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Purification and Characterization of an *Escherichia coli* Shiga-Like Toxin II Variant

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A Shiga-like toxin II variant was purified to homogeneity from *Escherichia coli* TB1(pCG6), which contained the toxin genes cloned in multicopy plasmid pUC18. The purification scheme involved polymyxin B extraction of the toxin from bacterial cells, followed by differential $(NH_4)_2SO_4$ precipitation, anion- and cation-exchange fast-protein liquid chromatography, and immunoaffinity exclusion chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified toxin revealed three protein bands that migrated with calculated molecular weights of 33,000, 27,500, and 7,500. These bands correspond to values for the A, A_1, and B subunits, respectively, that would be expected on the basis of the nucleotide sequence and comparison with data for Shiga toxin and other Shiga-like toxins. Electrophoresis under nonreducing conditions resulted in disappearance of the 27,000-molecular-weight band. Western blot (immunoblot) analysis revealed three protein bands with molecular weights of 33,000, 27,500, and 7,500. The purified toxin induced typical signs of edema disease in pigs injected intravenously with doses as small as 3 ng/kg of body weight. The 50% cytotoxic doses for Vero, PK15, and Madin-Darby bovine and canine kidney cells were 0.5, 2.0, 8.0, and 8.0 pg, respectively. The 50% lethal dose of purified toxin for mice was 0.9 pg by the intraperitoneal route. Approximately 75 μg of purified toxin was required to induce a 1-ml/cm fluid response in rabbit ileal loops. Antiserum to the Shiga-like toxin II variant neutralized homologous toxin, Shiga-like toxin II, and Verotoxin 2 but not Shiga-like toxin I.

Shiga-like toxins (SLT) are a family of cytotoxins produced by certain strains of *Escherichia coli* and are related in structure and function to Shiga toxin of *Shigella dysenteriae* type 1 (10, 15, 16, 18, 23–25, 29, 33). SLTs are also known as Vertotoxins (VT) because of their cytotoxicity for Vero cells (17, 21, 29). Members of the SLT family share similar biological activities, which include cytotoxicity for various cell cultures, enterotoxicity in rabbit intestinal loops, and lethality for mice and rabbits (10, 19, 25, 28). Three types of SLT produced by human isolates of *E. coli* have been characterized (24). SLT-I is identical to Shiga toxin (2, 15, 32) and is not neutralized by antibodies to other SLT types (10, 18, 24, 27, 28, 33). SLT-II (33) and VT2 (18, 29) are related on the basis of cross-neutralization. Production of SLT-I, like that of Shiga toxin, is iron regulated (25, 26, 34), while production of SLT-II is not iron regulated (L. M. Sung and M. P. Jackson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, H89, p. 184). SLT-I and SLT-II have been purified, and their biologic and physical properties have already been described (10, 19, 25, 28, 33). The properties of SLTs are consistent with their having a role in disease, and SLT-producing *E. coli* strains of human origin have been implicated in diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (17, 24).

Interest in SLT-producing *E. coli* of porcine origin preceded the discovery of SLT. As early as 1957 (31), a toxin produced by certain strains of *E. coli* was implicated in edema disease (ED) of pigs, and several researchers have attempted to purify this toxin (4, 5, 7, 11). In 1960 (12), similarities between ED toxin and Shiga toxin were recognized and exploited in attempts to purify the toxin from ED strains of *E. coli*. In 1977 (21), the first report on VT (SLT) indicated that an *E. coli* strain from ED was one of several strains which produced a cytotoxin active on Vero cells. Later, Dobrescu (6) and Smith and co-workers (30) suggested that ED toxin belonged to the SLT family and Marques and co-workers (23) noted that SLT-IIv was produced by ED strains of *E. coli*. V. P. J. Gannon and C. L. Gyles (Vet. Microbiol., in press) showed that signs of ED could be reproduced by partially purified extracts of *E. coli* K-12 with the SLT-IIv genes but not by similar extracts of *E. coli* K-12 with the SLT-IIv genes inactivated by transposon mutagenesis. Recently, the genes for SLT-IIv have been cloned and sequenced (14, 35).

Purification of SLT-IIv is particularly important because all previous attempts at reproduction of ED with toxin have used impure preparations. In particular, these studies have been complicated by the effects of endotoxin which contaminated the preparations (4, 5, 7, 11, 12, 31). Furthermore, pure SLT-IIv offers the opportunity to conduct experiments with a toxin of the SLT family in its natural host. The purpose of this study was to develop a scheme for purification of SLT-IIv, to determine whether ED could be reproduced in pigs by intravenous injection of pure SLT-IIv, and to determine the biological features of pure SLT-IIv.

MATERIALS AND METHODS

Bacteria. *E. coli* TB1(pUC18), TB1(pCG6), H30, C600 (933W), and E32511 were used in this study. TB1(pUC18) was *E. coli* TB1 with multicopy plasmid pUC18 (14). TB1 (pCG6) was identical to TB1(pUC18), except that it contained the genes for SLT-IIv on a 2.4-kilobase fragment of DNA derived from an O139:K82 ED isolate and cloned in plasmid pUC18 (14). Both strains were maintained at 4°C on tryptic soy agar plates containing 100 μg of ampicillin per ml. Strains H30, C600(933W), and E32511 (11) were used as sources of SLT-I, SLT-II, and VT2, respectively, and were stored on tryptic soy agar slants at 4°C.

