on nucleotide sequencing and predicted amino acid composition, SLT-I and Shiga toxin are identical or nearly identical proteins (4, 11, 31). Although SLT-II and SLT-I are reported to have similar biological activities (33), SLT-II is not neutralized by anti-SLT-I or anti-Shiga toxin antibodies. Conversely, SLT-II neutralizing antibodies do not neutralize SLT-I or Shiga toxin.

In addition to O157:H7, *E. coli* strains of several other serotypes isolated from persons with HUS or hemorrhagic colitis produce either SLT-I or SLT-II or both (1, 15). In the 10 outbreaks of hemorrhagic colitis and HUS investigated by the Centers for Disease Control to date, the presumptive epidemic strains have been serotype O157:H7 and have produced SLT-II alone or SLT-I and SLT-II. Organisms received at the Centers for Disease Control that produce SLT-I alone have been recovered from sporadic cases of diarrhea, hemorrhagic colitis, or HUS. Although SLT-II was recognized later, the epidemiologic data suggest that SLT-II is at least as important as SLT-I as a potential virulence factor (30). To facilitate the development of immunodiagnostic assays and to investigate the role of SLT-II in the pathogenesis of HUS and hemorrhagic colitis, we needed pure toxin. We report here the purification of SLT-II and the production of SLT-II-specific monoclonal antibodies (MAbs).

MATERIALS AND METHODS

Crude-toxin preparation. *E. coli* NM522(pEB1) (J. S. Spika, L. W. Mayer, and F. P. Downes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B-67, p. 36) was used as the source of toxin. *E. coli* NM522(pEB1) contains the cloned genes for SLT-II on the recombinant plasmid pEB1 (manu-

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script in preparation). Strain NM522(pEB1) was handled in accordance with the guidelines set forth by the Recombinant Advisory Committee (Appendix, Fed. Regist. **51**:16972, 1986). Since it produced less toxin than *S. dysenteriae* type 1 60R, all manipulation of strain NM522(pEB1) were performed under P2 containment.

Strain NM522(pEB1) was grown for 16 to 18 h at 37°C in Penassay broth (Difco Laboratories, Detroit, Mich.) supplemented with 35 μg of ampicillin per ml. Aliquots of the overnight growth were spread onto 65 (150 by 15 mm) Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.), each containing 35 μg of ampicillin per ml, and incubated overnight at 37°C. The bacterial growth was gently scraped from the plates at room temperature and washed twice by centrifugation at 4°C with 0.15 M NaCl. The bacteria were then suspended in 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) and lysed by two passages in a French pressure cell (American Instruments, Urbana, Ill.) at 20,000 $lb/_{in}^2$. Bacterial cells and the resulting lysate were kept on ice during the lysing procedure. To limit the viscosity of the preparation, RNase and DNase (Sigma Chemical Co., St. Louis, Mo.), each at 0.1 mg/ml, and 0.001 M $MgCl_2$ were added to the lysate. The lysate was then centrifuged at 10,000 × *g* for 1 h at 4°C. Crude toxin was extracted from the resulting supernatant fluid by precipitation with 60% saturated ammonium sulfate at 4°C. The resulting precipitate was collected by centrifugation, redissolved in approximately 40 ml of distilled water (28), and dialyzed three times at 4°C against 150 volumes of 0.1 M Tris hydrochloride (pH 6.5).

Anion-exchange chromatography. Manipulations of all chromatography columns were performed at room temperature. The dialyzed crude toxin was applied to a 50-ml (1.6 by 25 cm) DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column equilibrated with 0.1 M Tris hydrochloride (pH 6.5). The column was washed with the equilibration buffer until the optical density at 280 nm (OD_{280}) of the eluate returned to base-line levels. The column was developed with 400 ml of a 0 to 0.6 M NaCl gradient in equilibration buffer, and the OD_{280} and cytotoxicity of 3-ml fractions were monitored. Fractions with greater than 10^4 50% cytotoxic doses/ml (CD_{50}/ml) were pooled, concentrated to approximately 20 ml with a stirred-cell Amicon Concentrator (Amicon Corp., Danvers, Mass.) by using YM10 Diaflo Ultrafiltration Membrane Filters (Amicon Corp.), and dialyzed (Spectrapor, 6,000- to 8,000-molecular-weight cutoff; Spectrum Medical Industries, Inc., Los Angeles, Calif.) at 4°C against 300 volumes of 0.025 M histidine hydrochloride (pH 6.2).

Chromatofocusing chromatography. PBE 94 resin (Pharmacia) (21 ml) was equilibrated with 0.025 M histidine hydrochloride (pH 6.2) in a column (1.0 by 26 cm). The pooled, concentrated material from the anion-exchange column was applied to the chromatofocusing column, and Polybuffer 74 (Pharmacia), adjusted to pH 4.0 with 10 N HCl, was then applied to form a pH gradient between pH 6 and 4. The OD_{280} and cytotoxicity of 3-ml fractions were monitored, and fractions with greater than 10^4 CD_{50}/ml were pooled, concentrated as above, and dialyzed against 150 volumes of 0.2 M citrate buffer (pH 4.0).

