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Activation of Shiga-Like Toxins by Mouse and Human Intestinal Mucus Correlates with Virulence of Enterohemorrhagic *Escherichia coli* O91:H21 Isolates in Orally Infected, Streptomycin-Treated Mice

ANGELA R. MELTON-CELSA, STEPHEN C. DARNELL, AND ALISON D. O'BRIEN*

Department of Microbiology and Immunology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

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The enterohemorrhagic *Escherichia coli* (EHEC) O91:H21 isolates B2F1 and H414-36/89 are virulent in an orally infected streptomycin-treated mouse model. Previous studies demonstrated that B2F1 and H414-36/89 grow to high levels in mucus isolated from the mouse small intestine and colon and that growth in small-intestinal mucus is related to virulence. We measured the levels of Shiga-like toxins (SLTs) SLT-IIvha and SLT-IIvhb produced by B2F1 after growth in Luria-Bertani (LB) broth supplemented with mouse intestinal mucus by assaying the cytotoxicity of culture supernatants on Vero cells. Culture supernatants from B2F1 grown in mouse intestinal mucus, but not EHEC strains that produce SLT-II or SLT-IIc, were approximately 35- to 350-fold more toxic for Vero cells than supernatants from B2F1 grown in LB broth. This increased toxicity was not reflected by a concomitant increase in SLT antigen content. Furthermore, when culture supernatants from B2F1 or K-12 strains carrying plasmids encoding SLTs cloned from H414-36/89 or purified SLT-IIvhb from B2F1 were incubated with mouse intestinal mucus, the samples exhibited greater cytotoxicity than when they were incubated with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer alone. These toxin preparations also showed increased cytotoxicity after incubation with human colonic mucus. In contrast, culture supernatants from LB-grown EHEC isolates that produced SLT-I, SLT-II, SLT-IIc, or SLT-IIe did not show increased cytotoxicity after incubation with mouse or human intestinal mucus. The A subunits of purified SLT-II and SLT-IIvhb that had been treated with mouse intestinal mucus or trypsin were cleaved to A_1 fragments by the mucus, but trypsin-mediated cleavage, unlike treatment with mouse intestinal mucus, did not result in increased Vero cell cytotoxic activity. This finding implies that the increased cytotoxicity of SLT-IIvhb detected after incubation with mucus is probably not due to cleavage of the A subunit into the A_1 and A_2 fragments. Taken together, these results indicate that mouse or human intestinal mucus directly activates SLT-II-related toxins from B2F1 and H414-36/89 and suggest that toxin activation may explain the low 50% lethal doses of B2F1 and H414-36/89 in streptomycin-treated mice.

Enterohemorrhagic *Escherichia coli* (EHEC) strains cause food-borne hemorrhagic colitis and the hemolytic uremic syndrome. All EHEC strains maintain a large plasmid and produce one or more Shiga-like toxins (SLTs; also called Vero toxins), and some express the product of the *eaeA* gene, intimin. SLTs are believed to be involved in the production of the more serious sequelae of both hemorrhagic colitis and the hemolytic uremic syndrome (5, 21, 34). SLTs are potent toxins that consist of a single A subunit and a pentamer of B subunits (21). The B subunit is responsible for binding the toxin to the eukaryotic cell receptor Gb_3 (11, 14). The A subunit cleaves an adenine residue from the 28S rRNA within the 60S ribosome and, consequently, halts protein synthesis in the target eukaryotic cell (4, 26). The A subunit has a trypsin-sensitive cleavage site near the C terminus which, when nicked, results in the formation of an approximately 28-kDa peptide that has the enzymatic activity of the toxin and an approximately 4-kDa A_2 peptide that links the A_1 peptide to the B pentamer (6). The A_1 and A_2 peptides remain linked to each other by the single disulfide bond present in the A subunit.

Our laboratory uses a streptomycin-treated, orally infected mouse model to study EHEC infection (36). Mice fed certain strains of EHEC or K-12 strains carrying high-level toxin-producing plasmids become lethargic, occasionally exhibit hind-limb paralysis, stop eating, and die within 4 to 10 days of infection (depending on the size of the inoculum) (15, 36, 37). Death of the mice is due to severe bilateral tubular necrosis of the kidney (15, 37). The capacity of an *E. coli* isolate to cause a lethal mouse infection requires that the mouse intestine become colonized by the bacterium and that the strain produce an SLT of the II type (15). We previously reported that the O91:H21 EHEC isolates B2F1 (produces SLT-IIvhb and SLT-IIvha) and H414-36/89 (contains one copy of *slt*-II and two copies of *slt*-II-variant genes) are the most virulent (oral 50% lethal dose [LD_{50}] of <10 CFU) of the EHEC strains that we have tested in the streptomycin-treated mouse (15). In that same study, we showed that the capacity of B2F1 to grow in mouse small-intestinal mucus in vitro correlates with, but is not sufficient to explain, the low LD_{50} of B2F1 (15). Indeed, EHEC isolate E32511/HSC replicates as well as B2F1 in mouse small-intestinal mucus but has an oral LD_{50} of 10^{10} CFU. We also concluded that SLT production is absolutely required for the marked virulence of B2F1 in this model, on the basis of the

* Corresponding author. Mailing address: Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, Department of Microbiology and Immunology, 4301 Jones Bridge Road, Bethesda, MD 20814-4799. Phone: (301) 295-3421. Fax: (301) 295-1545. Electronic mail address: OBRIEN@USUHSB.USUHS.MIL.

TABLE 1. Strains and plasmids used or described in this study

Strain	Serotype	SLT type(s)	Strain from which SLT originated	Reference or source
B2F1 S^{r}[a]	O91:H21	IIvha, IIvhb		10
H414-36/89 S^{r}	O91:H21	II, II variant[b]		2
E32511	O157:H^{-}	II, IIc		CDC[c]
E32511/HSC S^{r}	O157:H^{-}	IIc		9
86-24	O157:H7	II		8
933D	O157:H7	I		CDC
S1191	O139	IIe		18
DH5α(pSQ544)	Rough	IIvhb	B2F1	16
DH5α(pSQ343)	Rough	IIvha	B2F1	16
DH5α(pSQ135)	Rough	II variant	H414-36/89	16
DH5α(pSQ47)	Rough	II variant	H414-36/89	16
DH5α(pSQ81)	Rough	II	H414-36/89	16
DH5α(pCKS120)	Rough	IIc	E32511	16
DH5α(pCKS123)	Rough	II	E32511	16

[a] S^{r}, streptomycin resistant.
[b] Strain H414-36/89 has two copies of an SLT-II-variant-type toxin (15).
[c] CDC, Centers for Disease Control and Prevention.

observation that passive immunization of infected mice with anti-SLT-II but not anti-cholera toxin antibodies protects the mice from lethality and death but not from colonization by B2F1 (15). In this report, we sought to further define the mechanism of B2F1 virulence and found that mouse and human intestinal mucus significantly increased the toxicity of SLTs produced by mouse-virulent B2F1 for Vero cells.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. B2F1 is an O91:H21 strain that produces SLT-IIvha and SLT-IIvhb, maintains the EHEC large plasmid, is *eaeA* negative, and was isolated from a patient with the hemolytic uremic syndrome (15, 32). SLT-IIvha and SLT-IIvhb were recently classified as SLT-IIc toxins (38), but we will refer to them as SLT-IIvhb and SLT-IIvha for clarity in this report.