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Vero cell assay. The method for assay of SLT and VT2 was essentially that described by MacLeod and Gyles (22). African green monkey kidney cells (Vero cells; ATCC CCL81; American Type Culture Collection, Rockville, Md.) were grown in Eagle modified essential medium (EMEM) with Earle salts and glutamine supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL Canada, Burlington, Ontario, Canada), 100 μg of gentamicin sulfate (Schering, Pointe Claire, Quebec, Canada) per ml, and 60 μg of anti-pleuropneumonia-like organism agent (Gibco BRL Canada) per ml. Confluent monolayers were removed with trypsin-EDTA, washed once (1,000 × g for 10 min), and suspended to 4×10^5 cells per ml in EMEM. Toxin preparations were serially diluted twofold with EMEM in 96-well tissue culture plates (Gibco BRL Canada). A 100-μl volume of Vero cells was then pipetted into each well, and the plates were incubated in 5% CO_2 at 37°C for 72 h. Monolayers were checked with an inverted microscope daily for cytotoxic effect. After 72 h, monolayers were fixed in 7.5% Formalin and stained with crystal violet solution. Toxin titer was expressed as the highest dilution of the sample that killed 50% of the Vero cell monolayer after 72 h (CD_{50}). Each sample was tested in duplicate, and the mean titer of the two assays was determined.

Purification of SLT-IIv. Purification of SLT-IIv involved extraction of bacterial cells with polymyxin B, followed by differential $(NH_4)_2SO_4$ precipitation, anion- and cation-exchange fast-protein liquid chromatography, and immunoaffinity exclusion chromatography. All chromatographic procedures were performed at room temperature.

Polymyxin B extraction. Comparison of the yield of SLT-IIv from cell lysates and from culture supernatant of *E. coli* TB1(pCG6) showed that approximately 99% of toxic activity was cell associated (see Table 1). Small-scale polymyxin B extractions (3) of this strain were performed with different concentrations of polymyxin B and for different times (see Tables 2 and 3). Subsequently, large-scale extraction of cells with polymyxin B was performed as follows. A 500-μl volume of an overnight culture was inoculated into 500 ml of glucose-syncase broth (22) supplemented with 100 μg of ampicillin per ml in a 2-liter Erlenmeyer flask. Cultures were grown at 37°C for 24 h at 200 rpm on a G10 Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Culture supernatant was centrifuged at 3,500 × g for 20 min at 4°C, and the bacterial pellet was washed twice in phosphate-buffered saline, pH 7.4 (PBS). The bacterial pellet from a 6-liter culture was suspended in 500 ml of PBS containing 0.2 mg of polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml and gently shaken (75 rpm) at 37°C for 2 h. The suspension was centrifuged at 25,000 × g for 20 min at 4°C, and the supernatant (polymyxin B extract) was passed through a 0.45-μm-pore-size cellulose membrane filter (BDH Chemicals, Toronto, Ontario, Canada).

Ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ was added to the polymyxin B extract to 40% saturation, and the mixture was stirred at 4°C for 2 h. The precipitate was removed following centrifugation at 25,000 × g for 30 min at 4°C, and the supernatant was adjusted to 60% saturation and stirred at 4°C for 2 h. The second precipitate was collected by centrifugation as described above and dissolved in 20 ml of PBS. The solution was desalted by passage through a Sephadex G50 (Pharmacia LKB Biotechnology, Baie D'Urté, Quebec, Canada) column (1.6 by 50 cm) equilibrated with 10 mM Tris hydrochloride (pH 7.6). Fractions (5 ml) were collected and assayed for SLT-IIv. Fractions which contained SLT-IIv were pooled and dialyzed (Spectrapor-6; Spectrum Medical Industries, Inc., Los Angeles, Calif.) at 4°C for 18 h against three changes of 250 volumes of 10 mM Tris hydrochloride (pH 7.6).

Anion-exchange chromatography. The dialyzed pooled material (75 mg of protein) from the ammonium sulfate precipitation procedure was applied at 0.5 ml/min to a fast-protein liquid chromatography HR 5/5 Mono Q anion-exchange column (Pharmacia LKB Biotechnology) equilibrated in 10 mM Tris hydrochloride (pH 7.6). The column was washed with 10 ml of the same buffer and developed at 0.5 ml/min with a 45-ml 0.0 to 0.5 M NaCl gradient in 10 mM Tris hydrochloride (pH 7.6). Elution of protein was monitored at 280 nm. One-milliliter volumes were collected and assayed for SLT-IIv. Fractions with peak levels of SLT-IIv activity from two chromatographic runs were pooled and adjusted to pH 6.0 with 100 mM HCl.

Cation-exchange chromatography. The pooled material from anion-exchange chromatography was applied at 0.5 ml/min to a fast-protein liquid chromatography HR 5/5 Mono S cation-exchange (Pharmacia LKB Biotechnology) column equilibrated with 0.1 M NaCl in 10 mM Tris hydrochloride (pH 6.0). The column was washed with 10 ml of the same buffer and developed at 0.5 ml/min with a 45-ml 0.1 to 0.6 M NaCl gradient in 10 mM Tris hydrochloride (pH 6.0). Elution of protein was monitored at 280 nm. One-milliliter volumes were collected and assayed for SLT-IIv. Fractions containing peak levels of SLT-IIv activity were pooled and subjected to immunoaffinity chromatography.