Cation-exchange chromatography. The concentrated toxin preparation from the chromatofocusing column was applied to a 25-ml CM-Sepharose CL-6B (Pharmacia) column (1.0 by 30 cm) equilibrated with 0.2 M citrate buffer (pH 4.0). The column was washed with equilibration buffer until the OD_{280} of 3-ml fractions returned to base-line levels. Toxin was eluted with a salt gradient (400 ml of 0 to 0.6 M NaCl in equilibration buffer), and fractions with greater than 10^5 CD_{50}/ml were pooled and concentrated as above. The concentrate was then dialyzed at 4°C against 150 volumes of 0.01 M phosphate-buffered saline (PBS) (pH 7.2).

Affinity chromatography. MAb BC5 BB12 was purified from mouse ascitic fluid by protein A chromatography with an Affi-Gel protein A MAPS II Kit (Bio-Rad Laboratories, Richmond, Calif.) as specified by the manufacturer. Pure immunoglobulin G(4 mg) in 0.1 M HEPES buffer (pH 7.5) was linked to Affi-Gel Active Ester Agarose resin (Bio-Rad Laboratories) by incubation of the antibody with 5 ml of resin at 4°C with gentle agitation. After 4 h of incubation, unbound resin sites were blocked with 0.5 ml of 1.0 M ethanolamine hydrochloride (pH 8) for 1 h at room temperature. The resin was packed into a column and washed with PBS to remove unbound antibody and blocking reagent. The concentrated toxin from the CM-Sepharose column was applied to the affinity column and incubated overnight at 4°C. The column was then washed with PBS at room temperature until the OD_{280} returned to base-line levels, and the toxin was eluted with approximately 15 ml of 3.5 M $MgCl_2$. The eluted fractions were dialyzed at 4°C against 200 volumes of PBS to remove $MgCl_2$. The dialyzed affinity-purified protein was assayed for cytotoxicity and protein content and used for further characterization of toxin.

MAb production. The antigen used for all immunizations was a toxoid of partially purified toxin from *E. coli* NM522(pEB1). The toxoid was prepared with toxin from the chromatofocusing column (21 μg/ml of protein, 10^6 CD_{50}/ml) as previously described (2). On day 1 each of five female BALB/c mice aged 6 to 8 weeks was injected intraperitoneally with 0.1 ml of an emulsion containing equal parts of toxoid and complete Freund adjuvant (Sigma). Each mouse was injected intraperitoneally on day 17 with 0.1 ml of an emulsion containing equal parts of toxoid and incomplete Freund adjuvant (1:2) (Sigma). Mice were bled from the orbital plexus on day 31, and the SLT-II-specific neutralizing titer of the resulting serum was determined. Two mice with the highest neutralizing titers were boosted intravenously with 0.1 ml of toxoid without adjuvant on days 50, 51, and 52. On day 54 one mouse was sacrificed and the spleen was removed.

Fusion of splenocytes and SP2/0 Ag14 myeloma cells was performed by the method of Zola and Brooks (36). Culture supernatant fluids were screened for neutralizing antibody with toxin from the chromatofocusing column. Hybridomas that produced culture supernatant fluids with neutralizing antibody titers of 1:25 or greater were subcloned twice by limiting dilution at 1 and 0.1 cell per well and reassayed for neutralizing antibody.

MAb subclasses were determined by the immunodiffusion assay (8) with concentrated monoclonal culture supernatant fluids and mouse immunoglobulin chain-specific antisera (Litton Bionetics Laboratory Products, Charleston, S.C.).

Western immunoblot. Affinity-purified toxin was separated into subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% polyacrylamide gel. The toxin proteins and molecular weight standards were then transferred electrophoretically from the gel to a nitrocellulose membrane (pore size, 0.45 μm; Trans Blot Transfer Medium; Bio-Rad Laboratories) at room temperature by using 100 mA current overnight or 250 mA for 3 h (3).

The protein molecular weight standards were cut from the nitrocellulose and stained with amido black (0.2%, wt/vol). The portion of the nitrocellulose containing the toxin protein

was sliced into strips approximately 5 mm wide and stained immunochemically to determine the antibody subunit specificity. Nitrocellulose strips were first blocked with 0.5% (wt/vol) evaporated milk in PBS for 30 min and then incubated for 4 h at room temperature with monoclonal culture supernatants diluted 1:10 in PBS with 0.05% (vol/vol) Tween-20 (PBS-Tween). After being washed three times in 20 ml of PBS-Tween, the nitrocellulose strips were incubated for 1 h at room temperature in diluted (1:3,000 in PBS-Tween) goat anti-mouse antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories). Immune complexes were visualized by developing with a freshly prepared solution of 0.5% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in 0.003% H_2O_2 and PBS (16).

