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The Specific Activities of Shiga-Like Toxin Type II (SLT-II) and SLT-II-Related Toxins of Enterohemorrhagic *Escherichia coli* Differ When Measured by Vero Cell Cytotoxicity but Not by Mouse Lethality

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Characteristically, enterohemorrhagic *Escherichia coli* (EHEC) strains produce Shiga-like toxin type I (SLT-I), SLT-II, or both of these immunologically distinct cytotoxins. No antigenic or receptor-binding variants of SLT-I have been identified, but a number of SLT-II-related toxins have been described. Because EHEC O91:H21 strain B2F1, which produces two SLT-II-related toxins, is exquisitely virulent in an orally infected, streptomycin-treated mouse model (oral 50% lethal dose [LD_{50}], <10 organisms), we asked whether the pathogenicity of strain B2F1 was a consequence of SLT-II-related toxin production. For this purpose, we compared the lethality of orally administered *E. coli* DH5α(Str^r) strains that produced different cytotoxic levels of SLT-II, SLT-IIvha (cloned from B2F1), SLT-IIvhb (also cloned from B2F1), or SLT-IIc (cloned from EHEC O157:H7 strain E32511) on Vero cells. We also calculated the specific activities of purified SLT-IIvhb and SLT-II in intraperitoneally injected mice and on Vero cells. The two purified toxins were equally toxic for mice, but SLT-IIvhb was approximately 100-fold less active than SLT-II on Vero cells and bound to the glycolipid receptor Gb_3 with lower affinity than did SLT-II. In addition, characterization of SLT-II-related toxin-binding (B) subunit mutants generated in this study revealed that the reduced in vitro cytotoxic levels of the SLT-II-related toxins were due to Asn-16 in the B subunit. Taken together, these findings do not support the idea that B2F1 is uniquely virulent because of the in vivo toxicity of SLT-II-related toxins but do demonstrate differences in in vitro cytotoxic activity among the SLT-II group produced by human EHEC isolates.

Infection with enterohemorrhagic *Escherichia coli* (EHEC) is associated with food-borne outbreaks of diarrhea (47), hemorrhagic colitis (28, 41, 42), and the hemolytic uremic syndrome (17, 24, 28, 39). One such outbreak occurred recently in the Pacific Northwest (3, 34). A key characteristic of these EHEC strains is that they produce Shiga-like toxins (SLTs) (for reviews, see references 10, 17, 33, 52, and 53). These SLTs, also known as verotoxins (VT) (18), inhibit protein synthesis by cleaving a specific adenine residue in the 28S subunit of eukaryotic rRNA (6). They are multisubunit toxins that consist of one enzymatically active A subunit and five receptor-binding B subunits and are cytotoxic for Vero tissue culture cells (33).

Two groups of SLTs that differ significantly in immunological reactivity have been described. They have been designated Shiga-like toxin type I (SLT-I) and Shiga-like toxin type II (SLT-II). The SLT-I group contains the prototype toxin SLT-I in addition to Shiga toxin from *Shigella dysenteriae* type I. SLT-I and Shiga toxin differ by only one amino acid (49) and are considered to be the same toxin. SLT-I/Shiga toxin uses the glycolipid globotriaosylceramide (Gb_3; Galα1-4 Galβ1-4Glcβ1-1Cer) as the functional eukaryotic cell surface receptor (20, 23).

The members of the SLT-II group cannot be neutralized by anti-SLT-I/Shiga toxin monoclonal or polyclonal antiserum (15). Unlike the SLT-I/Shiga toxin group, the SLT-II group exhibits sequence and antigenic variation. SLT-II produced by EHEC O157:H7 strains is considered the prototype toxin for this group, which also includes the toxin responsible for edema disease of swine (25, 29, 58), SLT-IIv (recently renamed SLT-IIe [see Materials and Methods for discussion of nomenclature]). In contrast to SLT-II, SLT-IIv binds more avidly to globotetraosylceramide (Gb_4; GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) than to Gb_3 (4, 43).

Since 1990, a number of new SLTs have been identified that are considered members of the SLT-II group on the basis of sequence homology and immunological cross-reactivity with SLT-II. These SLT-II group members include SLT-IIvha (VT2vha), SLT-IIvhb (VT2vhb), and SLT-IIc, all of which are nearly 97% homologous to SLT-II at the deduced amino acid sequence level (14, 45). Other SLT-II-related toxins have been described (8, 12, 21, 30, 37, 44, 54, 59), but many of these toxins were detected by PCR analysis and only a few have been isolated and sequenced.

Several clinical EHEC isolates that contain more than one copy of *slt*-II have been reported. Schmitt et al. described five such strains (45), including *E. coli* O157:H strain E32511, which contains *slt*-II and *slt*-IIc, and *E. coli* O91:H21 strain B2F1, which contains *slt*-IIvha and *slt*-IIvhb (14). We recently cloned and characterized three genes for SLTs from the EHEC O91:H21 isolate H414-36/89 (21). Antigenic analysis showed that two of the toxins from EHEC H414-36/89 were SLT-II related and that one was similar to the prototype SLT-II toxin.