Immunoaffinity exclusion chromatography. Antiserum from rabbits injected with a polymyxin B extract of strain TB1(pUC18) was saturated to 33% with $(NH_4)_2SO_4$ and centrifuged at 25,000 × g for 30 min at 4°C. The precipitate (ligand) was dissolved in 100 mM $NaHCO_3$ in 500 mM NaCl (pH 8.3) and dialyzed for 12 h at 4°C in two changes of 250 volumes of the same buffer. Cyanogen bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology) equilibrated in 1 mM HCl was incubated with the ligand at 4°C with gentle shaking overnight. Excess ligand was washed away with coupling buffer on a sintered glass filter, and the remaining active groups were blocked by incubation at 4°C with 100 mM Tris hydrochloride (pH 8.0) for 2 h. The resulting gel slurry was washed with alternating cycles of 100 mM acetate buffer in 500 mM NaCl (pH 4.0) and 100 mM Tris buffer in 500 mM NaCl (pH 8.0). Five milliliters of the gel was packed in a 10-ml pipette, washed extensively with PBS, and stored at 4°C.

Pooled fractions from the cation-exchange step were applied to the column at 0.5 ml/min and incubated at room temperature for 1 h. Protein was eluted at 0.5 ml/min with PBS, and 1-ml fractions were collected and assayed for SLT-IIv activity and protein. This material was the purified SLT-IIv used in further studies.

Analytical polyacrylamide gel electrophoresis (PAGE). A 500-μl sample from each step of the purification procedure was dialyzed against 250 volumes of distilled water and concentrated by evaporation at 37°C. Protein samples and low-molecular-weight markers (Sigma) were mixed with 5 μl of dissociating buffer (0.5 M Tris hydrochloride [pH 6.8], 2.0% sodium dodecyl sulfate [SDS], 5.0% 2-mercaptoethanol, 10.0% glycerol, 0.1% bromphenol blue) and heated at 100°C for 10 min. When nonreducing conditions were desired, 2-mercaptoethanol was omitted from the sample buffer. Ten-microliter samples were loaded onto individual lanes of 4% stacking gels (0.75 mm thick) and electrophoresed at a constant 150 V until the stacking dye reached the top of the resolving gel (15%) and then at 200 V until the

dye front reached the bottom of the resolving gel. Gels were fixed and protein bands were detected by silver staining (Bio-Rad Laboratories, Richmond, Calif.) as described previously (10).

Polyclonal antibody production. Antiserum to a polymyxin B extract of *E. coli* TB1(pUC18) was prepared in rabbits. The extract (1 mg of protein in 1 ml of PBS) was emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and 1 ml of the emulsion was injected subcutaneously into each of two adult New Zealand White rabbits. At 2-week intervals, the rabbits were injected intravenously with the extract without adjuvant. Two weeks after the fourth injection, the animals were killed and bled. Serum was collected and stored at −20°C.

Antiserum to purified SLT-IIv was prepared in pigs. Samples of 100 μg of SLT-IIv protein in 1 ml of PBS were detoxified by incubation with 0.4% glutaraldehyde at 4°C overnight and stored at −20°C. As needed, samples were thawed and emulsified in 1 ml of $Al(OH)_3$ gel, and 1 ml of the emulsion (50 μg of SLT-IIv) was injected intramuscularly into each of two Yorkshire-Landrace pigs (15 to 20 kg). Six injections of the same material were given at 1-week intervals. Two weeks after the last injection, the animals were killed and bled. Serum was separated and stored at −20°C.

Western blotting (immunoblotting). Purified SLT-IIv was subjected to SDS-PAGE in 15% gels as described previously (10). Dissociated toxin subunits and molecular weight (MW) markers were electrophoretically transferred (2 h at a constant current of 125 mA) at room temperature from the gel to nitrocellulose (Hybond-C; Amersham, Oakville, Ontario, Canada). An additional 20% methanol was included in the transfer buffer to enhance detection of the B subunit. The portion of the nitrocellulose blot corresponding to the MW marker lane was cut off and stained with 0.2% amido black. The rest of the strip was blocked with 3.0% gelatin for 2 h at 37°C or at 4°C overnight and then washed twice with PBS. Individual strips (0.5 cm wide) of nitrocellulose were incubated with the primary antibody at 37°C for 2 h or at 4°C overnight and then washed twice with PBS. The strips were incubated with a 1/2,000 dilution of protein A-horseradish peroxidase (Bio-Rad) in PBS for 2 h at 37°C, washed twice with PBS, and developed with a solution of 4-chloro-1-naphthol in methanol and 0.002% H_2O_2 in PBS.

Protein and lipopolysaccharide determination. Protein contents of samples from the different steps of the purification scheme were assayed by the method of Bradford (1) with bovine serum albumin as the standard. Lipopolysaccharide was determined throughout the purification procedure by the *Limulus* amoebocyte lysate assay (sensitivity, 0.1 ng of endotoxin per ml) with purified *E. coli* lipopolysaccharide (Sigma) as the standard.