PAGE. In a determination of toxin purity and subunit structure, samples from individual chromatography fractions and concentrated pools were mixed with an equal volume of sample treatment buffer (0.125 M Tris hydrochloride [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated at 100°C for 4 to 10 min. The reduced and denatured samples were subjected to PAGE on 1.0-mm SDS–15% polyacrylamide gels as described by Laemmli (13). After electrophoresis was complete, the gels were silver stained to visualize the protein bands (17). Unless otherwise specified, all gels were silver-stained to visualize the proteins. Concentrated toxin from the CM-Sepharose column was mixed at room temperature with sample treatment buffer without both 2-mercaptoethanol and SDS and electrophoresed under nondenaturing conditions on a 15% polyacrylamide gel. After electrophoresis, the gel was cut into 2-mm slices. The protein in each slice was passively eluted into PBS and assayed for cytotoxicity.

Protein determination. The protein content of the purification pools was monitored at the completion of each step of purification by using the Pierce BCA Protein Reagent or Pierce Coomassie Blue G-250 reagent (Pierce Chemical Co., Rockford, Ill.) (29) and bovine serum albumin as a standard.

HeLa cell cytotoxicity assays. Cytotoxicity was measured in 96-well HeLa cell microdilution cultures by the method of Gentry and Dalrymple (7). The toxin titer was defined as the reciprocal of the dilution that resulted in death of 50% of the HeLa cells (CD_{50}). The neutralization assay was performed by mixing equal volumes of antiserum and toxin, both diluted in cell culture medium. The toxin-antitoxin mixture was incubated at 37°C for 1 to 4 h, and 0.1 ml of the mixture was assayed on HeLa cells for residual cytotoxicity as previously described (7). When titrating antiserum or monoclonal culture supernatants for neutralizing antibody, various dilutions of the antibody samples were mixed with a fixed dilution of toxin containing approximately 10 to 50 CD_{50}. Cytotoxic activity in crude bacterial extracts or purified toxin preparations was neutralized by mixing various dilutions of the toxin preparations with a fixed dilution of antibody capable of neutralizing approximately 200 CD_{50} of the homologous toxin. MAb 13C4 (32; kindly supplied by A. D. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md.) and rabbit anti-SLT-I were used as the SLT-I-specific antibody. MAb BC5 BB12 and rabbit anti-SLT-II were used as SLT-II-neutralizing antibodies.

Preparation of rabbit antisera. Polyclonal rabbit antisera were prepared against SLT-I purified from *E. coli* O157:H7 strain 931 as described by O'Brien and LaVeck (22) and against partially purified SLT-II (CM-Sepharose fraction) from *E. coli* NM522(pEB1). Toxoids were prepared from the purified toxins by treatment with glutaraldehyde as described by Brown et al. (2), and portions (20 μg of protein per ml) were emulsified with an equal volume of complete Freund adjuvant (Sigma Chemical Co.). A 1-ml sample of the emulsion was injected intradermally in multiple sites (20 to 25 sites, approximately 0.05 ml/site) on the clipped backs of New Zealand albino rabbits. A second injection of the antigen was given intramuscularly into the thigh muscle 14 days after the first injection. The rabbits were boosted with an intravenous injection of toxoid (15 μg of protein) 40 days after the first injection. Volume bleedings were obtained at 5, 7, and 9 days after the booster injection, and the serum samples from a given rabbit were pooled.

Isoelectric-point determination. The pI of affinity-purified toxin was determined by using thin-layer electrofocusing acrylamide gels (PAG-plate; LKB Instruments, Inc., Rockville, Md.). An anode buffer of 1 M phosphoric acid and a cathode buffer of 1 M sodium hydroxide were used for a pH range of 3.5 to 9.5. Gels were electrophoresed for 90 min at 50 mA. Iso-gel pI Markers (FMC Corp., Marine Colloids Div., Rockland, Maine) were used as standards.

RESULTS

Toxin purification. As a source of toxin we used an *E. coli* K-12 strain transformed with the recombinant plasmid pEB1 carrying the SLT-II genes from *E. coli* EDL880 (serotype O157:H7), which produces both SLT-I and SLT-II. We chose the transformed strain to avoid contamination of our toxin preparation with SLT-I and because it produced 100-fold more SLT-II than the wild-type parent did.

As an initial purification step, the bacterial cell lysate was precipitated with 60% saturated ammonium sulfate. The precipitated crude toxin was then subjected to anion-exchange (Fig. 1), chromatofocusing (Fig. 2), cation-exchange (Fig. 3), and monoclonal anti-toxin affinity chromatography. Using this scheme, we obtained highly purified toxin with a specific activity of 9.1×10^8 CD_{50}/mg (Table 1).