A number of epidemiological studies have correlated infection with EHEC strains that produce high levels of SLT and

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TABLE 1. Bacterial strains and plasmids used or discussed in this study

Strain or plasmid	Description or genotype	Source or reference
E. coli strains		
DH5α	*supE44 ΔlacU169* (φ80*lacZ*ΔM15)*hsdR17 recA1 endA1 gyrA thi-1 relA1*	11
CJ236	*dut-1 ung-1 thi-1 relA1*(pCJ105)	19
B2F1	O91:H21 EHEC isolate; *slt*-IIvha, *slt*-IIvhb	14
H414-36/89	O91:H21 EHEC isolate; two *slt*-II-related toxins, one *slt*-II	21
E32511	O157:H7 EHEC isolate; *slt*-II, *slt*-IIc	45
Plasmids		
pCKS120	5.5-kb *Eco*RI *slt*-IIc fragment from E32511 in pBR328	This study
pCKS123	4.8-kb *Eco*RI *slt*-II fragment from E32511 in pBR328	This study
pCKS184	1.8-kb *slt*-IIc fragment from E32511 in pBR328	This study
pCKS188	pCKS184 with B subunit mutation N16D	This study
pCKS189	pCKS184 with B subunit mutation A24D	This study
pJES120	*slt*-II in pSK(−)	21
pSQ12	*slt*-IIvha from B2F1 in pHC79	21
pSQ343	*slt*-IIvha in pKS(−)	21
pJES54	*slt*-IIvhb from B2F1 in pHC79	This study
pSQ543	2.8-kb *Pst*I-*Eco*RI *slt*-IIvhb fragment from pJES54 in pSK(−)	This study
pSQ545	2.8-kb *Pst*I-*Eco*RI *slt*-IIvhb fragment in pKS(−)	This study
pSJ5431	pSQ543 with B subunit mutation N16D	This study
pSJ5432	pSQ543 with B subunit mutation A24D	This study
pSJ5433	pSQ543 with B subunit double mutation N16DA24D	This study
pSQ549	2.8-kb *Bam*HI-*Hin*dIII *slt*-IIvhb fragment from pSQ543 in pBR328	This study
pSJ5491	2.8-kb *Bam*HI-*Hin*dIII *slt*-IIvhb/N16D fragment from pSJ5431 in pBR328	This study
pSJ5492	2.8-kb *Bam*HI-*Hin*dIII *slt*-IIvhb/A24D fragment from pSJ5432 in pBR328	This study
pSJ5493	2.8-kb *Bam*HI-*Hin*dIII *slt*-IIvhb/N16DA24D fragment from pSJ5433 in pBR328	This study

the development of the hemolytic uremic syndrome (17, 24, 28, 39). Some of these investigators have incriminated the SLT-II group more than the SLT-I group in the development of microangiopathic sequelae. Specifically, epidemiological data from both Seattle (36) and England (46) indicate that strains that produce SLT-II alone are more likely to cause hemorrhagic colitis that results in the hemolytic uremic syndrome or thrombotic thrombocytopenic purpura than are strains that produce SLT-I and SLT-II or those that produce SLT-I alone. Furthermore, animal model EHEC studies by Wadolkowski et al. (57), Barrett et al. (1), and Francis et al. (7) have shown a similar correlation between SLT-II production and severity of disease in mice, rabbits, and gnotobiotic piglets, respectively. Recently, we reported that the EHEC O91:H21 strains B2F1 and H414-36/89 isolated from patients with the hemolytic uremic syndrome and hemorrhagic colitis, respectively, were highly virulent (50% lethal dose [LD_{50}], less than 10 organisms) in an orally inoculated, streptomycin-treated mouse model (21). We also found that EHEC strains that contain *slt*-II alone or *slt*-II in conjunction with only one *slt*-II-related toxin gene or with one *slt*-I gene are significantly less virulent in the same model system (21). The aim of the present investigation was to determine whether differences in toxicity between SLT-II and SLT-II-related toxins might explain the exquisite virulence of EHEC B2F1 in orally challenged mice. To test this hypothesis, the effects of the toxins were compared after delivery of toxin by *E. coli* DH5α(Str^r) in the orally infected mouse model and after purification and injection intraperitoneally into mice.

(A portion of this work was presented at the 92nd General Meeting of the American Society for Microbiology, New Orleans, La., 26 to 30 May 1992 [22].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and recombinant plasmids used or discussed in this study are listed in Table 1. Table 2 describes the amino acid relationship among relevant SLT-II-related toxins used. The new nomenclature for SLT-II-related toxins that appear to be closely related to SLT-II by DNA hybridization and sequence homology and exhibit only minor variations in other properties is "SLT-IIc," and the name for the toxin produced by *E. coli* that causes edema disease in swine has been changed from SLT-IIv to SLT-IIe (58a). However, the original designations for the "SLT-IIc" toxins, SLT-IIvha, SLT-IIvhb, and SLT-IIc, will be retained in this study so as to precisely identify the particular clones and toxin sequences. Collectively, these toxins will be called SLT-II-related toxins.

Media, enzymes, and biochemicals. Bacterial strains were routinely grown in Luria-Bertani medium (27). When selective pressure was required, the medium was supplemented with antibiotics (Sigma Chemical Co., St. Louis, Mo.) at the following concentrations per milliliter: ampicillin, 200 μg for pBluescript vectors and 100 μg for pBR328 and pHC79; streptomycin, 30 μg. Restriction enzymes, RNase, Klenow DNA polymerase, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Sequenase

TABLE 2. Amino acid sequence differences among SLT-II toxins[a]

Toxin	Amino acid sequence of[b]:	
	A subunit	B subunit
SLT-II	S19, H45, V252, F291, K297	D16, D24
SLT-IIc	* * * * *	N16, A24
SLT-IIvha	T19, P45, * S291, E297	N16, A24
SLT-IIvhb	* P45, M252, S291, E297	N16, A24

[a] Amino acids at the positions listed in the processed A subunit (297 total amino acids) or B subunit (70 total amino acids) of SLT-II (15), SLT-IIc (45), SLT-IIvha (14), and SLT-IIvhb (14).

[b] S, serine; H, histidine; V, valine; F, phenylalanine; K, lysine; D, aspartate; T, tyrosine; P, proline; E, glutamine; M, methionine; *, amino acid identity with the prototype SLT-II toxin at that position.

enzyme and Sequenase sequencing kits were purchased from U.S. Biochemical Corp., Cleveland, Ohio. Silver grade fetal calf serum was purchased from ICN Biomedical Inc., Irvine, Calif., and Eagle's minimum essential medium was purchased from Whittaker Bioproducts, Inc., Walkersville, Md.