Reproduction of ED with SLT-IIv. Doubling dilutions of purified SLT-IIv were administered intravenously to five groups of six weaned (10 to 15 kg) Yorkshire-Landrace pigs. The pigs were observed for 4 days for signs of ED. The pigs were killed after 4 days, or earlier if they became very ill. A postmortem examination was performed on each animal, and samples of stomach, small intestine, cecum, colon, spleen, liver, kidney, heart, muscle, eye, and brain tissues were collected for histopathological examination.

Neutralization studies. Doubling dilutions of serum were made with EMEM in 96-well microtiter plates. Ten CD_{50} s of purified SLT-IIv or polymyxin B-extracted SLT-I, SLT-II, or VT2 was pipetted into each well, and the plates were incubated at 37°C for 1 h and then at 4°C overnight. A 100-μl volume (4×10^5 cells per ml) of Vero cells was pipetted into each well, and the plates were incubated at 37°C in 5% CO_2 for 72 h. The plates were stained as described previously (22).

Cytotoxicity. The susceptibilities of Vero, Madin-Darby bovine and canine kidney, porcine kidney 15 (PK15), and human cervical carcinoma (HeLa) cells to the cytotoxic activity of purified SLT-IIv were determined. All cell lines, with the exception of HeLa cells, were maintained as described previously for Vero cells. For HeLa cell culture, 10% fetal bovine serum was used. Doubling dilutions of SLT-IIv were made with EMEM in 96-well microtiter plates and 100 μl of cells (4×10^5 cells per ml) was pipetted into each well. The plates were incubated as described previously (22).

Mouse lethality. Five groups of eight adult (4- to 6-week-old) Swiss Webster mice (Animal Care Facility, University of Guelph, Guelph, Ontario, Canada) were injected intraperitoneally with doubling dilutions of purified SLT-IIv. The mice were observed at 4-h intervals, and deaths were recorded over 7 days. The 50% lethal dose was calculated as described previously (25).

Enterotoxicity. Purified SLT-IIv was tested for enterotoxicity in rabbit intestinal loops. Male New Zealand White rabbits were starved for 24 h and anesthetized. In each rabbit, eight jejunal loops 9 to 10 cm long were constructed with 2 cm between adjacent loops. One-milliliter volumes of doubling dilutions of SLT-IIv and control materials were injected into four animals. Polymyxin B extracts of *E. coli* H30, C600(933W), and E32511 served as positive controls, and PBS was used as a negative control. Rabbits were killed after 18 h by barbiturate overdose (Euthanyl Forte; M. T. C. Pharmaceuticals, Cambridge, Ontario, Canada), and the volumes of fluid and lengths of ileal loops were measured. Fluid accumulation of 1 ml/cm of intestine was considered a positive response.

TABLE 1. Effect of polymyxin B[a] concentration on release[b] of SLT-IIv from *E. coli* TB1(pCG6)

Polymyxin B concn (mg/ml)[c]	Total no. of CD_{50}s	Total protein (μg)	No. of CD_{50}s/μg of protein
0.00	3.2×10^3	4.0	8.0×10^2
0.05	3.4×10^4	40.0	8.6×10^2
0.10	3.0×10^5	40.0	7.5×10^3
0.20	1.9×10^6	92.0	2.0×10^4
0.50	1.9×10^6	136.0	1.4×10^4
1.00	1.9×10^6	164.0	1.2×10^4

[a] Cell lysate produced 2.1×10^6 CD_{50}s/ml of culture, with a total protein content of 1,840.0 μg and 1.1×10^3 CD_{50}s/μg of protein.
[b] Cytotoxicity per milliliter of culture.
[c] Cells were incubated with polymyxin B for 2 h.

TABLE 2. Effect of incubation time with polymyxin B[a] on release[b] of SLT-IIv from *E. coli* TB1(pCG6)

Incubation time (min)	Total no. of CD_{50}s	Total protein (μg)	No. of CD_{50}s/μg of protein
15	1.0×10^5	56.0	1.9×10^3
30	1.0×10^5	52.0	2.0×10^3
45	4.0×10^5	60.0	6.7×10^3
60	4.0×10^5	72.0	5.6×10^3
120	1.9×10^6	96.0	2.0×10^4

[a] Cells were incubated with 0.2 mg of polymyxin B per ml.
[b] Cytotoxicity per milliliter of culture.

TABLE 3. Purification of SLT-IIv from *E. coli* TB1(pCG6)

Purification step	Vol (ml)	Total no. of CD_{50}s	Total protein (mg)	No. of CD_{50}s/mg of protein	% Yield	Fold purification
Polymyxin B extraction	450	1.1×10^{10}	540.0	2.0×10^{7}	100	
$(NH_4)_2SO_4$ precipitation	25	1.0×10^{10}	186.0	5.4×10^{7}	94	2.7
Anion exchange	5	8.2×10^{9}	20.5	4.0×10^{8}	75	20.0
Cation exchange	4	6.6×10^{9}	1.8	3.7×10^{9}	60	183.0
Immunoaffinity chromatography	7	6.6×10^{9}	1.5	4.4×10^{9}	60	222.0

RESULTS

Purification of SLT-IIv. Optimal conditions for extraction of SLT-IIv from strain TB1(pCG6) were incubation with 0.2 mg of polymyxin B per ml at 37°C for 2 h (Tables 1 and 2). Under these conditions, the extract contained 90% of cell-associated SLT-IIv and represented a 20-fold enrichment in toxic activity per milligram of protein compared with cell lysates. Incubation of cells with polymyxin B at less than 0.2 mg/ml resulted in incomplete release of SLT-IIv, while higher concentrations of polymyxin B did not increase recovery of toxin but increased total protein (Table 1). Incubation periods shorter than 2 h resulted in incomplete release of SLT-IIv from bacterial cells (Table 2).