In initial attempts to purify the toxin, molecular sieve chromatography with Bio-Gel P-100 resin (Bio-Rad Laboratories) was introduced after chromatofocusing chromatography. This technique did not increase the purity or yield of toxin and was omitted in subsequent experiments. In contrast, cation-exchange chromatography proved to be a very efficient method of purification. Initial attempts to simplify the procedure by omitting the DEAE and chromatofocusing chromatography steps were unsuccessful. Crude toxin precipitated by ammonium sulfate did not bind efficiently to the CM-Sepharose column. It is possible that contaminants which would have been removed by the DEAE and chromatofocusing columns interfered with the binding of the toxin to the cation-exchange column.

After CM-Sepharose chromatography, the toxin had been purified over 3,000-fold. Nondenaturing PAGE of the toxin preparation after this step revealed only one major protein band that coincided with the peak of cytotoxic activity eluted from the gel (Fig. 4). The two lightly staining bands near the cathode were attributed to contaminants in the Tris buffers, since they appeared in lanes without samples as well as in lanes containing samples. In later gels (Fig. 5), the bands disappeared when a new lot of Tris base was used for the preparation of the gels.

The immunoaffinity column was made with MAb BC5 BB12, which recognized the B subunit of SLT-II (see Fig. 7). The affinity column step resulted in approximately a 12% increase in specific activity (Table 1). Since toxin from the

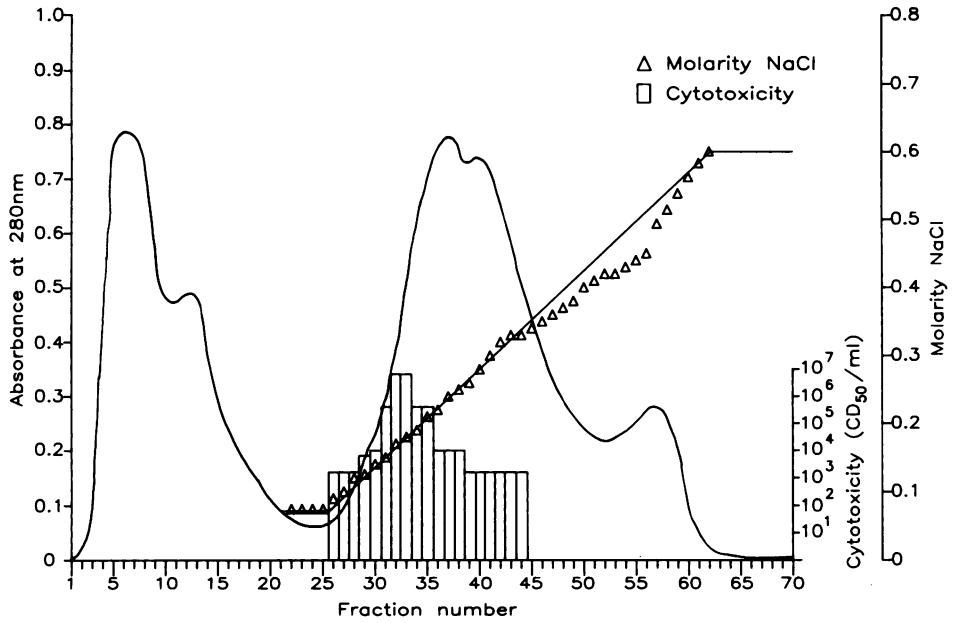

FIG. 1. Representative anion-exchange (DEAE) column elution profile of approximately 200 mg of total protein of crude SLT-II (ammonium sulfate precipitate) collected in 3-ml volumes. Fractions 26 to 39, which had a cytotoxic activity greater than 10^4 CD_{50}/ml, were pooled.

affinity column could conceivably have been a mixture of holotoxin and free B subunits, the pure toxin was subjected to gel filtration chromatography on Bio-Gel P-100 resin. Fractions were monitored for cytotoxic activity and the presence of B subunit by SDS-PAGE. No fractions containing only B subunit were detected (data not shown).