Mouse feeding and LD_{50} studies. The method for mouse feeding was described previously (56). CD-1 male mice aged 5 to 6 weeks old (Charles River Laboratories, Wilmington, Mass.) were used throughout this study. Throughout each experiment, mice were given drinking water which contained streptomycin (5 g/liter) and ampicillin (5 g/liter). Oral streptomycin treatment reduces the level of facultative anaerobic bacteria that normally colonize the mouse intestine (31). The ampicillin was given orally to prevent segregation of plasmids that contained ampicillin resistance markers. A spontaneously streptomycin-resistant (Str^r) *E. coli* K-12 strain [DH5α(Str^r)] transformed with vector alone or with vector containing a cloned toxin gene was fed to groups of three to five mice at levels of approximately 10^{10} CFU. Mouse death was monitored daily for 14 days. Kidneys were isolated from moribund mice or healthy controls for histological analysis. The specimens were fixed in 10% buffered formalin, processed routinely for histologic examination, and stained with hematoxylin and eosin.

The quantity of purified toxin required to kill mice was assessed by LD_{50} studies. Various dilutions of purified SLT-II or SLT-IIvhb in 0.2 ml of sterile nonpyrogenic 0.9% saline (Kendall McGaw Laboratories, Inc., Irvine, Calif.) were administered intraperitoneally to groups of five CD-1 mice. Deaths were monitored daily, and LD_{50}s were determined by the method of Reed and Muench (40). The experiments reported here were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (32).

Toxin purification. Both SLT-II and SLT-IIvhb were purified from *E. coli* DH5α that contained the appropriate toxin gene on a high-copy pBluescript plasmid (Stratagene). Saturated overnight cultures of *E. coli* DH5α/pJES120 (SLT-II) or *E. coli* DH5α/pSQ543 (SLT-IIvhb) were inoculated into a total of 6 liters of enriched medium (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol). The bacteria were cultured for 24 h, harvested by centrifugation, and lysed by sonic disruption. The bacterial lysates were clarified by centrifugation and concentrated with 60% ammonium sulfate. The resultant crude toxin preparations were dialyzed against 0.05 M Tris (pH 7.0) (SLT-II) or 0.05 M Tris (pH 8.0) (SLT-IIvhb), subjected to CL-6B DEAE-Sepharose anion-exchange chromatography (Pharmacia, Uppsala, Sweden) with the same buffer, and eluted with a 0 to 1.0 M NaCl gradient. The cytotoxic fractions were pooled and dialyzed against chromatofocusing starting buffer (for SLT-II, 0.025 M histidine-HCl [pH 6.2]; for SLT-IIvhb, 0.025 M imidazole-HCl [pH 7.4]). The toxin preparations were then applied to a chromatofocusing column (Polybuffer-exchanger 94 [Pharmacia]) that had been equilibrated with the appropriate starting buffer. Fractions were eluted from the column with Polybuffer 74-HCl (pH 4.0) (Pharmacia). The cytotoxic fractions were pooled and dialyzed against phosphate-buffered saline (PBS) and then subjected to immunoaffinity chromatography with monoclonal anti-SLT-II B subunit antibody (designated BC5BB12, kindly supplied by N. Strockbine, Centers for Disease Control, Atlanta, Ga. [5]) as the adsorbent. Fractions were eluted from the immunoaffinity column with 0.1 M glycine (pH 2.8) and immediately neutralized with 50 μl of 1 M Tris (pH 9.0). The cytotoxic activity and the homogeneity of each fraction were assessed by Vero cell cytotoxicity and denaturing polyacrylamide gel electrophoresis, respectively. The fraction(s) that contained the highly purified toxin protein was dialyzed against PBS and stored at 4°C. Purified toxin protein concentrations were determined in triplicate with a Micro-BCA protein assay reagent kit (Pierce).

Cytotoxicity and toxin immunoreactivity assay. Cytotoxicity assays of bacterial cell lysates and purified toxin preparations were done with Vero cells by the method of Gentry and Dalrymple (9). The 50% Vero cell cytotoxic dose (CD_{50}) is expressed per milliliter of culture and is the reciprocal of the highest dilution of toxin that caused 50% Vero cell death. Toxin immunoreactivity assays were done by incubating a single dilution of antibody with serial twofold dilutions of clarified bacterial cell lysates for 4 h at 37°C. The toxin-antibody mixture was then placed on Vero cells and incubated for an additional 48 h. The cells were then fixed in formalin and stained with crystal violet, and the A_{620} was measured in a Titertek Multiskan MC microplate reader (Flow/ICN Biomedicals, Costa Mesa, Calif.) to assess cell death. The antibodies used for cytotoxin immunoreactivity experiments included polyclonal anti-SLT-II (designated AJ65 [50]), ascitic fluid of monoclonal anti-SLT-II B subunit (designated BC5BB12, [5]), and culture fluid of monoclonal anti-SLT-II A subunit (designated 2E1 [38]).

Quantification of toxin protein from crude toxin preparations. A comparative immuno-dot blot analysis was used to determine the amount of SLT protein present in various crude toxin preparations. Serial dilutions of either sonically disrupted bacterial cultures or purified toxin preparations were spotted onto BAS-NC nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) through a 96-well dot blot apparatus (Schleicher & Schuell) connected to a vacuum. The nitrocellulose membrane was air dried and incubated for 1 h at room temperature in PBS–0.1% Tween 20 (Bio-Rad Laboratories, Richmond, Calif.) (PBS-T) which contained 5% nonfat dried milk (Carnation Co., Los Angeles, Calif.). The membrane was washed with PBS-T and then incubated with a 1:5 dilution of anti-SLT-II A subunit monoclonal antibody (designated 11E10 [38]) in PBS-T for 1 h. The membrane was washed three times with PBS-T to remove unbound antibody and then incubated for 1 h with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobin G antibody (Bio-Rad) in PBS-T. After incubation, the membrane was washed five times in PBS-T, immersed in ECL Western immunoblotting detection reagent (Amersham International, Amersham, United Kingdom) for 1 min, and then immediately exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) for 15 s to 3 min.

