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Translocation of Shiga Toxin across Polarized Intestinal Cells in Tissue Culture

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Escherichia coli **strains producing Shiga toxins (Stx) 1 and 2 colonize the lower gastrointestinal tract in humans and are associated with gastrointestinal and systemic diseases. Stx are detectable in the feces of infected patients, and it is likely that toxin passes from the intestinal tract lumen to underlying tissues. The objective of this study was to develop an in vitro model to study the passage of Stx across intact, polarized cell monolayers. Translocation of biologically active Stx was examined in four cell lines grown on polycarbonate filters. Stx1 translocated across intestinal cell monolayers (CaCo2A and T84 cells) in an energy-requiring and saturable manner, while the monolayers maintained a high level of electrical resistance. Stx1 had no effect on electrical resistance or inulin movement across these cell lines for at least 24 h. Induction of specific Stx receptors with sodium butyrate reduced the proportion of toxin translocated across CaCo2A monolayers but had no major effect on the movement of horseradish peroxidase or [^{3}H]inulin. We have shown that biologically active Stx1 is capable of moving across intact polarized intestinal epithelial cells without apparent cellular disruption, probably via a transcellular pathway. The data also suggest that the presence of Stx receptors on the apical surface of intestinal epithelial cells may offer some protection against the absorption of luminal Stx1.**

Diarrheagenic *Escherichia coli* can be divided into five main types (19): enteroinvasive *E. coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli*, and the Shiga toxin 1 or 2 (Stx1 or Stx2, respectively) (also known as SLT-I or -II, respectively, and Verotoxin 1 or 2, respectively) (5)-producing enterohemorrhagic *E. coli* (EHEC) strains of multiple serotypes, the most well known of which is O157:H7. Stx-producing *E. coli* strains have been linked with both major epidemics and sporadic disease and are responsible for a wide spectrum of illnesses, including both bloody and nonbloody diarrhea, which may be complicated by severe systemic complications in the form of hemolytic-uremic syndrome (HUS) (a triad of renal failure, thrombocytopenia, and hemolytic uremia) or thrombotic thrombocytopenic purpura. HUS is now the most common cause of acute renal failure in children in the United States, and EHEC strains are the most common cause of HUS (24).

EHEC strains are ingested orally from contaminated food, most commonly beef products (especially ground beef), and milk, or water or by person-to-person transmission. EHEC strains are known to colonize the large bowel and distal small bowel in a mouse model (39) and are thought to cause predominantly right-sided colonic lesions in humans (15). Members of the family of Shiga-like toxins are genetically, biochemically, and biologically related to Stx from *Shigella dysenteriae* type 1. Two structurally and functionally related, yet immunologically distinct, toxins (Stx1 and Stx2) are most frequently associated with human disease (2, 13, 25). Two other members of the Stx family have also been described: Stx2c, which is also associated with disease in humans (35), and Stx2e, which is associated with edema disease of swine (21). Readily detectable amounts of free Stx1 and Stx2 are present in the feces of patients infected with EHEC (1, 14, 32). It is likely, therefore, that the gastrointestinal intraluminal levels of Stx are at least as high as, and probably higher than, the levels detected in fecal samples. The principal systemic manifestations of Stx-related disease are believed to be due to the action of the toxins on endothelial cells, which results in endothelial cell swelling and the development of thrombotic microangiopathy, hemorrhagic colitis, HUS, or thrombotic thrombocytopenic purpura (23).

Despite reports that EHEC strains invade certain tissue culture cell lines (28), they are not generally considered to be invasive organisms like the prototypic Stx-producing *S. dysenteriae* type 1. The question of how Stx from *E. coli* gains access to the underlying tissues thus arises. There are several possible routes the toxin may take, including transcellular or paracellular routes or via "holes" in the mucosal epithelial cell layer caused by the destruction of the epithelial cells. Such cellular destruction may occur either through the effects of the toxin itself or as a consequence of some other pathological process, such as the formation of attaching and effacing lesions (7).