Characterization of SLT-II. When applied to SDS-PAGE, affinity-purified toxin separated into three bands (Fig. 5). The most darkly staining band (B subunit) had a molecular weight of 10,200 ± 800. The intensity of staining of this band indicated that the toxin molecule may be composed of multiple B subunits. The larger subunit (A subunit) had a molecular weight of approximately 32,000. A putative A_1 subunit was also observed as a lightly staining band with a molecular weight of approximately 25,000. This band was similar in size to protease-nicked A_1 subunit from SLT-I and

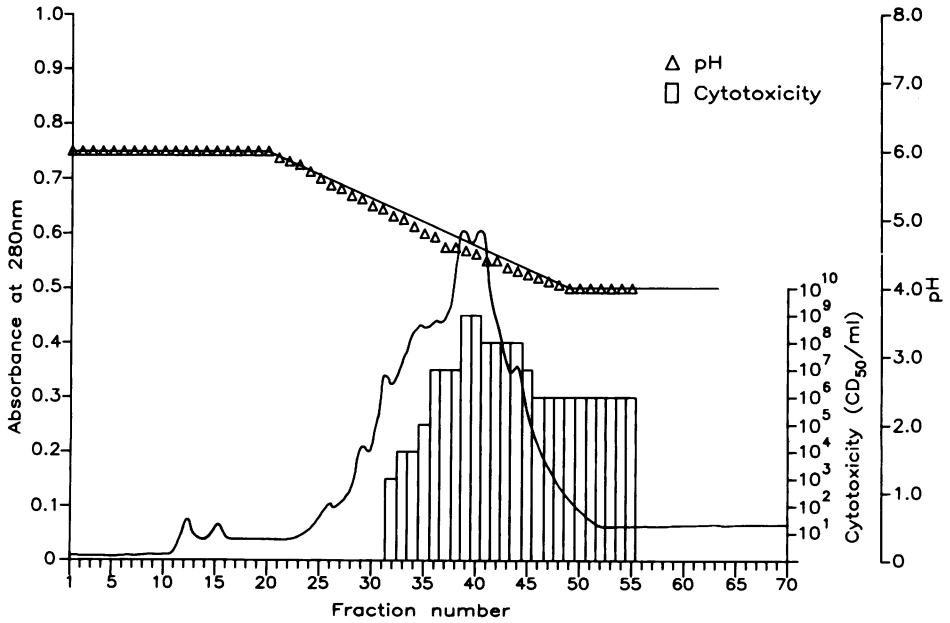

FIG. 2. Representative chromatofocusing column elution profile of 158 mg of total protein of anion-exchange pool concentrate collected in 5-ml volumes. Fractions 35 to 55, which had a cytotoxic activity greater than 10^4 CD_{50}/ml, were pooled.

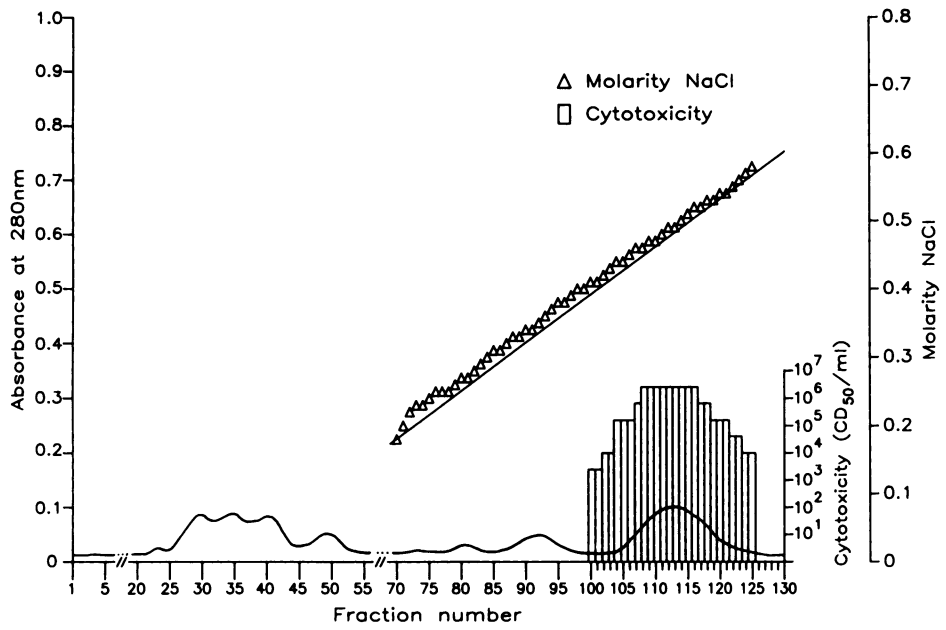

FIG. 3. Cation-exchange (CM-Sepharose) column elution profile of 22 mg of total protein of chromatofocusing pool concentrate collected in 3-ml volumes. Fractions 104 to 125, which had a cytotoxic activity greater than 10^5 CD_{50}/ml, were pooled.

Shiga toxin (20). Western blot analysis showed that the A and the putative A_1 subunits were both recognized by SLT-II specific MAbs DC1 EH5 and GB6 BA4 (see Fig. 7).