Recombinant DNA techniques. Plasmid DNA was purified by alkali lysis (2) or by Qiagen column preparation (Qiagen Inc., Chatsworth, Calif.). DNA fragments used for subcloning were separated by agarose gel electrophoresis (0.9% agarose) and eluted from the gel matrix with the GeneClean system (Bio 101, La Jolla, Calif.). Ligated fragments were transformed into *E. coli* K-12 strains made competent for uptake of DNA by calcium chloride treatment and heat shock (26).

Site-directed mutagenesis of *slt*-IIvhb and *slt*-IIc B subunits. Two oligonucleotides designed to introduce point mutations were synthesized on the basis of the sequence for *slt*-IIvhb (14) and *slt*-IIc (45) with a model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Oligonucleotide N16D, G TAT AAT GAG GAT GAT ACA TTC, altered codon 16 of the mature B subunit of *slt*-IIvhb and *slt*-IIc from AAT to GAT of *slt*-II. Oligonucleotide A24D, GTA AAA GTG GAC GGA AAA GAG, altered codon 24 of the mature B subunit of *slt*-IIvhb and *slt*-IIc from GCC to GAC of *slt*-II. Uracil-incorporated, single-stranded pSQ543 or pCKS184 DNA was mutagenized according to the directions supplied with the Bio-Rad Muta-Gene in vitro mutagenesis kit. The double

TABLE 3. Virulence in orally inoculated mice of *E. coli* DH5α(Str^r) that produce SLT-II-related toxins

Plasmid	Toxin	Cytotoxicity (Vero CD_{50}/ml of culture)[a]	Mouse death[b] (no. dead/no. fed)
Expt 1			
pSQ343	IIvha	1×10^4	10/10
pSQ543	IIvhb	1×10^6	23/23
pCKS120	IIc	1×10^3	9/10
Expt 2			
pSQ12	IIvha	5×10^2	10/10
pSQ545	IIvhb	5×10^4	9/9
pSQ549	IIvhb	1×10^2	1/10
pJES120	II	1×10^7	5/5
pCKS123	II	1×10^5	0/10
Controls			
pKS(−)	None	$<1 \times 10^2$	0/10
pHC79	None	$<1 \times 10^2$	0/10
pBR328	None	$<1 \times 10^2$	0/10

[a] Cytotoxicity values were determined on the cultures that were fed to the mice. The lowest level of detection for this assay was 10^2 CD_{50}/ml.

[b] Mice were fed approximately 10^{10} CFU bacteria and monitored for death for 14 days. Each ratio represents pooled data from one to six experiments.

mutant SLT-IIvhb/N16DA24D was generated with the single mutant SLT-IIvhb/N16D as the template and the A24D oligonucleotide as the mutagenic primer. All of the nucleotide changes used to generate the B subunit mutations were confirmed by DNA sequence analysis.

Toxin-receptor affinity analysis. The affinities of SLT-II and SLT-IIvhb for Gb_3 were determined by an in vitro binding assay done as previously described (43) except that purified Gb_3 and purified SLTs were used instead of crude preparations of these reagents.

RESULTS

Virulence of *E. coli* DH5α producing SLT-II-related toxins in streptomycin-treated, orally infected mice. Recent studies in our laboratory demonstrated that both EHEC O91:H21 strains B2F1 and H414-36/89 have LD_{50}s of less than 10 bacteria in orally infected, streptomycin-treated mice (21). Both of these O91:H21 strains contain two copies of *slt*-II-related toxin operons (14, 21). Furthermore, passive immunization of mice with anti-SLT-II antibody prior to oral inoculation with B2F1 or H414-36/89 protects mice from death (21). These data indicate that production of SLT-II-related toxins is critical for the virulence of these strains in mice. To determine whether production of SLT-II-related toxin by an *E. coli* K-12 strain was sufficient to cause the death of orally infected mice, ampicillin-resistant plasmids that contained *slt*-IIvha, *slt*-IIvhb, or *slt*-IIc were separately transformed into *E. coli* DH5α(Str^r) and fed to mice treated with streptomycin and ampicillin. Production of the SLT-II-related toxins by *E. coli* DH5α(Str^r) was lethal for the orally inoculated mice (Table 3, experiment 1). Moreover, mice that were fed *E. coli* DH5α(Str^r) that elaborated SLT-II-related toxin developed renal lesions similar to those found in mice that were fed the O91:H21 strains (Fig. 1) (21). These data support the hypothesis that the elaboration of SLT-II-related toxin even by a laboratory-attenuated *E. coli* K-12 strain is sufficient to cause renal lesions and death of orally inoculated mice. None of the control mice fed *E. coli* DH5α(Str^r) transformed with vector alone died (Table 3).

Previous studies by Wadolkowski et al. (56) demonstrated that *E. coli* DH5α(Str^r) that contained cloned *slt*-II toxin operons also caused kidney lesions, but levels of at least 10^6 Vero CD_{50} SLT-II per ml were required for these recombinant strains to be lethal in the orally inoculated mice. To assess the relative oral toxicity of the nearly homologous (Table 2) SLT-II-related toxins in mice, *E. coli* DH5α(Str^r) strains that produced lower levels of these toxins, as determined by Vero cell cytotoxicity, were fed to streptomycin-treated mice (Table 3, experiment 2). These toxin-producing *E. coli* DH5α(Str^r) strains were constructed by cloning the *slt*-IIvha and *slt*-IIvhb toxin operons into lower-copy-number vectors or into pBluescript vectors in the reverse orientation of the P_{lac} promoter (Table 1). Results from this set of feeding experiments demonstrated that *E. coli* DH5α(Str^r) strains that produced SLT-II-related toxins at Vero cell cytotoxicity titers of 5×10^2 to 1×10^3 Vero CD_{50}/ml remained lethal in mice (pSQ12, pCKS120 [Table 3]). The toxin-expressing strains had an apparent threshold of lethality at Vero cell cytotoxicity levels of approximately 10^2 (pSQ549 [Table 3]). By contrast, *E. coli* DH5α(Str^r) strains that made SLT-II were consistently lethal only when the Vero CD_{50} per milliliter produced were 10^7 (pJES120 [Table 3]).