Cytotoxicity assay. The cytotoxicity of culture supernatants or sonic lysates for Vero cells was determined as described previously (7, 28). Briefly, samples were serially diluted in tissue culture medium. One hundred microliters of each dilution was added to individual wells of a 96-well microtiter plate which contained approximately 10^4 Vero cells in 100 μl of medium. The tissue culture plates were then incubated at 37°C in the presence of 5% CO_2 for 48 h and then fixed and stained with crystal violet. The intensity of color of the fixed and stained cells was measured with a Titertek Multiscan MC reader at 620 nm. The 50% cytotoxic dose (CD_{50}) was determined as the reciprocal of the highest dilution that caused 50% Vero cell death compared with control untreated cells.

Isolation of mouse intestinal mucus. Intestinal mucus was isolated from 22- to 24-g male CD-1 mice essentially as described previously (36). The mice were pretreated with streptomycin (5 g/liter) in their drinking water to eliminate facultative anaerobes from the intestine. Food was removed from the mice 18 h prior to mucus isolation to reduce the amount of fecal material. The mice were sacrificed, and their intestines were removed the following day. Fecal material was gently pushed out of the intestines and discarded. The mucus was then scraped into HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 2.4 g/liter) buffered to pH 7.4. The mucus sample was clarified by centrifugation at 12,000 × *g* for 10 min followed by centrifugation for 15 min at 27,000 × *g*. Mucus recovered in this way is free of aerobes, as indicated by the lack of viable organisms after plating the mucus on Luria-Bertani (LB) (17) agar plates. Total protein content of the mucus was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.).

Isolation of human colonic mucus. Human colonic mucus was isolated from the pooled effluent obtained after flexible sigmoidoscopies or colonoscopies performed on at least eight patients. Such pooled intestinal effluent was collected on two separate occasions. The pooled effluent was stored on ice, and the upper layer of dilute effluent was removed and discarded. The colonic mucus, which settled to the bottom of the container, was further separated from the effluent by a 10-min centrifugation at 12,000 × *g*. The protein concentration of the mucus preparation was then measured with a bicinchoninic acid protein assay kit (Pierce). Human colonic mucus was diluted with HEPES buffer to give a final mucus protein concentration of 1 mg/ml in the activation experiments.

Effect of mouse intestinal mucus on growth of EHEC in vitro and on SLT cytotoxicity. LB broth was supplemented with mouse mucus at a final mucus protein concentration of 1 or 2 mg/ml. The mucus-supplemented LB or unsupplemented LB broth tubes were inoculated from a diluted overnight culture to achieve a final concentration of 10^2 CFU/ml and incubated in a shaking water bath at 37°C for 20 h. The CFU per milliliter of culture were determined by making serial dilutions of the sample into phosphate-buffered saline (PBS) and plating the dilutions onto LB agar. Additionally, an aliquot was removed from the culture for subsequent assessment of Vero cytotoxic activity. The bacteria were removed from the sample by centrifugation at 27,000 × *g* for 15 min, and the clarified supernatant was stored at −20°C. For comparison of the cytotoxic activities of EHEC O157:H7 strain 86-24 and B2F1 grown in LB supplemented with mucus, supernatants or sonically disrupted lysates of the cultures were prepared. Cytotoxicity data generated in the experiments in which the EHEC strains were grown in LB broth or LB with mouse intestinal mucus were normalized for growth by dividing the CD_{50} per milliliter by the CFU per milliliter and multiplying by 10^{10}. The effect of growth of bacteria in the presence of mucus was then calculated as the ratio of the CD_{50} per CFU after growth in LB with mouse intestinal mucus to the CD_{50} per CFU after growth in LB alone.

SLT antigen level determination. SLT antigen levels were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Meridian Diagnostics, Inc., Cincinnati, Ohio) according to the manufacturer's instructions. Immunoblotting was used as an alternative method as follows. Culture supernatants were concentrated with a Centriprep-30 (Amicon, Beverly, Mass.) column and then subjected to native polyacrylamide gel electrophoresis (PAGE) with a continuous 10% gel. The proteins separated on the gel were transferred to BAS-NC nitrocellulose (Schleicher & Schuell Inc., Keene, N.H.) by electroblotting. The nitrocellulose was then blocked with 5% nonfat dry milk in Tris-buffered saline that contained 0.1% Tween 20 (TBST). After the blocked nitrocellulose filter was washed with TBST, the filters were incubated with polyclonal antisera to B2F1 toxins (32) at a dilution of 1:3,300. The antibody-treated filters were then washed extensively with TBST and incubated with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin antiserum (Amersham, Arlington Heights, Ill.) at a dilution of 1:1,000. Antigen-antibody complexes were detected by incubating the blot with enhanced chemiluminescence Western blot (immunoblot) detection reagents (Amersham) and then exposing the blot to X-Omat film (Eastman Kodak Co., Rochester, N.Y.).

Cytotoxic activity of crude SLT preparations treated with mouse or human intestinal mucus. Bacterial strains were grown overnight in LB broth. The cultures were centrifuged at 12,000 × *g* for 10 min to remove the bacteria. The supernatant was sterilized by passage through a 0.45-μm-pore-size filter. The sterile supernatant was then incubated at 37°C for 2 to 4 h with HEPES buffer alone or in mouse proximal-small-intestinal mucus (MPSIM) or human colonic mucus that was adjusted to a final concentration of 1 mg/ml. The CD_{50} per milliliter of sample was then determined on Vero cells as described above.