Isoelectric focusing gels of affinity-purified SLT-II have shown one or two bands in the pH range of 5.1 to 5.3. Isoelectric focusing of the affinity-purified toxin in thin-layer polyacrylamide gels showed a sharp, intensely staining band with a PI of 5.2 and fainter bands with pIs around 5.1 (Fig. 6). In some purified toxin preparations after electrofocusing on agarose gels, we observed two sharp bands with pIs of 5.2 and 5.3. Cytotoxicity assays on material eluted from slices of the agarose gel revealed that virtually all the cytotoxic activity applied to the gel migrated in the same location as the protein bands at pH 5.2 and 5.3 (data not shown). The two bands may represent holotoxin and free B subunit, which has a predicted pI of 5.38 (10) (processed B subunit). Alternatively, the two bands may represent heterogeneity in the holotoxin owing to various numbers of B subunits associated with the A subunit. Heterogeneity in the holotoxin is more likely, since no free B subunit was detected in that affinity-purified toxin preparation by gel filtration.

The pure toxin was cytotoxic to HeLa cells and Vero cell monolayers (see Table 3). As little as 1.1 pg of pure toxin killed 50% of the HeLa cells in the microdilution cytotoxicity assay (Table 1). SLT-II-specific polyclonal antibodies and MAbs neutralized the pure toxin, whereas anti-SLT-I MAb 13C4 did not neutralize the toxin (Table 2). Preliminary experiments with purified SLT-II showed that the toxin was lethal for mice and rabbits (data not shown).

MAb production. The use of a toxoid of partially purified toxin as the antigen facilitated the production of five hybridoma cell lines which produced SLT-II neutralizing antibodies (Table 2). All monoclones characterized were of the IgG1 heavy-chain and kappa light-chain classes.

Two monoclones recognized the A and A_1 subunits of Western-blotted toxin proteins, whereas the remaining three monoclones recognized the B subunit (Fig. 7; Table 3). None of the five SLT-II-specific antibodies described here bound to the A or B subunits of Western-blotted crude Shiga toxin. MAb 13C4 did not bind to the subunits of SLT-II; however, it did recognize the B subunit of Western-blotted crude Shiga toxin (data not shown).

DISCUSSION

Using the procedures presented here, we purified SLT-II to apparent homogeneity. Recently, Padhye et al. (26) and Yutsudo et al. (35) reported purification schemes for VTs that are immunologically unrelated to Shiga toxin. Padhye et al. present a scheme for purifying a unique Vero cytotoxin

TABLE 1. Purification yield of SLT-II from *E. coli* NM522(pEB1)

Fraction	Vol (ml)	Total cytotoxicity (CD_{50})	Total protein (mg)	Sp act (CD_{50}/mg)	Fold purification	% Yield
Bacterial cell lysate	69	2.8×10^8	1,070	2.6×10^5		
Ammonium sulfate precipitate	40	2.6×10^8	424	6.1×10^5	2.3	92.8
Anion-exchange pool concentrate	20	1.3×10^8	164	7.9×10^5	3.0	46.4
Chromatofocusing pool concentrate	26	1.0×10^8	22.1	4.5×10^6	17.3	35.7
Cation-exchange pool concentrate	5	8.0×10^7	0.1	8.0×10^8	3,077	28.6
Affinity column pool concentrate	18	7.2×10^7	0.079	9.1×10^8	3,500	25.7

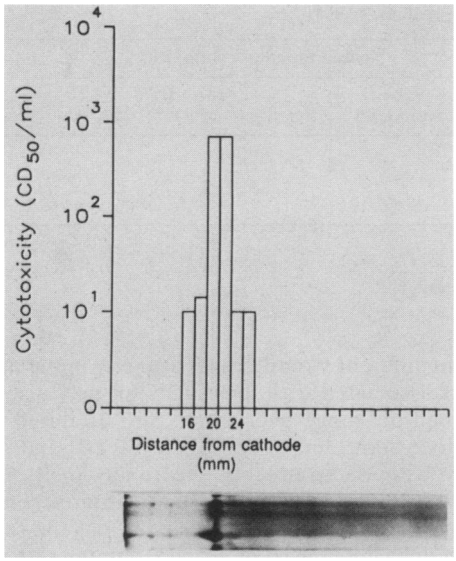

FIG. 4. Nondenaturing PAGE of SLT-II eluted from the cation-exchange column. Approximately 30 μg of cation-exchange-concentrated pool protein was run in each of two lanes of a nondenaturing polyacrylamide gel. One lane was silver stained, and the other lane was sliced into 2-mm segments which were passively eluted into 1 ml of PBS. Eluates from each slice were assayed for cytotoxic activity. The darkly staining bands correspond to the peak of cytotoxicity.

from culture filtrates by ultrafiltration and anion exchange chromatography. Yutsudo et al. describe a scheme for purifying VT2 from culture filtrates by ammonium sulfate fractionation, DEAE-cellulose chromatography, repeated chromatofocusing chromatography, and repeated high-performance liquid chromatography (35). Our method differed from those previously reported in that we purified cell-associated toxin. We used a scheme similar, in part, to the