Specific activity of purified SLT-IIvhb on Vero cells and in mice given intraperitoneal injections. One explanation for the observation that *E. coli* DH5α(Str^r) transformants that produce 10^2 to 10^3 Vero CD_{50} of SLT-IIvha, SLT-IIvhb, or SLT-IIc per ml kill orally inoculated mice, whereas *E. coli* DH5α(Str^r) transformants that make as much as 10^5 Vero CD_{50} of SLT-II per ml fail to do so, is that the toxins differ in specific activity at the in vitro or in vivo level, or both. To test this hypothesis, SLT-IIvhb and SLT-II were purified and the specific activities of the toxins were compared on Vero cells and in CD-1 mice. SLT-IIvhb was selected for purification because SLT-IIvha, SLT-IIvhb, and SLT-IIc have identical B subunit sequences and a clone of *slt*-IIvhb that produced high levels of toxin was available. The in vitro specific activity of SLT-II on Vero cells was approximately 5 pg/CD_{50}, a value comparable to published results (60). However, the Vero cell specific activity of purified SLT-IIvhb was about 100-fold lower than that of SLT-II (approximately 500 pg/CD_{50} for three SLT-IIvhb toxin preparations). When the purified toxins were intraperitoneally injected into CD-1 mice, SLT-II and SLT-IIvhb were equally toxic. The in vivo specific activity of both SLT-II and SLT-IIvhb was 1 to 2 ng/LD_{50}. Thus, SLT-II was more toxic for Vero cells than SLT-IIvhb was, but the toxins were equally toxic in mice. Therefore, the apparent difference in the in vivo toxicity of *E. coli* DH5α(Str^r) transformants that produce SLT-II-related toxins versus SLT-II reflects the use of Vero CD_{50} as a measure of activity.

Comparison of the ratio of Vero cell active toxin (as measured by CD_{50}) to total toxin (as measured by immunoreactivity) in crude preparations of SLT-II-related and SLT-II toxins. One explanation for the lower specific activity of SLT-II-related toxins on Vero cells is that the ratio of Vero cell active toxin to Vero cell inactive toxin is lower for the SLT-II-related toxins. A two-step experimental approach was used to test this hypothesis. First, the amount of toxin antigen per unit protein in crude extracts of SLT-II-related and SLT-II toxins was compared by immunoblot analysis with a monoclonal antibody specific for the A subunit of SLT-II. This monoclonal antibody was chosen as a probe because minor amino acid changes in the A subunit of SLTs appear to cause little or no variation in the reactivity of the toxins with antibodies (13). As a control, the immunoreactivities of purified SLT-II and purified SLT-IIvhb were compared and found to be similar.