Toxin purification and treatment of purified toxin with mouse or human mucus and/or trypsin. SLT-II and -IIvhb were purified as described previously (16). SLT-I was purified by the same method (16), with the following modifications. *E. coli* DH5α(pLPSH3), an SLT-I-producing strain, was grown in LB broth with 100 μg of ampicillin per ml. Plasmid pLPSH3 is a derivative of pNAS10 in vector pBR329 (30). The antibody used for the affinity column was 13C4, an anti-SLT-I B subunit monoclonal antibody (31).

Purified toxin was incubated for 1 to 2 h at 37°C with a HEPES buffer control, a bovine serum albumin (BSA) control, MPSIM, or human colonic mucus at a final concentration of 1 mg/ml. The CD_{50} of the treated toxin per milliliter was then measured on Vero cells as described above. For the trypsin treatment studies, purified toxin was incubated with trypsin at a final concentration of 20 μg/ml for 2 h at 37°C. Trypsin inhibitor was then added to a final concentration of 28 μg/ml, and the cytotoxicity of the sample was determined on Vero cells. Purified treated and untreated toxin preparations were subjected to sodium dodecyl sulfate (SDS)-PAGE (12% gel) and then to the immunoblot procedure described above.

Mouse feeding studies. The mouse feeding studies were done essentially as described previously (15, 36). Male 22- to 24-g CD-1 mice were treated with streptomycin sulfate (5 g/liter) in their drinking water 18 h prior to feeding. Food was removed from the mice 15 to 18 h prior to feeding. The bacterial strain to be fed was grown with aeration overnight in LB broth. The bacterial strain was then diluted into 20% sucrose, and the mice were fed the approximate dose in 0.8 ml. The actual dose fed was determined by colony count as described above. The experiments reported here were conducted according to the principles set forth by the National Institutes of Health (20).

Bacterial counts, associated Vero cell activity, and SLT antigen levels in mouse intestinal contents, serum, and kidney extracts after feeding of B2F1 to streptomycin-treated mice. B2F1 was fed at a dose of approximately 4×10^9 CFU to two groups of five mice each as described above. After 9 hours or 4 days, blood was collected by exsanguination of the mice following anesthesia with metofane. Mice were sacrificed, and the kidneys were removed, placed in cold PBS, and disrupted in a stomacher. Next, the entire cecum, a 10-cm segment from the distal small intestine, the colon, and the kidney were placed individually into cold PBS and disrupted in a stomacher. The CFU per sample were determined by plate count on LB agar as described above. Because these mice had been treated with streptomycin to remove normal bacterial flora, essentially pure cultures of the streptomycin-resistant bacterial inoculum were recovered from the intestines. After removal of the bacteria from the homogenate of the intes-

TABLE 2. Ratios of cytotoxicity of supernatants or lysates of EHEC strains grown in LB supplemented with mouse intestinal mucus to the cytotoxicity after growth in LB alone

Strain	Fold increase[a] in cytotoxicity (range, *n*) following growth in mouse mucus isolated from:	
	Proximal small intestine	Colon
B2F1	36.6[b] (3.1–672, 12)	362[b] (317.6–1,666.7, 3)
E32511/HSC	1.2[c] (0.4–3.1, 4)	ND[d]
86-24	ND	2.5[c] (1–3.9, 4)

[a] Calculated by the following formula: (CD_{50}/CFU in LB with mucus)/(CD_{50}/CFU in LB).
[b] Geometric mean.
[c] Arithmetic mean.
[d] ND, not determined.

tinal segment by centrifugation at 12,000 × *g* for 10 min followed by centrifugation at 27,000 × *g* for 15 min, the level of Vero cell cytotoxic activity associated with the clarified sample or from sonic lysates of the bacterial pellet resuspended in an equal volume of PBS was determined. SLT antigen levels were measured in serum (separated from clotted blood), kidney, and intestinal samples by an ELISA kit as described above.

Comparison of mouse colonization capacities of B2F1 and E32511/HSC. To compare intestinal colonization of strain B2F1 with that of strain E32511/HSC, approximately 5×10^6 organisms of each strain were fed to a group of five mice as described above. After 14 h of infection, the intestines were removed and the number of bacteria per segment was determined as described above.

RESULTS

Cytotoxicity of B2F1 culture supernatants after growth in mouse intestinal mucus. B2F1 grows equally well in (i) HEPES buffer supplemented with mouse intestinal mucus isolated from any site along the mouse intestinal tract (17), (ii) LB broth (data not shown), and (iii) LB broth supplemented with mouse intestinal mucus (data not shown). Because growth in mouse intestinal mucus correlates with but is not sufficient to explain the striking virulence of B2F1 in orally infected, streptomycin-treated mice, we asked whether there might be an increase in the cytotoxicity for Vero cells of B2F1 culture supernatants after growth in LB supplemented with mouse small-intestinal mucus compared with growth in LB. Preliminary experiments showed that there was an increase in the level of cytotoxicity in the supernatant of cultures of B2F1 grown in LB broth supplemented with mucus isolated from any site along the small intestine (data not shown). Because growth of EHEC in MPSIM correlates with virulence, we used MPSIM for most of our remaining studies. The average increase in cytotoxicity of supernatants from B2F1 grown in LB with MPSIM added compared with supernatants from B2F1 grown in LB broth alone is shown in Table 2. Mouse intestinal mucus alone was not toxic for Vero cells at the levels used in these experiments.

Cytotoxicity of culture supernatants or bacterial lysates of EHEC isolate B2F1, 86-24, or E32511/HSC after growth in mouse intestinal mucus. We next determined whether EHEC strains E32511/HSC and 86-24 exhibited similar increases in Vero cell cytotoxicity after growth of the strains in the presence of mouse intestinal mucus. We assessed the level of cytotoxicity for Vero cells of E32511/HSC culture supernatants after growth in LB broth with MSPIM. E32511/HSC is an O157:NM isolate that produces SLT-IIc only (9) and grows as well as B2F1 in MPSIM (15). The cytotoxicity in culture supernatants of E32511/HSC was not increased after growth in LB with MPSIM (Table 2).

We next assessed the level of cytotoxicity for Vero cells in culture supernatants or lysates from strains 86-24 and B2F1 that were grown in LB broth or LB broth with mouse colonic mucus. Strain 86-24 is an O157:H7 isolate that produces SLT-II (8). Colonic mucus rather than MPSIM was used because 86-24 does not grow well in MPSIM (19a). The cytotoxicity of SLT-II from 86-24 was not appreciably increased after growth in the presence of mouse colonic mucus, but the cytotoxicity of the SLT(s) from B2F1 was markedly increased (Table 2).