The purpose of the current study was to develop and utilize an in vitro tissue culture model to study the movement of Stx1 across polarized intestinal epithelial cell monolayers of CaCo2A and T84 cells. These intestinal cell lines, derived from human colon carcinoma, form polarized monolayers when grown on collagen-coated polycarbonate filters and develop a high level of transepithelial electrical resistance (6, 30). In this paper we report that biologically active Stx1 is able to translocate from the apical to the basolateral surfaces of both intestinal cell lines without apparent loss of monolayer integrity.

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TABLE 1. Tissue culture cell lines used in the current study

Cells	Formation of tight junctions	Globotriaosylceramide content (pmol/mg of cell protein)[a]	Amt of Stx1 to kill 50% of a monolayer
Not gut derived			
CHO	No	UD (16)	—[b]
HeLa	No	1,136 ± 118 (16)	1–10 pg/ml
Vero	No	1,329 ± 216 (16)	1–10 pg/ml
Gut derived			
T84	Yes	UD# (11)	—
CaCo2A	Yes	868 ± 61 (11)	>10 μg/ml
CaCo2A-bu[c]	Yes	2,410 ± 25 (11)	0.1–1 μg/ml

[a] Reference numbers are given in parentheses. Results are expressed as means ± SEM. UD, undetectable.
[b] —, cells totally insensitive.
[c] Sodium butyrate exposed.

We anticipate that the model described will allow further study of the mechanisms involved in Stx translocation.

MATERIALS AND METHODS

Tissue culture. A variety of tissue culture cells with various levels of sensitivity to Stx were used (Table 1). All cell lines were grown at 37°C in 5% CO_2 with penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). Chinese hamster ovary K-1 (CHO) cells were grown in F12 medium supplemented with 5% fetal calf serum. HeLa 229 cells and Vero cells were grown in modified McCoy's 5a medium with 10% fetal calf serum. CaCo2A cells were grown in Dulbecco's modified Eagle's medium containing 4,500 mg of L-glutamine per liter, D-glucose, 10% fetal calf serum, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). T84 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (low glucose) and F12 medium with 10% fetal calf serum and 15 mM HEPES. CHO, Vero, and HeLa cells were obtained from the American Type Culture Collection (Rockville, Md.), and CaCo2A and T84 cells were obtained from the GRASP tissue culture core, New England Medical Center.

Polycarbonate filter inserts (0.33 cm^2) (Transwell membranes; Costar, Cambridge, Mass.) in 24-well plates were coated with rat tail collagen prior to being seeded with one of the cell lines. The cells were fed every 2 days, and experiments were conducted at between 8 and 10 days following seeding.

Experimental procedures. Purified Stx1 (10 ng to 10 μg per well) (8), [*methoxy*-^{3}H]inulin (125.6 mCi/g; 3 to 6 $\times$ 10^5 cpm per well) (New England Nuclear, Boston, Mass.), or horseradish peroxidase (HRP) (type VI-A) (50 μg per well) (Sigma Chemical Co., St. Louis, Mo.) was prepared in the appropriate tissue culture medium for the cells under investigation. The total volume added to the Transwells (100 μl to the top chamber and 600 μl to the lower chamber) was per the manufacturer's instructions to avoid the effects of hydrostatic pressure. In experiments in which [^{3}H]inulin was used, nonradioactive inulin was also added to the medium (final concentration, 1 mM). Following the addition of the various agents to the upper chamber, the plates were incubated at 37°C for 24 h. Samples were removed from the lower chamber at various times up to 24 h and immediately replaced with an equal volume of medium to maintain hydrostatic pressure equilibrium. In certain experiments, Stx was added to the lower chamber and aliquots were removed from the upper chamber for assay. In these comparative experiments, the concentrations of toxin used in either the upper or lower chamber were the same.

Electrical resistance across selected cell monolayers grown on 0.33-cm^2 polycarbonate filter inserts in 24-well plates was determined as described previously (29). Measurements of electrical resistance were done by using either a voltage clamp (University of Iowa) or a Millicell-ERS (Millipore Corporation, Bedford, Mass.). There were no differences in the results obtained by the two methods of measuring electrical resistance.