Differential ammonium sulfate precipitation was used as the initial purification and concentration step. At 40% saturation, approximately 1% of the toxin was precipitated, along with 20% of the starting total protein. The precipitate from 60% saturation contained 94% of the starting toxic activity and gave 2.7-fold purification (Table 3). The desalting step on Sephadex G50 was implemented because dialysis after $(NH_4)_2SO_4$ precipitation resulted in an insoluble precipitate and loss of SLT-IIv activity.

Next, the toxin preparation was subjected to anion-exchange chromatography using a 0.0 to 0.5 M NaCl gradient. The peak of cytotoxic activity eluted between 0.05 and 0.10 M NaCl (Fig. 1) and resulted in 20-fold purification with a 75% yield of cytotoxic activity (Table 3).

During cation-exchange chromatography, the peak of cytotoxic activity eluted between 0.15 and 0.20 M NaCl (Fig. 2). Pooled material was purified 183-fold with a 60% yield.

The final purification step, immunoaffinity exclusion chromatography, resulted in a preparation that was purified 222-fold with a recovery of 60%. This purification scheme produced 1.5 mg of purified SLT-IIv from a 30-g bacterial pellet (Table 3). The entire purification scheme from extraction of the bacterial cells to the end of the immunoaffinity procedure was completed in 3 days. Purified SLT-IIv preparations were free of endotoxin.

Analytical PAGE. SDS-PAGE of cell lysate, polymyxin B extract, $(NH_4)_2SO_4$ precipitate, and anion and cation exchange chromatography and immunoaffinity-purified preparations from TB1(pCG6) is shown in Fig. 3. Cell lysate (Fig. 3A, lane 1) had a greater number of bands and a higher concentration of protein than the polymyxin B extract (lane 2), and the $(NH_4)_2SO_4$ precipitate (lane 3) had fewer bands and a reduction in intensity of other bands compared with the polymyxin B extract. Only three protein bands were detected in the highly cytotoxic material recovered after cation-exchange chromatography (Fig. 3B, lane 2) and immunoaffinity exclusion chromatography (lane 3). These protein bands were also observed after anion-exchange chromatography (lane 1), but several additional protein bands were present at this stage of purification. In some preparations of the cation-exchange material, three weak bands (not present in Fig. 3B) were detected. In no case were these bands still present after immunoaffinity exclusion chromatography. Examination of corresponding preparations from *E. coli* TB1(pUC18) showed that there were no discernible differences in the patterns obtained with lysates, polymyxin B extracts, or ammonium sulfate precipitates. However, the three protein bands shown in the anion- and cation-exchange and immunoaffinity exclusion chromatography material from TB1(pCG6) were not present in the corresponding material from TB1(pUC18). The three bands associated exclusively with TB1(pCG6) migrated as protein bands with MWs of 33,000, 27,500, and 7,500, which were consistent with the expected MWs of the A, A_1, and B subunits, respectively.

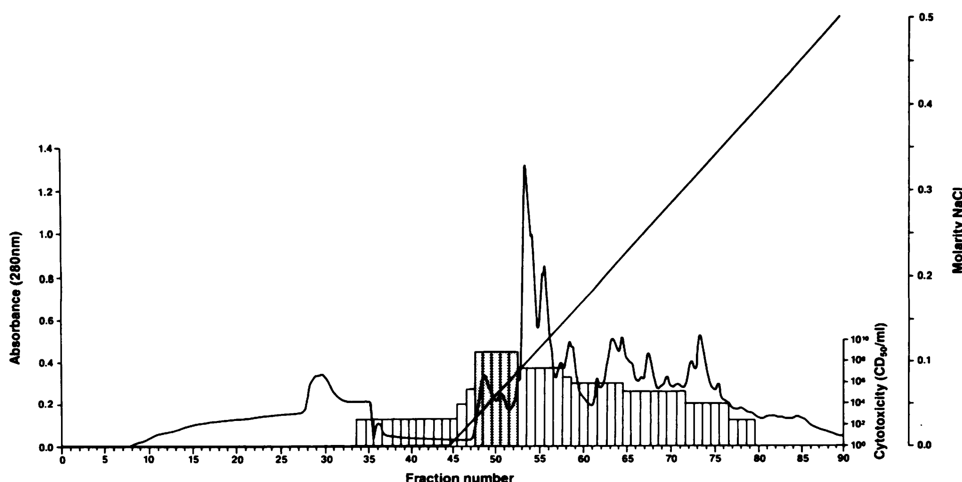

FIG. 1. Representative protein elution profile from anion-exchange chromatography of $(NH_4)_2SO_4$-precipitated polymyxin B extract of *E. coli* TB1(pCG6). Approximately 75 mg of protein was chromatographed over a 0.0 to 0.5 M NaCl gradient at a flow rate of 0.5 ml/min. One-milliliter volumes were collected and assayed for SLT-IIv activity.