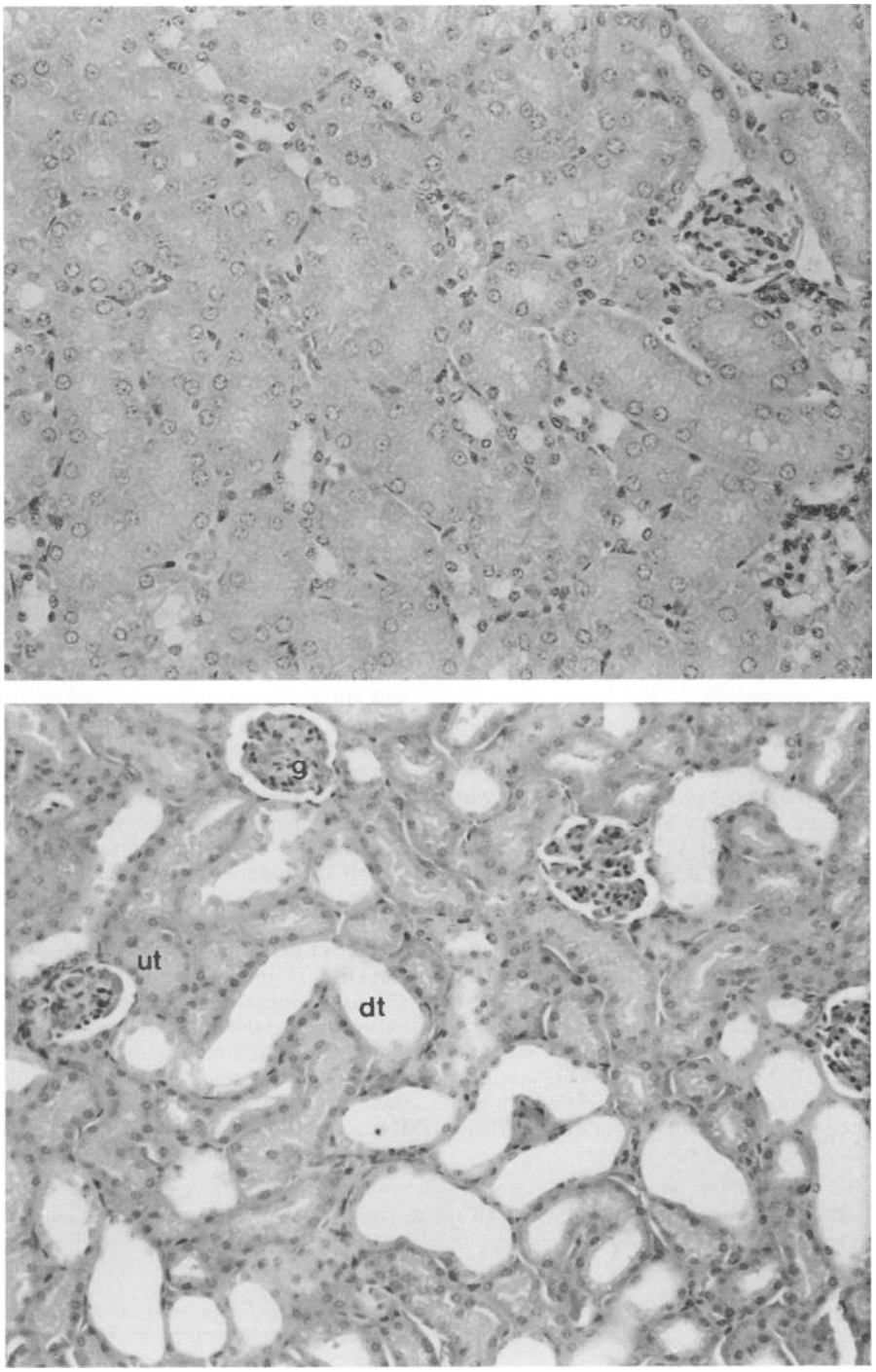

FIG. 1. (A) Photomicrograph of the renal cortex from a normal CD-1 mouse. No lesions were evident in the renal cortex of this or other control animals. (B) Photomicrograph of the renal cortex from a moribund, streptomycin-treated CD-1 mouse fed approximately 10^{10} CFU of *E. coli* DH5α(Str^r)/pSQ543 (expresses SLT-IIvhb). The moribund mouse was sacrificed on day 3 postinoculation, and the tissue was prepared as described in Materials and Methods. The lumens of several of the tubules were dilated and contained necrotic tubular epithelial cells. The dilated tubules (dt) were lined with fewer, more flattened, tubular epithelial cells than were the lumens of unaffected tubules (ut) as a result of loss of the adjacent cells. The glomeruli (g) appeared histologically normal. This photomicrograph is representative of renal sections obtained from several mice infected with *E. coli* DH5α that expresses SLT-II-related toxin. For both panels, hematoxylin and eosin stained; magnification, ×200.

This latter observation supports our supposition that the two toxins react equally well with this monoclonal antibody (Fig. 2) in the immunoblot assay. The crude toxin preparations from *E. coli* DH5α that expressed SLT-II(pJES120), SLT-IIvha-(pSQ343), or SLT-IIvhb(pSQ543) and produced 1×10^7, 5×10^4, and 5×10^5 Vero CD_{50}/ml, respectively, appeared to contain similar amounts (within a twofold difference) of toxin antigen (Fig. 2). Thus, comparable amounts of toxin protein

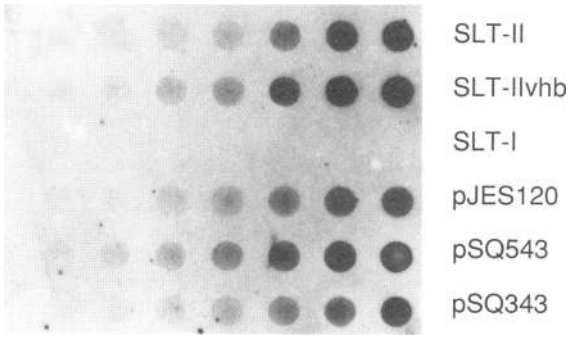

FIG. 2. Immunoblot of crude toxin preparations for quantitation of toxin antigen. Serial twofold dilutions of 100 ng of purified SLT-II, SLT-IIvhb, and SLT-I and 100 μl of a 10-fold dilution of sonically derived lysates of *E. coli* DH5α/pJES120 (SLT-II), *E. coli* DH5α/pSQ543 (SLT-IIvhb), or *E. coli* DH5α/pSQ343 (SLT-IIvha) were spotted onto nitrocellulose and hybridized with anti-SLT-II A monoclonal antibody (11E10). The intensities of the dot-blot color reactions were estimated visually.

were produced by *E. coli* DH5α transformed with each of the *slt* clones.

Next, the cytotoxic activity (toxicity) of crude lysates was compared with the cytotoxic activity of the lysates that remained after incubation with antibody (the latter is designated antigen unit) to determine whether toxin that was inactive on Vero cells was present in the lysates. The ratio of toxicity to antigen unit indicated whether most of the toxin present was active on Vero cells (high ratio) or whether a significant amount of the toxin was inactive on Vero cells (low ratio), since high levels of inactive toxin would titrate the neutralizing antibody and result in a low ratio. As demonstrated in Table 4, the cytotoxic activity (CD_{50}) per antigen unit was significantly higher for SLT-II than for SLT-IIvhb or SLT-IIc. For the assays summarized in Table 4, we used either monoclonal antibody to the SLT-II B subunit or polyclonal antisera to SLT-II. These antibodies were selected because they neutralize cytotoxicity more effectively than do anti-SLT-II A subunit monoclonal antibodies (45). Nonetheless, the CD_{50}/antigen unit ratios were also significantly higher for SLT-II than for the SLT-II-related toxin preparations when the immunoreactivity assays were done with monoclonal antibody to the SLT-II A subunit (data not shown). Together, the results described in this section indicate that per microgram of toxin protein SLT-II is more Vero cell active than are SLT-II-related toxins. One caveat to this conclusion is that the SLT-II-related toxins may not react as well in this type of immunoreactivity assay with any antisera elicited against SLT-II.