Stx1 activity was measured by quantitating the incorporation of [^{3}H]leucine into protein as previously described (8). Bioactivity was converted to toxin protein by comparison with a control dose-response curve run concurrently in all assays. The amount of tritiated inulin in a particular sample was determined by adding 10 μl of the sample material to 4 ml of liquid scintillation fluid and counting the radioactivity in a scintillation counter. HRP was measured as described by Hecht et al. (9).

We have previously found that sodium butyrate increases the expression of the Stx1 receptor on CaCo2A cells (12). Therefore, in certain experiments various concentrations of sodium butyrate (Sigma Chemical Co.), were added at day 6 to 8, and the experiments were conducted 2 days later. In other experiments we determined the effect of the uncoupler of oxidative phosphorylation 2,4-dinitrophenol (2,4-DNP) (Sigma Chemical Co.), which depletes cellular energy reserves (22). 2,4-DNP was used as a 0.2 M stock made up in ethanol. Preliminary experiments showed that when CaCo2A cells were exposed for 3 or 6 h to various concentrations of 2,4-DNP, a concentration of 5 to 7.5 mM 2,4-DNP was required to inhibit protein synthesis to at least 50% of that in control wells, as determined by tritiated leucine incorporation (data not shown). Therefore, 2,4-DNP was used at a concentration of 5 mM in the present study. Because the 2,4-DNP was dissolved in ethanol, an equivalent volume of plain ethanol was used as a control in the 2,4-DNP experiments. The 2,4-DNP was added to the cells at the same time as the Stx1 and tritiated inulin in these experiments.

RESULTS

Physiological studies. There was no detectable resistance across the nonpolarized CHO or HeLa cells grown on polycarbonate filters. In contrast, the two polarized intestinal cell monolayers developed resistance levels (means ± standard errors of the mean [SEM]) of 2,020 ± 491 and 838 ± 290 $\Omega \cdot cm^2$ for T84 (n = 8) and CaCo2A (n = 11) cells, respectively, after between 8 and 10 days of growth on collagen-coated polycarbonate filters. When CaCo2A and T84 monolayers were exposed to concentrations of Stx1 up to 10 μg/ml for up to 24 h, we detected no decrease in electrical resistance in either cell line. The resistance for three sets of T84 cells was 1,612 ± 142 and 2,024 ± 129 before and after exposure to Stx1, respectively, and that for three sets of CaCo2A cells was 632 ± 147 and 782 ± 200 before and after exposure to Stx1, respectively. Resistances in all six wells actually rose during the 24 h of toxin exposure, but no more than in non-toxin-exposed wells. Exposure of CaCo2A cells to Stx1 for 24 h had no apparent effect on the morphology of the cells as determined by electron microscopy by standard techniques (Fig. 1).

Translocation of tritiated inulin was used as a marker of monolayer integrity, since only small amounts of inulin are translocated by polarized cells unless cell injury occurs or the tight junction is altered. CaCo2A cells grown on polycarbonate filters for various times (1 to 8 days) were therefore tested for electrical resistance and inulin translocation. There was an

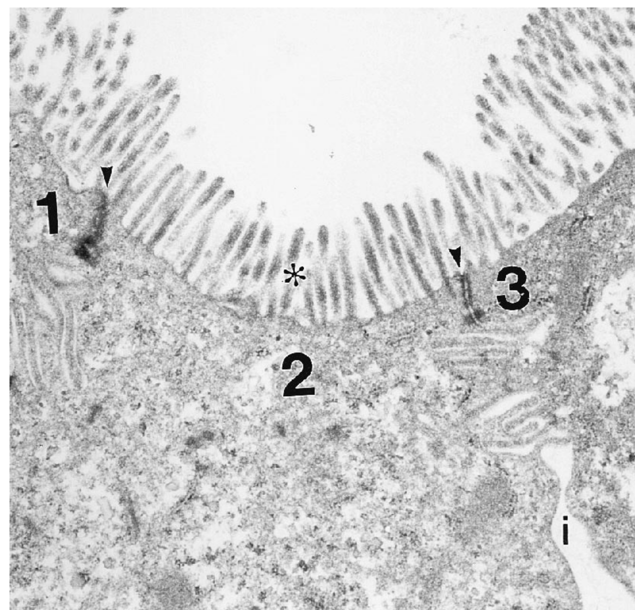

FIG. 1. Electron micrograph of CaCo2A monolayer after incubation overnight in the presence of Stx1. A prominent brush border (asterisk) is present on each of three epithelial cells (1 to 3). Two intact apical junction complexes between the three cells are indicated by arrowheads. The extensively interdigitating lateral cell membranes of cells 2 and 3 are evident between the apical junction complex and the intercellular space (i). A portion of the nucleus is present in cell 1. Magnification, ×12,000.