SLT antigen levels in B2F1 culture supernatants after growth in mouse intestinal mucus. The higher levels of toxicity in culture supernatants of B2F1 after growth in LB supplemented with either mouse small-intestinal or colonic mucus than in culture supernatants from B2F1 grown in LB suggested either that mouse intestinal mucus serves as a signal to B2F1 to up-regulate toxin expression, that there is direct activation of the toxin by a factor in the mucus, or that there is more SLT secreted in the presence of the mucus. If mouse intestinal mucus does serve as a signal to increase production of SLT from B2F1, then a concurrent increase in the level of toxin antigen should be detectable. We first attempted to measure the toxin antigen levels by an immunoblot procedure (16), and on the basis of results obtained with that method, we reported in an abstract that we had detected an increase in SLT antigen content (19). Subsequently, we found that there was cross-reactivity between mouse intestinal mucus in our samples and the secondary antibody used to detect the mouse monoclonal primary antibody in the immunoblot protocol. To readdress the question of whether there was more toxin antigen in the B2F1 cultures grown in the presence of mouse intestinal mucus, we measured SLT antigen levels by ELISA and by native PAGE of culture supernatants followed by Western blot analysis. Both the ELISA and the Western analysis showed that the toxin antigen contents in LB- or mucus-grown B2F1 cultures were essentially equivalent (data not shown). These findings strongly suggest that the increased cytotoxicity of the B2F1 culture supernatant or lysates after growth in mouse intestinal mucus is due to an activation of one or both of the B2F1 toxins, SLT-IIvha and SLT-IIvhb.

Activation of crude SLT preparations by MPSIM. To test the hypothesis that a direct activation of SLT(s) from B2F1 occurs in the presence of mouse intestinal mucus, culture supernatant from B2F1 grown in LB was incubated in the presence of mouse intestinal mucus diluted in HEPES buffer or HEPES buffer alone for 4 h at 37°C. The cytotoxicity for Vero cells of the bacterium-free culture supernatant of B2F1 incubated with MPSIM was significantly higher than when the culture supernatant was incubated with HEPES alone (Fig. 1). This observation supports our supposition from the SLT antigen level experiments that the mucus-promoted elevated cytotoxicity reflects an increase in the activity of SLT-IIvha or SLT-IIvhb or both toxins. The increased cytotoxicity of SLT(s) after incubation with MPSIM will hereafter be referred to as activation.

We next determined if incubation with MPSIM had an effect on the cytotoxicity of toxin present in the supernatants from other EHEC isolates. Supernatants of LB-grown cultures of B2F1, three O157 isolates (86-24, E32511/HSC, and 933D), and the O139 edema disease isolate S1191 were incubated with MPSIM or HEPES buffer. The CD_{50} per milliliter for Vero cells of the treated supernatants was then determined. Only supernatant from B2F1 exhibited greater toxicity after incubation with MPSIM than after incubation with HEPES buffer (Fig. 1). The fact that only B2F1 supernatant showed this increase indicated either (i) that there is a component present in B2F1 supernatant in addition to toxin that is critical for toxin activation and that this putative component is absent from

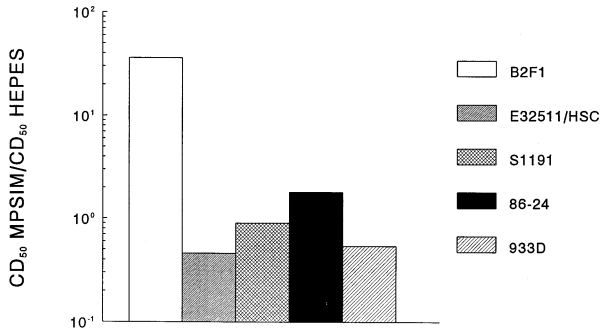

FIG. 1. Activation of SLT from EHEC strains. Culture supernatant from the EHEC isolates after growth in LB broth was incubated with MPSIM or HEPES buffer at 37°C for 2 to 4 h, and the CD_{50} per milliliter was determined on Vero cells. The data are presented as the ratio of the CD_{50} of the supernatant after incubation with MPSIM to the CD_{50} of the supernatant after incubation with HEPES buffer. The ratios are the averages from three or more experiments. The toxins produced by these strains are as follows: B2F1, SLT-IIvha and SLT-IIvhb; E32511/HSC, SLT-IIc; S1191, SLT-IIe; 86-24, SLT-II; and 933D, SLT-I.

other EHEC strains or (ii) that the toxins from B2F1 are uniquely activatable by MPSIM. To determine if the B2F1 toxins in the absence of the B2F1 background have the capacity to be activated, culture supernatants containing SLT-IIvha from DH5α(pSQ343) or SLT-IIvhb from DH5α(pSQ544) were incubated with MPSIM or HEPES. The level of cytotoxicity associated with the supernatants after these treatments was then measured on Vero cells. The CD_{50} per milliliter of each toxin culture supernatant incubated with MPSIM was at least 10-fold higher than the cytotoxicity of the respective culture supernatant incubated with HEPES alone (Fig. 2). These data indicate that there is no B2F1 cofactor required for toxin activation.

Next, supernatants from bacteria containing recombinant plasmids expressing either SLT-II or SLT-IIc from strain E32511 or recombinant plasmids expressing one of the three SLTs from mouse-virulent strain H414-36/89 were tested for the capacity to be activated. Only supernatants from two of the toxin genes cloned from H414-36/89 [DH5α(pSQ47) or DH5α(pSQ135)] were activated (Fig. 2). For a toxin to be classified as an activatable toxin, the mucus-treated toxin preparation had to have an average (after three or more experiments) of at least a 10-fold increase in Vero cell cytotoxicity compared with the HEPES-treated toxin preparation. That only some SLTs were activated indicates that there must be specific attributes of the toxins produced by these recombinant plasmids that are necessary for activation.