Identification of amino acids in the SLT-IIvhb and SLT-IIc B subunits responsible for reduced Vero cell activity. As illustrated in Table 2, there are four amino acid differences between SLT-II and SLT-IIvha/SLTvhb in the A subunit and two amino acid differences in the processed B or receptor-binding subunit (14). However, SLT-IIc, which has the same in vitro phenotype as SLT-IIvha/SLT-IIvhb, is identical to SLT-II in the A subunit and to SLT-IIvha/SLT-IIvhb in the processed B subunit (45). To determine whether one or both of the amino acid differences in the B subunit of these toxins is integral to the differential cytotoxic phenotype of the SLT-II-related toxins, we created two single and one double amino acid substitution(s) in SLT-IIvhb and two single mutations in SLT-IIc. These mutations, SLT-IIvhb/N16D, SLT-IIvhb/A24D, SLT-IIvhb/N16DA24D, SLT-IIc/N16D, and SLT-IIc/A24D, were designed to make the toxins more like SLT-II at the amino acid level and therefore correspond to the amino acid codons present in the mature B subunit of SLT-II at those positions. Toxin preparations from *E. coli* DH5α transformed with the B subunit mutants cloned into pBR328 were subjected to cytotoxicity and immunoreactivity analysis (Table 4). The N16D mutation in both SLT-IIvhb and SLT-IIc resulted in a 200-fold increase in cytotoxicity, whereas the A24D mutation had little or no effect on SLT-II-related toxin cytotoxicity levels. Similarly, the N16D mutation but not the A24D mutation altered the cytotoxin immunoreactivity profile of the SLT-II-related toxins. The CD_{50}/antigen unit ratios (the ratio of cytotoxicity to cytotoxicity after incubation with antisera) were low for the A24D mutants and the parent SLT-II-related toxins but much higher for the N16D mutants, the SLT-IIvhb/N16DA24D mutant, and the prototype SLT-II toxin. The absolute values for the ratios depended on the specificity of the antiserum and the arbitrarily chosen standardized dose for that antiserum. Preliminary mouse-feeding experiments were undertaken with strains expressing the mutant toxins; however, these experiments were terminated after we found that SLT-IIvhb and SLT-II have the same specific activity in vivo (see

TABLE 4. Specific toxicity of SLT-IIvhb and SLT-IIc B subunit mutant toxin preparations

Toxin[a]	Toxicity (CD_{50}/ml)	Anti-SLT-II[b]		Anti-SLT-II B[c]	
		Antigen units/ml[d]	CD_{50}/antigen unit[e]	Antigen units/ml[d]	CD_{50}/antigen unit[e]
SLT-II	6.6×10^5	1.3×10^3	512	4.8×10^2	1,365
SLT-IIvhb	6.4×10^2	1.6×10^2	5	4.2×10^2	2
SLT-IIvhb/N16D	1.6×10^5	1.6×10^2	1,000	4×10^1	4,000
SLT-IIvhb/A24D	5.2×10^3	6.4×10^2	8	1.3×10^3	1
SLT-IIvhb/N16DA24D	1.6×10^5	3.2×10^2	500	1.6×10^2	1,000
SLT-IIc	5×10^3	3.2×10^2	16	5.1×10^3	1
SLT-IIc/N16D	9.8×10^5	9.6×10^2	1,024	3.2×10^2	3,072
SLT-IIc/A24D	2.6×10^3	1.6×10^2	16	3.2×10^2	8

[a] Toxin preparations were from DH5α transformed with pBR328-derived plasmids pSQ549 (SLT-IIvhb), pSJ5491 (SLT-IIvhb/N16D), pSJ5492 (SLT-IIvhb/A24D), pSJ5493 (SLT-IIvhb/N16DA24D), pCKS120 (SLT-II), pCKS184 (SLT-IIc), pCKS188 (SLT-IIc/N16D), and pCKS189 (SLT-IIc/A24D).
[b] AJ65, 1:25 dilution.
[c] BC5 ascites, 1:100 or 1:200 dilution.
[d] Antigen units/ml, CD_{50} of toxin antigen per milliliter that remains after incubation with antibody.
[e] CD_{50}/antigen unit, CD_{50} of a toxin preparation per milliliter divided by the CD_{50} of toxin antigen units of the homologous toxin preparation per milliliter. Units given are arbitrary. Absolute values vary depending on the antibody used and cannot be compared between heterologous antibodies.

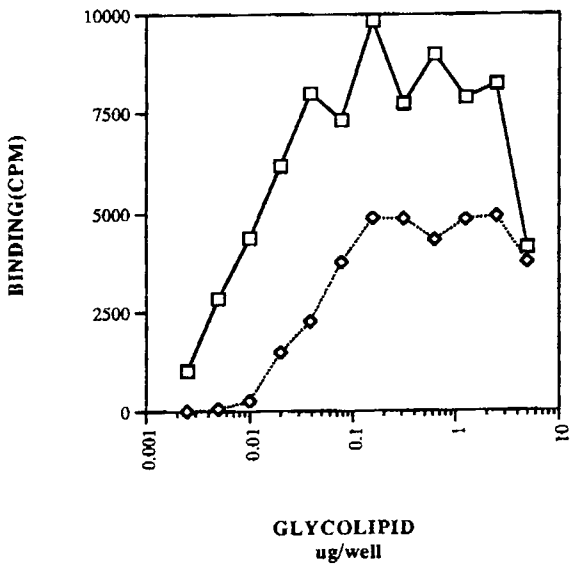

FIG. 3. Receptor-binding affinities of SLT-II and SLT-IIvhb. Saturating concentrations of purified toxin were incubated with serial dilutions of purified Gb_3. Receptor-bound toxin was detected by incubation with saturating amounts of monoclonal antibody directed against the SLT-II A subunit (designated 11E10 [38]) and ^{125}I-labeled sheep anti-mouse secondary antibody. Binding is expressed as counts of ^{125}I-labeled secondary antibody per minute. The receptor affinity assay was done four times. Data shown are from a single representative experiment. Symbols: □, SLT-II; ◇, SLT-IIvhb. Similar results were obtained when a bovine anti-SLT-II antibody was used to detect the Gb_3-bound toxin (not depicted).

above). Taken together, the cytotoxicity and immunoreactivity data indicate that the asparagine residue at position 16 is a critical determinant of the SLT-II-related cytotoxin phenotype.