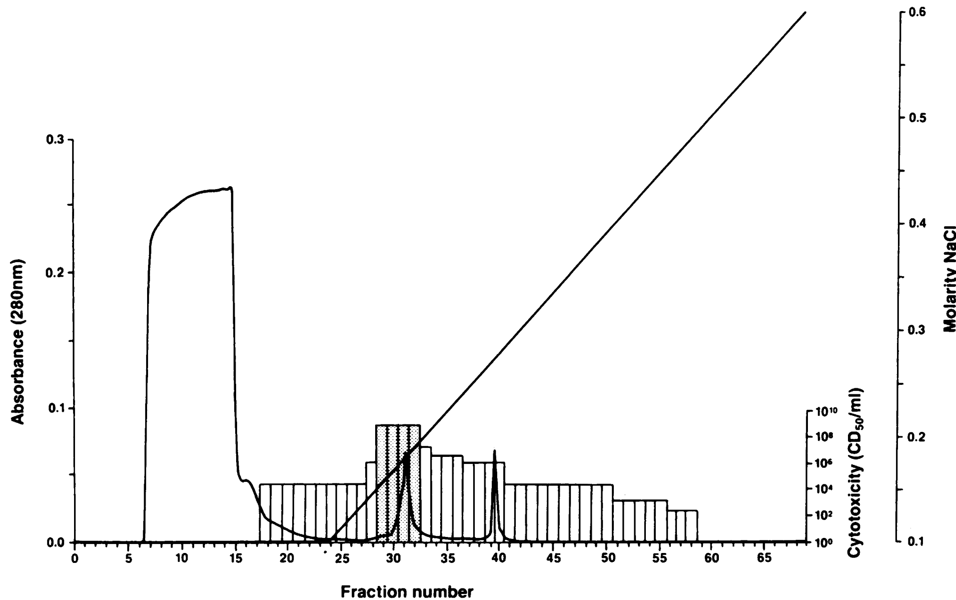

FIG. 2. Representative protein elution profile from cation-exchange chromatography of pooled TB1(pCG6) anion-exchange fractions which contained SLT-IIv activity. Approximately 20 mg of protein was chromatographed over a 0.1 to 0.6 M NaCl gradient at a flow rate of 0.5 ml/min. One-milliliter volumes were collected and assayed for SLT-IIv activity.

When immunoaffinity-purified SLT-IIv was electrophoresed under nonreducing conditions (Fig. 3C, lane 2), bands with MWs of 33,000 and 7,500 were observed.

Western immunoblot. Western blot analysis with anti-SLT-IIv sera showed three bands with MWs of 33,000, 27,500, and 7,500, which were consistent with the A and A_1, and B subunits (Fig. 4). When methanol was omitted from the transfer buffer, the band corresponding to the B subunit was not detected. No bands were demonstrated with preimmunization or anti-TB1(pUC18) serum.

Reproduction of ED. Typical ED developed in pigs which received as little as 3 ng of SLT-IIv per kg of body weight. Speed of onset and severity of clinical signs and pathologic lesions were directly related to the toxin dose. The first signs of ED occurred as early as 7 h postinjection and consisted of edema of the eyelids and inappatence. Neurological signs consisting of incoordination, confusion, and ataxia were the first signs of severe illness. Paralysis, tremors, paddling of the limbs, convulsions, dyspnea, altered squeal, and coma were observed in advanced stages of the disease. Gross and microscopic lesions characteristic of ED were observed in the stomach, cecum, colon, and brain, and edema was noted in the subcutaneous tissues of the eyelids, throats, and foreheads of affected animals.

Neutralization studies. Results of the neutralization studies are summarized in Table 4. Anti-SLT-I neutralized only homologous toxin. Both anti-SLT-IIv and anti-SLT-II sera neutralized homologous toxin at high titers and neutralized