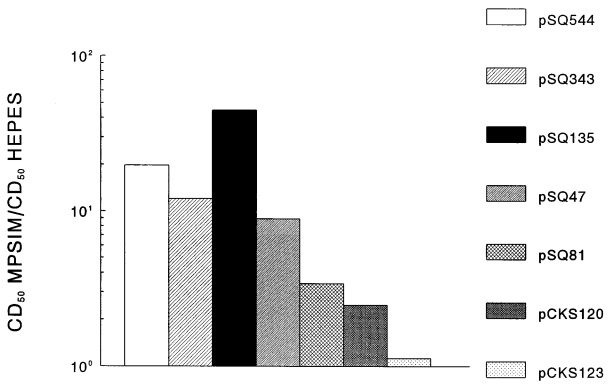

FIG. 2. Activation of SLT from *E. coli* K-12 transformed with recombinant plasmids expressing *slt* genes. Supernatant from cultures grown in LB broth was incubated with MPSIM or HEPES buffer at 37°C for 2 to 4 h. The CD_{50} per milliliter was then determined on Vero cells. The data are presented as the ratio of the Vero cell CD_{50} in the supernatant after incubation with MPSIM to the Vero cell CD_{50} in the supernatant after incubation with HEPES buffer. The ratios are the averages from three or more experiments. The toxin types produced from these plasmids are as follows: pSQ544, SLT-IIvhb; pSQ343, SLT-IIvha; pSQ135, SLT-II variant; pSQ47, SLT-II variant; pSQ81, SLT-II; pCKS120, SLT-IIc; and pCKS123, SLT-II.

Activation of purified SLTs. Next, we tested whether purified toxins can be activated. Purified SLT-I, SLT-II, or SLT-IIvhb was incubated with MPSIM or HEPES buffer for 1 h at 37°C, and the CD_{50} per milliliter for Vero cells was determined. The cytotoxicity of SLT-IIvhb was activated more than 1,000-fold in the presence of MPSIM, whereas the cytotoxicity of SLT-II decreased somewhat (Fig. 3). The reason for the decrease in the cytotoxicity of SLT-II is not known. The cytotoxicity of SLT-I increased approximately sevenfold after incubation with MPSIM (Fig. 3), an observation that suggests that SLT-I has a slight capacity to be activated or that the toxin is less stable after dilution into HEPES buffer than after dilution into mucus. We favor the latter hypothesis because the SLT-I present in the culture supernatant from strain 933D was not activated in the presence of mouse intestinal mucus (Fig. 1). To determine if activation was specifically due to mouse intestinal mucus, purified SLT-I or SLT-IIvhb was incubated with bovine serum albumin (BSA) or HEPES buffer at 37°C for 2 h, and the CD_{50} per milliliter for Vero cells was measured. The cytotoxicity of the purified toxins was not altered by the presence of the BSA (data not shown). The fact that purified SLT-II variant toxin or SLT-II variant toxin present in bacterial supernatants or extracts is activatable indicates that the source of the toxin does not alter whether the toxin is activated or not.

Immunoblot analysis of purified toxins treated with MPSIM or trypsin. To determine if an observable change in the molecular weight of the toxin occurred after incubation with MPSIM, purified SLT-IIvhb that had been incubated with either MPSIM or HEPES buffer was subjected to SDS-PAGE followed by immunoblotting with polyclonal antisera to toxin purified from B2F1 (32). SLT-IIvhb that had been incubated with MPSIM showed a shift in molecular weight (Fig. 4A) that was consistent with cleavage of the A subunit to the A_1 fragment. This result suggests that mouse intestinal mucus has the capacity to nick the SLT-IIvhb A subunit. Although the size of

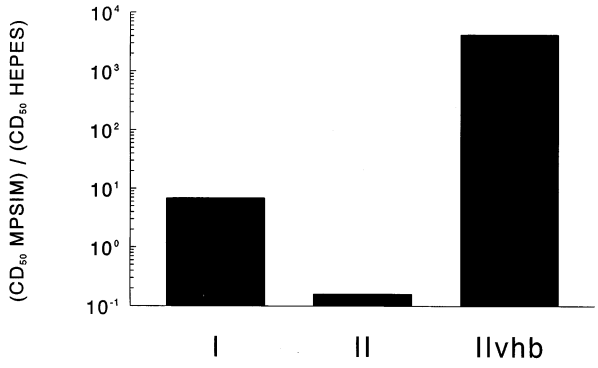

FIG. 3. Activation of purified SLTs. Purified SLT was incubated with MPSIM or HEPES buffer for 1 to 2 h at 37°C. The Vero cell CD_{50} per milliliter was then measured. The data are presented as the ratio of cytotoxicity of the SLT preparation after incubation with MPSIM to the cytotoxicity after incubation with HEPES buffer. The ratios represent the averages from five or more experiments.

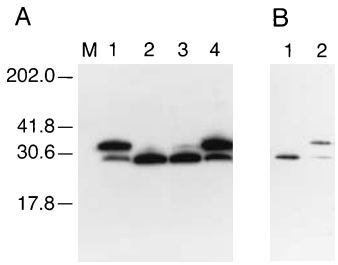

FIG. 4. Immunoblot of SDS-PAGE analysis of SLTs incubated with MPSIM or trypsin. (A) Purified SLT-IIvhb was treated with MPSIM, HEPES, or trypsin and incubated at 37°C for 2 h. The samples were separated by SDS-PAGE on a 12% gel and then subjected to immunoblot analysis as described in Materials and Methods. Lane 1, SLT-IIvhb treated with HEPES; lane 2, SLT-IIvhb treated with MPSIM; lane 3, SLT-IIvhb treated with trypsin; lane 4, SLT-IIvhb treated with HEPES. (B) Purified SLT-II was treated with MPSIM or HEPES and incubated at 37°C for 2 h. Lane 1, SLT-II treated with MPSIM; lane 2, SLT-II treated with HEPES. The numbers at the left indicate molecular masses of protein standards (in kilodaltons).

the A_1 fragment appeared to be the same as the size of the A_1 seen in the HEPES- or trypsin-treated toxin (Fig. 4A), these results do not rule out the possibility that the mucus-nicked toxin was cleaved at a slightly different site.

To determine if nicking is sufficient for activation, purified SLT-IIvhb was incubated with trypsin or HEPES buffer. The CD_{50} per milliliter was then measured, and a sample of the trypsin- or HEPES-treated material was subjected to SDS-PAGE followed by immunoblotting as described above. Trypsin-treated toxin showed a similar shift from the intact A subunit to the A_1 subunit (Fig. 4A). The A_2 subunit was not evident in the blot, either because it was unstable under SDS-PAGE conditions or because it was not recognized by the antiserum. However, the Vero cell cytotoxicities of the trypsin-treated toxin and the HEPES-treated toxin were the same, whereas the MPSIM-treated toxin was 667-fold more cytotoxic for Vero cells than the HEPES-treated toxin. The trypsin-treated toxin was still cytotoxic because the disulfide bond that links A_1 and A_2 was not reduced prior to addition of the toxin to Vero cells. When the trypsin-treated toxin was next incubated with MPSIM or HEPES buffer, the MPSIM-treated, trypsin-nicked toxin was activated 167-fold. That the trypsin-treated toxin had activity equivalent to that of HEPES-treated toxin and could subsequently be activated provides evidence that nicking is not sufficient for activation. Additional support for our conclusion that nicking is not sufficient for activation is the finding that SLT-II is nicked (Fig. 4B), but not activated (Fig. 3), by MPSIM. These findings do not address the question of whether nicking is necessary for SLT-IIvha and SLT-IIvhb activation.