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TABLE 2. Electrical resistance and movement of tritiated inulin across various cell lines grown on polycarbonate filters[a]

Cell line	Addition of Stx1 (10 ng/well)	Transepithelial resistance (Ω · cm²)	% [³H]inulin translocation at:		
			60 min	150 min	16 h
T84	No	2,431	0.3	0.6	1.0
	Yes	2,483	0.4	0.6	1.1
		2,236	0.3	0.5	1.2
CaCo2A	No	1,196	0.4	0.9	1.1
	Yes	1,001	0.9	1.1	2.4
		1,154	0.5	0.7	1.0
HeLa229	No	34	3.5	8.3	39.4
	Yes	143	4.2	8.4	47.3
		135	4.4	8.8	49.4
CHO	No	104	3.8	NT[b]	52.1
	Yes[c]	107	3.7	NT	46.7
	Yes	57	3.6	NT	49.0

[a] Four different cell lines were grown on polycarbonate filters in triplicate; each row represents a single well. Electrical resistance was measured at the beginning of the experiment. Tritiated inulin (6×10^5 cpm) was added to the top chamber of each well; and the percentage of this amount which translocated to the lower chamber at various times is shown. Stx1 (10 ng per well) was added to the top chamber of two wells at the same time as the tritiated inulin in each set of triplicates. The data for CHO cells were obtained from a separate experiment in which the 150-min time point was omitted.

[b] NT, not tested.

[c] 100 ng of Stx1 was added to this well.

inverse correlation between the percentage of inulin movement from the top chamber to the bottom chamber at 3 h and the transmonolayer resistance ($r^2 = 0.4$). Similar results were found 24 h after the addition of inulin. When the translocation of inulin in four different cell lines was examined, [³H]inulin was found to move much more rapidly across HeLa and CHO cells, which lack tight junctions, than across the polarized intestinal cells (Table 2). These experiments demonstrated that [³H]inulin movement reflects the integrity of the monolayer, and we used this simple assay routinely in place of measured electrical resistance in several of the subsequent experiments. Stx1 (10 ng per well) was added to pairs of wells as part of the same experiment for which results are shown in Table 2. This dose was chosen for two reasons: (i) we had found that this allowed a readily detectable amount of Stx to translocate to the lower chamber, and (ii) it approximates the concentration of Stx found in stool samples. A 16-h exposure to this dose of Stx1 did not alter movement of [³H]inulin in any of the four cell lines studied (Table 2).

Having shown that the presence of Stx1 in the upper chamber did not affect the translocation of [³H]inulin, we then examined the movement of [³H]inulin, HRP, and Stx1 added at the same time to the upper chamber of three cell lines. A series of wells containing either CHO cells, HeLa cells, or CaCo2A cells growing on polycarbonate filters was exposed to a mixture of tritiated inulin, HRP, and Stx1. As expected, there was rapid movement of inulin and HRP across both CHO and HeLa cells. In contrast there was little movement of either inulin or HRP across the CaCo2A cells (Table 3). Despite the high level of movement of both inulin and HRP across HeLa cells, Stx1 movement across HeLa cells was remarkably reduced compared with that across CHO cells, approximating the amount translocating CaCo2A cells (Table 3). In this experiment resistance levels were determined at the beginning and the end of the experiment and were high in the CaCo2A cells and low in the other two cell lines, as expected. Resistances did not change during the course of the experiment (Table 4). Stx and inulin were also able to move across T84 cells, with 0.2% (± 0.06%) and 3% (± 0.9%) of the respective dose added detectable in the lower chamber at 24 h. Although there were variations in the amounts of toxin translocated in different experiments and cell lines, polarized intestinal cells were clearly allowing Stx1 to move to the lower chamber.