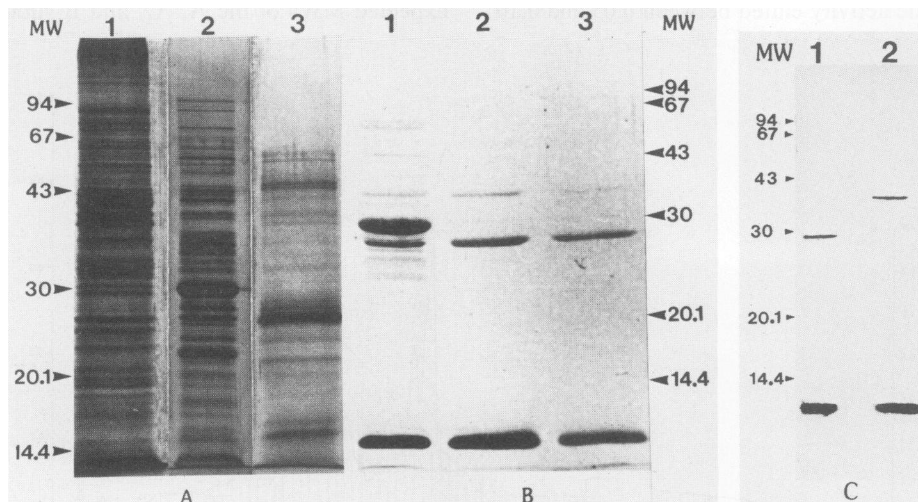

FIG. 3. Silver-stained SDS-PAGE preparations from *E. coli* TB1(pCG6). (A) Cell lysate (lane 1), polymyxin B extract (lane 2), and $(NH_4)_2SO_4$-precipitated material (lane 3). Each lane contained approximately 2×10^6 CD_{50}s. (B) Demonstration of protein bands associated with purified SLT-IIv. Lanes 1 to 3 show anion-exchange, cation-exchange, and immunoaffinity chromatography preparations, respectively, each at approximately 5×10^8 CD_{50}s. (C) Purified SLT-IIv electrophoresed under reducing (lane 1) and nonreducing conditions (lane 2). The MWs indicated are in thousands.

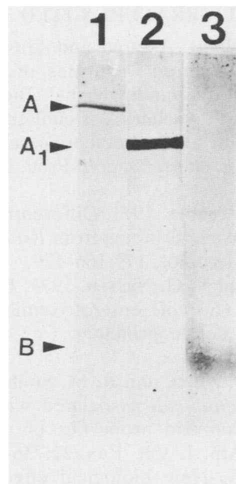

FIG. 4. Western blot of purified SLT-IIv immunostained with porcine polyclonal antibody to SLT-IIv. The A subunit (MW, 33,000) was detected under nonreducing conditions (lane 1), and the A_1 subunit (MW, 27,500) was detected under reducing conditions (lane 2). The B subunit (MW, 7,500) was detected only when an additional 20% methanol was included in the transfer buffer (lane 3).

heterologous toxins (other than SLT-I) to 1/16 to 1/4 of the homologous titer. Anti-VT2 serum, on the other hand, completely neutralized both homologous toxin and heterologous toxin (other than SLT-I) to the same high titer.

Cytotoxicity. The CD_{50}s of purified SLT-IIv for Vero, PK15, and Madin-Darby bovine and canine kidney cells were 0.5, 2, 8, and 8 pg, respectively. Rounding and detachment of cells were seen at 18 to 24 h after exposure to toxin. SLT-IIv did not have cytotoxic activity on HeLa cells.

Lethality for mice. The 50% lethal dose of purified SLT-IIv in mice was 0.9 pg per mouse (Table 5). The first signs of toxic activity appeared after 24 h and included ruffled fur, huddling, and disinclination to move. Hindlimb paralysis and rapid breathing were the first signs of severe illness, and mice usually died within 12 h of these signs. As the dose of toxin decreased, signs of intoxication developed more slowly and survival times increased. Diarrhea was not observed in any of the mice.

Enterotoxicity. SLT-IIv was enterotoxic in rabbit intestinal loops at 3.2×10^8 and 1.6×10^8 CD_{50}s (75 μg of protein) but not at 8×10^7 or 4×10^7 CD_{50}s. SLT-I, SLT-II, and VT2 gave positive responses at 1×10^6 CD_{50}s.

DISCUSSION

Previous attempts to purify the ED toxin were hindered by the need to use mice and pigs for detection of toxin (4, 5, 7, 12, 31). As soon as it appeared that ED toxin was toxic to Vero cells (6, 21, 30), convenient and sensitive cell culture assays were substituted. Even with these assays, however, the relatively low levels of VT characteristic of ED strains of *E. coli* (20, 30) constituted a formidable obstacle to purification. In this study, strain TB1(pCG6) was used as the source of toxin for purification because it produced up to 100 times more SLT-IIv than did the wild strain from which the genes were cloned (22) and because it allowed a direct comparison with negative-control strain TB1(pUC18) throughout purification. Culture conditions for optimal yield of SLT-IIv were based on results of a previous study (22), in which the highest yields of SLT-IIv were obtained with incubation at 37°C for 24 h with shaking in syncase broth adjusted to pH 8.0. Except for the starting pH, these culture conditions were similar to those used by Kongmuang and colleagues (19) in their work with SLT-I.

TABLE 4. Neutralization of SLTs with homologous and heterologous antisera

Antigen	Titer[a] (%)[b]			
	SLT-IIv	SLT-II	VT2	SLT-I
SLT-IIv	20,480 (100.0)	1,280 (6.3)	2,560 (12.5)	—[c] (0.0)
SLT-II	320 (25.0)	1,280 (100.0)	160 (12.5)	— (0.0)
VT2	5,120 (100.0)	5,102 (100.0)	5,120 (100.0)	— (0.0)
SLT-I	— (0.0)	— (0.0)	— (0.0)	10,240 (100.0)

[a] The titer is expressed as the reciprocal of the highest dilution of a 50-μl volume of serum which protected Vero cells from 10 CD_{50}s of SLT or VT.
[b] Percent neutralization of homologous toxin.
[c] —, No neutralization.

TABLE 5. Morbidity and mortality of eight mice injected intraperitoneally with purified SLT-IIv

SLT-IIv Amt (pg) of injected	No. sick	No. dead	Mean ± SD time (h) of death postinjection
7.20	8	8	44 ± 5
3.60	8	7	66 ± 22
1.80	6	6	70 ± 14
0.90	5	4	138 ± 20
0.45	2	0	NA[a]

[a] NA, Not applicable.