Binding affinity of purified SLT-IIvhb and SLT-II to Gb_3. A possible reason for the reduced specific activity (picograms of pure toxin per CD_{50}) of SLT-II-related versus SLT-II toxins is that the SLT-II-related toxins may bind with different affinities to the putative functional receptor Gb_3. Indeed, we previously demonstrated that crude preparations of SLT-IIvhb bind to Gb_3 receptor analogs but with less affinity than SLT-II does (43). Purified SLT-II and SLT-IIvhb used in the current study were compared for relative affinity to Gb_3 with a saturating amount of toxin. The concentration of Gb_3 required for half-maximal binding of SLT-II was approximately 0.01 μg of Gb_3 per well, whereas the Gb_3 concentration for half-maximal binding of SLT-IIvhb was approximately 0.05 μg of Gb_3 per well (Fig. 3). Thus, SLT-II had a fivefold-higher affinity for Gb_3 in this assay system.

DISCUSSION

Three principal conclusions were inferred from this study of SLT-II and SLT-II-related toxins produced by EHEC. First, the synthesis of these toxins by an avirulent, laboratory-derived *E. coli* K-12 strain was sufficient to cause kidney damage in and kill orally infected, streptomycin-treated mice. Thus, toxin alone is the key determinant of EHEC pathogenicity in this mouse model. Second, the type of SLT-II produced by an EHEC strain does not appear to affect the mouse virulence of the strain because SLT-II and SLT-II-related toxins when purified and injected parenterally into mice had comparable in vivo specific activities. This conclusion does not rule out the possibility that more SLT-II-related toxin than SLT-II is produced in vivo by wild-type EHEC. Third, more SLT-II-related toxin antigen than SLT-II toxin antigen is required to produce 1 CD_{50} on Vero cells. This lower specific cytotoxic activity of SLT-II-related toxin on Vero cells reflects the amino acid at position 16 of the B subunit and may be a consequence of the lower affinity of binding to Gb_3 on Vero cells.

Our observation that purified SLT-IIvhb had a lower specific cytotoxic activity on Vero cells than did the prototype SLT-II toxin differs from the findings reported by Takeda and colleagues (35). These investigators found that toxin purified from strain B2F1 (presumably a mixture of SLT-IIvha and SLT-IIvhb) has a Vero cell specific activity of 5.9 pg/CD_{50} (35), a value that is similar to the specific activity of their preparation of SLT-II (60), whereas we found the specific activity of SLT-IIvhb to be approximately 500 pg/CD_{50}. The discrepancy between our findings and those of Oku et al. (35) may reflect differences in the Vero cell lines used in the two laboratories. Despite these differences in cytotoxicity results, it should be emphasized that the in vivo specific activity of the toxin preparation purified from strain B2F1 by Oku et al. (35) (2.7 ng/LD_{50}) is comparable to that obtained for SLT-IIvhb in this study (1 to 2 ng/LD_{50}).

The finding that Asn-16 in the SLT-IIvhb and SLT-IIc B subunits was responsible for the reduction in cytotoxicity and altered immunoreactivity of the SLT-II-related toxins on Vero cells when compared with SLT-II suggests that Asn-16 is involved in carbohydrate binding. That A subunit differences between SLT-II and the SLT-II-related toxins are not important for the cytotoxicity and immunoreactivity profiles is indicated by the fact that SLT-IIc is identical to SLT-II in the A subunit (Table 2) but has the same cytotoxicity and immunoreactivity profile as SLT-IIvha/SLT-IIvhb (Table 4). We also found that mutant SLT-IIc/N16D is phenotypically similar to SLT-IIvhb/N16D. Therefore, the only difference observed between SLT-IIvha/SLT-IIvhb/SLT-IIc and SLT-II that is relevant to cytotoxicity appears to be the Asn-16 residue in the B subunit. We are currently purifying the mutant toxins for further receptor-binding analysis.

Additional lines of evidence in support of the importance of the amino acid at position 16 in SLT toxin binding to receptors are the site-directed mutagenesis studies of Jackson et al. (16) and the X-ray crystallographic results of Stein et al. (48). Both studies indicate that position 17 in the Shiga toxin/SLT-I B subunit (which corresponds to position 16 in the SLT-II-related toxins) is involved in toxin-receptor binding. The SLT-II-related toxins, SLT-IIvha, SLT-IIvhb, and SLT-IIc, contain an uncharged asparagine residue at position 16, whereas Shiga/SLT-I and SLT-II have a negatively charged aspartic acid residue at this location. The lack of a negatively charged amino acid at position 16 in the SLT-II-related toxins may affect the electrostatic interaction between the amino acids in the putative toxin-receptor binding cleft and result in the observed reduction in toxin-receptor binding. Additional studies which indicate that this region of the B subunit is important in receptor binding include those of Tyrell et al. (55). These investigators found that a D18N mutation in the B subunit of SLT-I resulted in binding of the mutant toxin to Gb_4 as well as to the functional receptor Gb_3. It should be noted that Ito et al. also demonstrated an antigenic dissimilarity between SLT-II (VT2) and SLT-IIvha (VT2vh) (13). However, in contrast to our findings, these investigators attributed the antigenic dissimilarity (which they observed by Ouchterlony double-gel diffusion analysis) between the two toxins to the alanine residue at position 24 in the B subunit of SLT-IIvha (13).

Several explanations are possible for the disparity we observed between the in vitro cytotoxic activity of SLT-II-related toxins and the activity of the toxins in animals. One possible reason for the apparent relative inactivity of the SLT-II-related toxins on Vero cells is the decreased affinity of these toxins for the SLT-II functional receptor, Gb_3 (Fig. 3). However, SLT-I and SLT-II also differ in binding affinity for Gb_3 but do not exhibit differences in specific activity on Vero cells (51). An alternative reason for the reduced toxicity of SLT-II-related toxins on Vero cells is that the functional receptor that leads from binding to internalization of these toxins is not Gb_3 but another receptor that is present at low levels on our Vero cells. A corollary might be that the SLT-II-related toxins do bind Gb_3 on Vero cells but are not internalized efficiently. Whatever the explanation for these in vitro findings, the toxicity of SLT-II-related toxins is unimpaired in animals.

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