Activation of SLTs with human colonic mucus. To evaluate whether human colonic mucus could activate SLTs, bacterial supernatants or purified SLT-I, SLT-II, or SLT-IIvhb was incubated with human colonic mucus or HEPES buffer for 2 h at 37°C. The cytotoxicity of each of the samples was then measured on Vero cells. The results obtained with human colonic mucus paralleled the findings with mouse intestinal mucus in five areas. First, supernatants containing SLT-IIvha and SLT-IIvhb, or an SLT-II variant, and purified SLT-IIvhb were activated by the human colonic mucus (Table 3). Second, purified SLT-IIvhb A subunit was nicked in the presence of the human mucus (data not shown). Third, supernatants from strains that produce SLT-I, SLT-II, SLT-IIc, or SLT-IIe were not activated (Table 3). Fourth, purified SLT-I appeared to be activated in one of three experiments (Table 3). However, as with the murine mucus, we believe that SLT-I is probably not activated because the SLT-I found in supernatants from 933D or DH5α(pNAS13) was not affected by mucus. Fifth, purified SLT-II was also not activated by the human colonic mucus (Table 3), although, as with the mouse mucus, there appeared to be a decrease in the cytotoxicity of SLT-II after incubation with human colonic mucus.

Bacterial counts, associated cytotoxicity, and SLT antigen levels in mouse intestinal contents, serum, and kidney extracts of mice fed B2F1. Our current in vitro data and previous in vivo results support a model whereby SLTs from B2F1 encounter mouse intestinal mucus in vivo, are activated in the gut, and are then delivered systemically, causing kidney damage and death. To test this model, we investigated how many B2F1 organisms could be recovered at various sites of the intestine after oral feeding of B2F1 and whether associated free SLT was present in the intestine, serum, or kidneys.

In a preliminary experiment, two mice were fed approximately 10^4 B2F1 cells. After 20 h of infection, approximately 10^5 to 10^6 B2F1 cells were recovered from the proximal small intestine, and 10^7 to 10^8 B2F1 cells were isolated from the distal small intestine. However, unlike the colonic and cecal contents from the mice, the proximal- and distal-small-intestinal contents were not cytotoxic for Vero cells. For subsequent experiments, we chose to test the distal rather than the proximal small intestine for SLT because 10- to 100-fold more bacteria were recovered from that site.

In a subsequent set of experiments, two groups of five mice were fed approximately 4×10^9 B2F1 cells, their blood, kidneys, and intestines were removed 9 h or 4 days later, and the number of B2F1 found in the blood, kidneys, or intestine was enumerated (Fig. 5A). As controls, two groups of five mice were fed sucrose alone. No B2F1 cells were recovered in the blood or kidneys 9 h postinfection. We did not test the 4-day blood or kidney samples for B2F1, but previous experiments revealed that even after 5 days of infection, no B2F1 cells are found in the blood or kidneys of the infected mice (data not shown). The number of B2F1 recovered from the distal small intestine was approximately 10^7 CFU/10-cm segment after 9 h or 4 days (Fig. 5A). The numbers of bacteria recovered from the cecum or 10-cm colonic segments were approximately 10^9

TABLE 3. Activation of SLTs by human colonic mucus

SLT type	Fold activation[a]		
	HCM[b] prepn 1	HCM prepn 2	Avg
Purified toxin			
SLT-IIvhb	14, 20	7, 25	16.5[c]
SLT-II	0.1	0.7	ND[d]
SLT-I	10	1, 1.5	ND
Culture supernatant			
SLT-IIvha and SLT-IIvhb	10, 10, 100, 23.3	11.7, 25, 50	23[e]
SLT-II variant	8.75, 8.3	16.7, 8.6	10.6[c]
SLT-II	0.5, 0.3, 1.25	0.3, 0.6	0.6[c]
SLT-IIc	0.7, 0.3	1.2, 0.15	0.6[c]
SLT-IIe	10, 7, 3	2.25, 1.4, 1.2	3[e]
SLT-I	1.5, 1	0.4, 0.1, 1.2	0.8[c]

[a] Calculated as (toxin CD_{50} after treatment with human colonic mucus)/(toxin CD_{50} after treatment with HEPES).
[b] HCM, human colonic mucus.
[c] Arithmetic mean of the data from four or more measurements from the two preparations.
[d] ND, not determined. The average was not calculated if fewer than three activation experiments were done.
[e] Geometric mean of the data from four or more measurements from the two preparations.

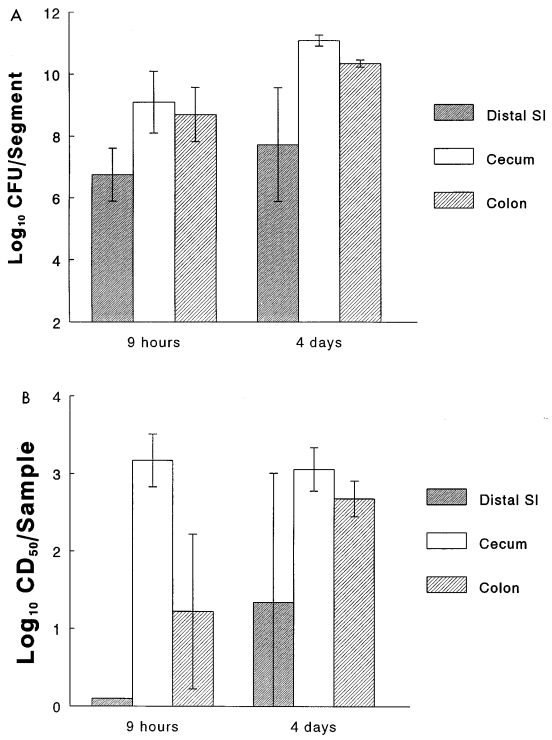

FIG. 5. Bacterial counts and associated cytotoxicity of mouse intestinal contents 9 h or 4 days after oral feeding of B2F1. Two groups of five mice each were fed 4×10^9 B2F1 cells, and the number of bacteria and Vero cell cytotoxicity of the entire cecum or 10-cm intestinal segments of the distal small intestine (SI) or colon were determined after disruption of the segments with a stomacher. (A) Log_{10} CFU recovered from intestinal segments of mice fed B2F1; (B) Vero cell cytotoxicity of intestinal contents of mice fed B2F1. The data are geometric means with 2 standard errors of the mean.