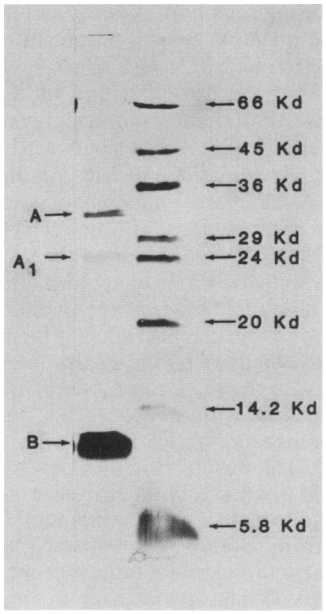

FIG. 5. Silver-stained SDS-PAGE analysis of affinity purified SLT-II. Lanes: 1, 12 μg of protein of affinity-purified SLT-II; 2, low-molecular-mass standards (Sigma). Kd, Kilodaltons.

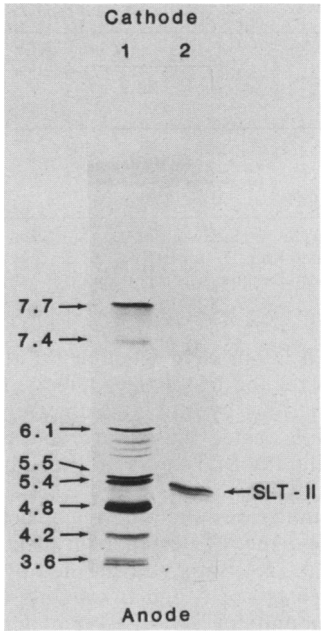

FIG. 6. Silver-stained thin-layer polyacrylamide isoelectric focusing gel of affinity-purified SLT-II. Lanes: 1, pI standards (FMC Corp.); 2, approximately 25 ng of affinity-purified SLT-II.

one reported by Yutsudo et al.; however, we differed from Yutsudo et al. in our use of a single chromatofocusing chromatography, cation-exchange chromatography, and monoclonal antitoxin affinity chromatography.

The strain (J-2) used by Yutsudo et al. (35) as a source of toxin is reported to produce only VT2. Other than the unique Vero cytotoxin that is not neutralizable with antibodies against Shiga toxin, Padhye et al. (26) do not comment on whether other cytotoxins are produced by their strain (EDL932). Strain EDL932, originally isolated at the Centers for Disease Control, was included in a study reported by Marques et al. (15) and was found to produce both SLT-I and SLT-II. Since only neutralization data with anti-Shiga toxin

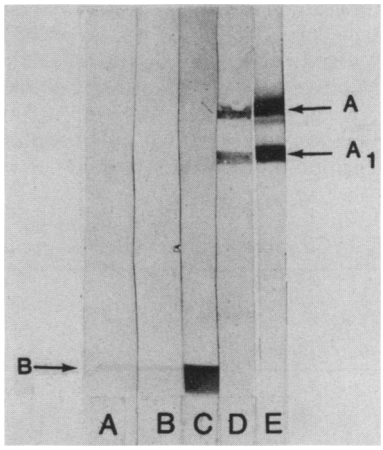

FIG. 7. Western blot analysis of affinity-purified SLT-II immunostained with MAb ED5 DF3 (lane A), MAb EA5 BA3 (lane B), MAb BC5 BB12 (lane C), MAb DC1 EH5 (lane D), or MAb GB6 BA4 (lane E). No reaction of MAb 13C4 specific for the B subunit of SLT-I with the A and B subunits of SLT-II was observed (data not shown). MAb 13C4 did recognize the B subunit of Shiga toxin.

TABLE 2. Antigenic characterization of purified SLT-II

Toxin	Cytotoxicity		Neutralization of cytotoxicity			
	HeLa cells	Vero cells	MAb 13C4	Rabbit anti-SLT-I	MAb BC5 BB12[a]	Rabbit anti-SLT-II
Pure SLT-II	+	+	−	−	+	+
Pure SLT-I[b]	+	+	+	+	−	−
Shiga toxin (crude)[c]	+	+	+	+	+	−

[a] Similar results were obtained with MAbs DC1 EH5, EA5 BA3, ED5 DF3, and GB6 BA4.
[b] SLT-I was purified from *E. coli* EDL931 as described by O'Brien and LaVeck (22).
[c] The Shiga toxin preparation was a polymyxin B extract from *Shigella dysenteriae* type 1 strain 60R.

were reported, it is not clear whether the toxin purified by Padhye et al. is related to, or the same as, SLT-II or VT2. The production of a cytotoxin distinct from SLT-I and SLT-II is possible, since the culture and assay conditions used for detecting the SLTs may not have been optimal for other Vero cytotoxins.