The association of ED toxin with bacterial cells has long been recognized by researchers who used either cell lysates or cell extracts as toxin sources (4–7). Other researchers have reported that SLT-IIv (11, 22, 23) is predominantly cell associated, and cell lysate has been used as the starting material for purification of cell-associated Shiga toxin (9), SLT-I (25), and SLT-II (10). A polymyxin B extract was selected in the present protocol for SLT-IIv because most of the toxin in the cell could be extracted and the specific activity of the extract was approximately 20 times that of the cell lysate. Polymyxin B extraction has been reported to be effective for the release of Shiga toxin (8, 13), SLT-I (28), and VT2 (S. C. Head, M. A. Karmali, M. Petric, and M. E. Roscoe, Int. Symp. Workshop on Verocytotoxin (Shiga-like toxin) Producing *Escherichia coli* (VTEC) Infections, abstr. no. STF-16, 1987). Ammonium sulfate precipitation is a common step in purification of ED toxin (4, 7, 11, 12) and SLTs (9, 10, 19, 28, 36, 37) and was effective for SLT-IIv in this study. Preliminary studies of precipitation of SLT-IIv with trichloroacetic acid gave highly variable results, while precipitation with acetone resulted in insoluble material and loss of toxic activity (unpublished data).

The overall purification scheme resulted in high recovery of toxin in a relatively short time. The yield of SLT-IIv in this study was comparable to yields reported for Shiga toxin (9, 25), SLT-I (19, 25, 28), and SLT-II (10). Several features of this purification scheme differ from those used in purification of other SLTs. (i) Anion- and cation-exchange fast-protein liquid chromatography was used to provide rapid (2 h) and highly reproducible separation of proteins. (ii) We did not use a chromatofocusing procedure because of loss of cytotoxic activity at this step (unpublished data). (iii) The immunoaffinity exclusion step in our studies used a polyvalent antiserum to remove contaminants instead of toxin, thereby simplifying toxin recovery.

The MWs of the A and B subunits of SLT-IIv indicated by SDS-PAGE (33,000 and 7,500) are in good agreement with

those predicted by DNA sequence analysis for the processed A and B subunits (14, 35). The A subunit of SLT-IIv, like those of Shiga toxin (9, 25), SLT-I (25), and SLT-II (10), was readily converted to the A_1 form under reducing electrophoresis conditions.

Western blot analysis of purified SLT-IIv revealed the presence of three protein bands with MWs of 33,000, 27,500, and 7,500 which correspond to the A, A_1, and B subunits demonstrated by SDS-PAGE. Similar results were obtained with two other batches of anti-SLT-IIv sera prepared by another protocol (unpublished data).

Details of the clinical picture, gross lesions, and histological changes and their relation to the SLT-IIv dose will be reported elsewhere. Purified SLT-IIv was effective in reproducing all the clinical and pathologic features of ED, thereby confirming the identity of SLT-IIv and ED toxin. Hemorrhagic colitis was observed in pigs which received large doses of SLT-IIv. This is not a common feature of naturally occurring ED, but it has been observed in some cases (11). None of the effects of endotoxin reported in previous studies (4, 5, 11) were observed. Much needs to be understood, however, about the pathogenesis of ED, including colonization of the intestines and absorption of toxin from the gut.

In this study, there were cross-neutralizations of SLT-II-related toxins by antisera to SLT-IIv, SLT-II, and VT2, whereas anti-SLT-I serum neutralized only homologous toxin. These results are consistent with findings by others (10, 18, 25–28) and suggest that the SLT family may be conveniently considered to consist of two groups, namely, SLT-I and Shiga toxin as group 1 and the others as group 2. SLT-IIv was cytotoxic to Vero cells at concentrations similar to those reported for Shiga toxin (19, 36), SLT-I (19, 28), and SLT-II (28). SLT-IIv was not cytotoxic to HeLa cells, a finding reported by some researchers (6, 23) but not by others (Gannon and Gyles, in press). This discrepancy may be due to differences in the sensitivities of the various HeLa cell cultures to SLT-IIv (36).

Purified SLT-IIv was lethal to mice at 0.9 pg, a dose similar to those reported for Shiga toxin (9, 25), SLT-I (19, 25, 28), and SLT-II (10).

The enterotoxic activity of SLT-IIv is of particular interest because of uncertainty as to whether this toxin causes diarrhea in pigs (11). Although purified SLT-IIv was enterotoxin in rabbit intestinal loops, the dose required was approximately 75 times the dose reported for Shiga toxin (9, 25, 36) and SLT-I (19, 25, 28).

In this report, we have described purification of SLT-IIv by a protocol which is relatively rapid and results in high recovery of toxin. We have demonstrated that the pure toxin, free of endotoxin, reproduces all the signs of ED. Both the similarities of SLT-IIv to SLT-II (14, 35) and the similarities in the vascular lesion which occurs in ED and in the SLT-associated diseases in humans (17) indicate that experimental ED may be a useful natural model of disease due to SLT. For this model to be fully exploited, however, studies need to be conducted on reproduction of disease in pigs to which toxin is administered orally or intraintestinally.

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