CFU after 9 h and approximately 10^{10} CFU after 4 days (Fig. 5A). No B2F1 organisms were recovered from the mice fed sucrose alone.

We then assessed the cytotoxicity for Vero cells of total intestinal contents that had been disrupted with a stomacher (Fig. 5B). No cytotoxicity was detected in the distal small intestine after 9 h of infection, whereas after 4 days, only one mouse had high levels of Vero cell cytotoxicity in the distal small intestine. However, Vero cell cytotoxicity was detected in sonic lysates of bacteria recovered from three of five of the distal-small-intestine samples from the 4-day group (data not shown), which indicates that B2F1 produces SLT while present in the distal small intestine. By 9 h postinfection, significant Vero cell cytotoxicity was recovered from the cecum but not the colon. Four days after infection, both the cecum and the colon had high levels of associated Vero cell cytotoxicity. We were also able to detect SLT antigen by ELISA from the intestinal samples (data not shown).

We next attempted to detect SLT in serum and kidney samples from the mice by the Vero cell assay and ELISA. We were unable to detect SLT in the serum by either assay (data not shown). Kidney extracts from both infected and uninfected mice were toxic at low dilutions, and no toxicity was detected at dilutions above 1:50. Thus, we were unable to determine whether there was low-level Vero cell SLT toxicity. Furthermore, no SLT antigen was detected in the kidney extracts by ELISA. However, we found that when kidney extracts from uninfected mice were incubated with crude SLT, we also could not detect the SLT antigen in the kidney sample (data not shown). One interpretation of our inability to detect toxin antigen in SLT-spiked kidney samples is that SLT bound the ubiquitous Gb_3 receptors in that organ (33) and was then unavailable for recognition by antibody.

Comparison of mouse colonization capacities of B2F1 and E32511/HSC. The observation that the toxins from B2F1 are activatable but the toxin from E32511/HSC is not, taken with the fact that both organisms can grow in MPSIM (15), suggests that the difference in virulence between these strains is attributable to the selective activation of the toxins from B2F1. However, the capacity to grow in MPSIM in vitro may not completely correlate with the capacity to colonize the mouse gut in vivo. Therefore, the levels of colonization of the streptomycin-treated mouse intestine after oral feeding of B2F1 or E32511/HSC were compared. Streptomycin-treated mice were fed approximately 5×10^6 CFU of B2F1 or E32511/HSC, and colonization of the intestines was determined 14 h later. B2F1 and E32511/HSC colonized the streptomycin-treated mouse intestine equally well (Fig. 6).

DISCUSSION

In this study, we found that the SLT-II variant toxins encoded by mouse-virulent EHEC strains B2F1 and H414-36/89 become activated, that is, more cytotoxic for Vero cells after incubation with mouse or human intestinal mucus. To determine which SLT amino acids might be important for activation, we compared the amino acid sequences of the activatable toxins, SLT-IIvha and SLT-IIvhb from B2F1, with the closely related but not activatable toxin, SLT-IIc from E32511/HSC. The sequence of the mature B subunits are identical among SLT-IIc, SLT-IIvha, and SLT-IIvhb. Therefore, the amino acids important for activation must be present in the A subunit. Both SLT-IIvha and SLT-IIvhb contain three amino acids that are different from SLT-IIc in the A subunit (Table 4). Because two of the three amino acids are the same in SLT-IIvha and SLT-IIvhb, one or both of these amino acids, serine at position 291 and glutamic acid at position 297 of the mature A subunit (which has a total of 297 amino acids), are probably important for activation.

Mucus not only activated SLT-IIvha and SLT-IIvhb but also cleaved the A subunit of purified SLT-IIvhb into a fragment with the apparent size of A_1. Although nicking purified SLT-IIvhb with trypsin also generated a fragment with the apparent

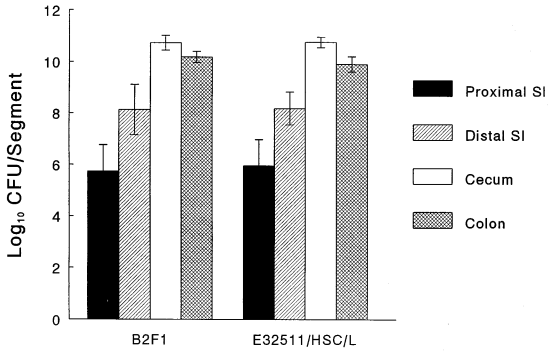

FIG. 6. Bacterial counts in mouse intestines after oral feeding of B2F1 or E32511/HSC. Groups of five mice were fed approximately 5×10^6 B2F1 or E32511/HSC cells, the number of bacteria in the cecum, colon, or 10-cm segments of the proximal or distal small intestine (SI) was enumerated after disruption of the sample with a stomacher, and serial dilutions of the sample were plated on LB agar. The data are geometric means plus or minus 2 standard errors of the mean.

TABLE 4. Amino acid differences in the A subunits of SLT-IIc, SLT-IIvha, and SLT-IIvhb

Toxin	Amino acid sequence differences[a]
SLT-IIc	S19, V252, F291, K297
SLT-IIvha	T19, V252, **S291**, **E297**
SLT-IIvhb	S19, M252, **S291**, **E297**

[a] The amino acid positions given are for the mature A subunit (297 amino acids). The amino acids that are the same for SLT-IIvha and SLT-IIvhb but different from those for SLT-IIc are indicated in boldface.

size of A_1, the toxin was not activated. Because trypsin-nicked SLT-IIvhb had the capacity to subsequently be activated by MPSIM, we believe that a mechanism other than cleavage from A to A_1 and A_2 is responsible for the activation of SLT-IIvha and SLT-IIvhb by MPSIM. Additional support for the theory that cleavage and activation by MPSIM are independent events is the observation that prenicking of Shiga toxin or SLT-I with trypsin also does not increase the cytotoxicity of those toxins for Vero cells (3, 13).