There were similarities and differences between the SLT-II we purified and the VTs reported by Yutsudo et al. (35) and Padhye et al. (26) Both VT2 purified by Yutsudo et al. and SLT-II consisted of A and B subunits of similar sizes. The A and B subunits of SLT-II have molecular weights of 32,000 and 10,200 ± 800, respectively, whereas the A and B subunits of VT2 have molecular weights of 35,000 and 10,700, respectively. The sizes of the subunits for SLT-II are also consistent with minicell and DNA sequence data reported for SLT-II (10, 18). From the DNA sequence analysis of SLT-II, the predicted molecular weights of the mature A and B subunits were 33,135 and 7,817.

The observed sizes of the B subunits for SLT-II and VT2 were larger than predicted for the mature B subunit from the nucleotide sequence data. The degree to which the B subunit is processed for SLT-II or VT2 is not known. Newland et al. (18) observed no processing of the SLT-II A and B subunits with polymyxin B treatment of labeled minicells.

In contrast, Padhye et al. (26) report that their toxin does not consist of subunits. The molecular weight of their toxin is 64,000 by SDS-PAGE and 54,000 by molecular-sieve chromatography. Although single bands are evident after Coomassie blue staining of SDS-polyacrylamide and isoelectric focusing gels, it is not clear from their data that the cytotoxicity migrated with these protein bands.

SLT-II purified in our study had a pI of 5.2. The pIs reported by Yutsudo et al. (35) and Padhye et al. (26) are 4.1 and 5.2, respectively. It is not clear why there is such a difference between our pI and that of Yutsudo et al. One possible explanation would be that the pIs differ owing to the number of B subunits which are associated with the holotoxins from different strains. Heterogeneity in the number of B subunits associated with the A subunit may also account for the broad pH range over which SLT-II eluted from the chromatofocusing column. The pIs of SLT-II and VT2 may also differ with the source of the toxins (cell associated versus extracellular) used in the purification schemes. It is interesting that the toxin of Padhye et al. has the same pI as SLT-II but does not consist of A and B subunits. In contrast, the pIs of Shiga toxin and SLT-I (7.03 ± 0.02 [22]) are quite different from those of SLT-II, VT2, and the unique Vero cytotoxin.

TABLE 3. Characterization of SLT-II-specific MAbs

Hybridoma cell line[a]	SLT-II subunit specificity	Culture supernatant neutralizating titer[b]
BC5 BB12	B	640
DC1 EH5	A	40
EA5 BA3	B	80
ED5 DF3	B	320
GB6 BA4	A	80

[a] The immunoglobulin isotype was immunoglobulin G1 kappa for all cell lines.
[b] Reciprocal of the dilution of hybridoma culture supernatant fluid which neutralized 50 CD_{50} of crude SLT-II.

The biologic activities of SLT-II and the toxins purified by Yutsudo et al. (35) and Padhye et al. (26) were similar; however, the specific activities differed. All three toxins were cytotoxic to either Vero or HeLa cells and lethal for mice. For both SLT-II and VT2, 1 CD_{50} unit was approximately 1 pg; however, for the toxin purified by Padhye et al., 1 CD_{50} unit was 25 pg. There was over a 200-fold difference between the 50% lethal doses of VT2 and the unique Vero cytotoxin for mice. Yutsudo et al. report that 1 ng of VT2 was a 50% lethal dose (20-g ddY mice, intraperitoneal injection), whereas Padhye et al. report that 270 ng of unique Vero cytotoxin was a 50% mouse lethal dose (20-g Sprague-Dawley mice, intraperitoneal injection) (25). Variation in specific activities could result from differing amounts of inactive toxin such as free B or A subunits, varying degrees of purity, or differing sensitivities of the cell lines or animals.

No cross-neutralization was apparent in reciprocal titrations of Shiga toxin and SLT-II against specific polyclonal antibodies and MAbs. On the basis of biochemical characteristics such as pI and immunological characteristics in neutralization experiments, Shiga toxin or SLT-I and SLT-II were dissimilar. However, biologic similarities between these toxins may be more important in determining their relevance in the pathogenicity of a newly recognized group of enterohemorrhagic *E. coli* strains (14).

The identification of SLT-producing bacteria by the cell culture toxicity assay is time-consuming and expensive, and requires a level of expertise and special facilities not generally available in clinical or public health microbiology laboratories. The development of rapid, simple assays for these toxins should facilitate more widespread testing for these organisms and subsequently lead to a better appreciation of their prevalence. The purification of SLT-II and the production of specific MAbs have provided reagents for the development of assays to detect this toxin and antibodies against it in specimens from patients. Using these reagents, we have developed an enzyme-linked immunosorbent assay for SLT-II (F. P. Downes et al., manuscript in preparation). The purified toxins and monoclones should also facilitate animal studies of hemorrhagic colitis and HUS and help define the role of SLTs in these diseases.

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