Since activation required preincubation with mucus and mucus alone was not toxic to Vero cells, we believe that the mucus somehow modifies the toxin to make it more cytotoxic. Although we did not observe a change in the molecular weight of the A_1 peptide in the immunoblot (Fig. 4), we probably would not have detected a subtle alteration in molecular weight under the SDS-PAGE conditions used. Alternatively, mucus modification may involve the A_2 peptide since the amino acid differences between the activatable and nonactivatable toxins occur in the A_2 peptide. In this study, we could not address possible effects of mucus on the A_2 peptide because it was not detected in our immunoblots.

Although the crystal structure of Shiga toxin has been solved (6), the last four amino acids of the A_2 fragment were not placed into the structure because they could not be clearly resolved. However, recent genetic studies suggest that the last six amino acids of the Shiga toxin A subunit extend beyond the B pentamer (12). Moreover, the A subunits of SLT-II, SLT-IIvha, SLT-IIvhb, and SLT-IIc are four amino acids longer at the C terminus than is Shiga toxin. These terminal amino acids may extend out of the B pentamer of the toxin, in a manner analogous to *E. coli* heat-labile toxin (29). Thus, the two amino acid residues that we identified in SLT-IIvha and SLT-IIvhb that are potentially important for activation may be external to the B pentamer and, therefore, available as a substrate for the activating factor(s) that is present in mouse and human intestinal mucus. Additionally, since these two residues are remote from the active site of the toxin, activation by mouse intestinal mucus probably does not affect the enzymatic activity of this toxin. Rather, activation probably affects cytotoxicity by altering another activity of the toxin, such as trafficking of toxin in the eukaryotic cell.

Of the EHEC strains that we have fed to streptomycin-treated mice, only B2F1 and H414-36/89 have LD_{50}s of less than 10 CFU, and only toxins from these strains are activatable. This correlation suggests that the presence of an activatable toxin is a requisite for the low mouse LD_{50}s exhibited by these strains. On the basis of this hypothesis, we propose a model for the pathogenesis in orally infected streptomycin-treated mice of EHEC strains B2F1 and H414-36/89 and EHEC strains with LD_{50}s of $\geq 10^{10}$ CFU, such as E32511/HSC or 86-24 (Fig. 7). The bacteria are ingested and colonize the small and/or large bowel, where they produce SLT (this study and reference 15). The SLTs produced by B2F1 and H414-36/89 become activated, but the SLTs produced by E32511/HSC and 86-24 do not (this study). Colonization of the small intestine leads to toxin production and systemic delivery of SLT as a result of the highly absorptive nature of this part of the intestine. In support of this systemic delivery hypothesis is the observation that passive intraperitoneal immunization with anti-SLT-II antibodies protects the animals from kidney pathology and death. The systemically delivered toxin collects in sites where Gb_3 is found in the mouse such as the renal tubule cells (33). The kidney damage caused by the toxin, perhaps in conjunction with cytokines (35), leads to the death of the mouse (15, 37).

The fact that the only human clinical EHEC isolates that are mouse lethal at doses of less than 10^1 CFU express an activatable toxin indicates that the presence of an activatable SLT may be required for the mouse lethality of clinical EHEC isolates. In contrast, streptomycin-treated mice fed K-12 strains transformed with recombinant plasmids expressing SLT-II or SLT-II variant toxins from strains of human origin (with or without the S291 and E297) are mouse lethal only if they produce relatively high levels of SLT (16, 22, 37). Paton et al. also reported that differences in A-subunit amino acids contribute to differences in mean time to death in streptomycin-treated mice (22), a result which is consistent with our finding that differences in A subunit amino acids have an effect on mouse virulence.

We found the S-291 and E-297 in a few other SLT-II variant toxins when we performed a BLASTP search (1) of the peptide sequence databases in the National Center for Biotechnology Information. The SLT-II variants identified in the search included those produced by *Citrobacter freundii* (27), EHEC strain 7279, an EHEC strain of the O111:H$^-$ serotype (24), an SLT-producing OX3:H21 strain from a patient with sudden infant death syndrome (23, 24), and SLT-IIe from pig edema disease-producing *E. coli* strains. Although SLT-IIe does have S-291 and E-297, it was not activated by mouse intestinal mucus (Fig. 2). This failure to activate may reflect the many other amino acid differences between SLT-IIe and the activatable toxins SLT-IIvha and SLT-IIvhb. SLT-IIe has other biological differences from most SLT-II variants; indeed, SLT-IIe binds preferentially to the receptor Gb_4, whereas SLT-IIvha and SLT-IIvhb bind Gb_3 (25). The fact that SLT-IIe was not activatable shows that the mere presence of S-291 and E-297 in the A subunit is not sufficient for SLT-II-related activation. We

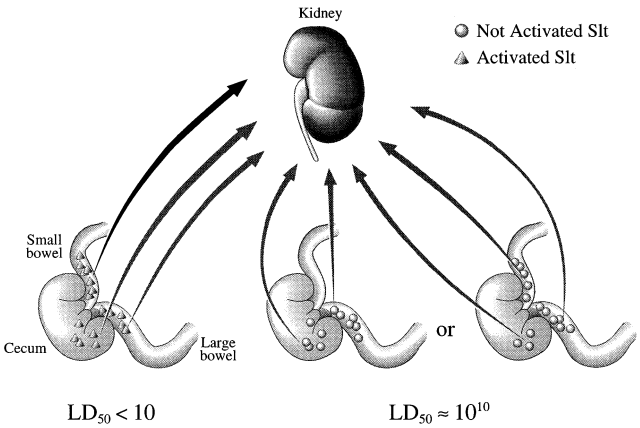

FIG. 7. Model of toxin delivery. Only the location of the SLT is shown, but the EHEC strain would be found in the same sites as the SLT. The SLT is shown as either activated or not activated. We cannot measure ratios of activated SLT to not activated SLT.

would predict that (i) the SLT-II variants produced by the human isolates described above are activatable and (ii) the strains synthesizing them are highly mouse virulent provided that the strain can grow in mouse small-intestinal mucus.

The observation that only certain SLTs had the capacity to be activated by human mucus suggests the possibility that in EHEC infection of humans, the presence in a strain of an activatable toxin makes that strain more virulent and/or compensates for the absence of another virulence attribute. For example, the absence in B2F1 of the genes for induction of the attaching and effacing lesion may be counterbalanced by the fact that the SLTs from B2F1 have the capacity to be activated. In contrast, SLT-I and SLT-II did not appear to be activated by human or mouse intestinal mucus, but these toxins are linked to EHEC illnesses in humans. Thus, activation of an SLT per se is not required for SLT to cause disease.

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