Translocation of Stx1 and [³H]inulin was considerably reduced in CaCo2A cells when the mixture (with the same concentration of Shiga toxin) was added to the bottom wells (serosal-to-mucosal transport), compared with addition to the upper chamber (mucosal-to-serosal transport). The percentages of inulin translocated were 5.9 ± 3.6 and 1.4 ± 0.1 from mucosal to serosal reservoirs (mean ± standard deviation for five experiments) and from serosal to mucosal reservoirs (four experiments), respectively, whereas the percentages of Stx translocated were 4.4 ± 2.7 and 0.7 ± 0.03 from mucosal to serosal and serosal to mucosal surfaces, respectively. Overall there was approximately a fourfold drop in inulin transport and a sixfold drop in Stx1 transport in the serosal to mucosal direction compared with the reverse.

In order to determine whether the movement of either [³H]inulin or Stx1 was dependent on an active metabolic process, we studied the effect of temperature and 2,4-DNP exposure on the translocation of the two molecules. The movement of both [³H]inulin and Stx1 was inhibited when the experiment was conducted at 4°C compared with 37°C (Table 5). However, after 4 h at 4°C, the movement of Stx1 was reduced about 70-fold and that of inulin was reduced only about 2-fold compared with that of the cells maintained at 37°C. When [³H]inulin and Stx1 were present in the upper chamber for 4 h at 4°C and then warmed to 37°C, the movement of both [³H]inulin and Stx1 to the lower chamber increased following the warming, and by 24 h the percentages of [³H]inulin and Stx1 which had translocated were the same in all wells irrespective of starting temperature (Table 5). There was no effect of 2,4-DNP on the movement of tritiated inulin across polarized CaCo2A cells compared with either the ethanol or medium control (Fig. 2A). However, there was a major reduction in the translocation of Stx1 in the presence of 2,4-DNP compared with both the

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TABLE 3. Movement of tritiated inulin (³H-I), HRP, and Stx1 across three different cell lines grown on polycarbonate filters

Time (h)	% of agent translocated to the lower chamber[a] for:								
	CHO cells			HeLa cells			CaCo2A cells		
	³H-I	HRP	Stx1	³H-I	HRP	Stx1	³H-I	HRP	Stx1
3	38 (3.3)	4.8 (1.7)	24 (4)	23 (0.3)	1.5 (0.2)	1.2 (0.9)	3.2 (0.3)	0.003 (0.0005)	0.8 (0.3)
6	56 (2.5)	7.6 (4.4)	30 (6)	39 (4)	4.3 (0.5)	2.2 (1.3)	3.6 (0.3)	0.004 (0.0005)	1.1 (0.3)
24	84 (2.3)	8.0 (0.4)	47 (4)	83 (1.4)	9.7 (1.3)	4.3 (1.7)	5.0 (0.1)	0.009 (0.002)	4.5 (1.2)

[a] Each datum point represents the mean (SEM) from two experiments.

TABLE 4. Individual resistance measurements in the paired wells from the three cell lines shown in Table 3 at the beginning and end of the experiment

Time (h)	Resistance ($\Omega \cdot cm^2$)		
	CHO cells	HeLa cells	CaCo2A cells
0	139, 152	169, 161	890, 880
24	190, 216	179, 159	886, 766

ethanol and medium controls (Fig. 2B). There were no major differences between the medium controls and the ethanol controls (Fig. 2).

CaCo2A cells were then grown on polycarbonate filters and exposed to various concentrations of sodium butyrate, which is known to induce the Stx1 receptor, globotriaosylceramide, in these cells in monolayer culture (12). We examined the effect of various concentrations of sodium butyrate on the movement of tritiated inulin, Stx1, and HRP when all were added at the same time. There was little effect of butyrate on the movement of [^{3}H]inulin, and if anything, the higher butyrate doses caused an increase in inulin translocation at 24 h (Table 6). In contrast there was a marked butyrate dose-related reduction in the translocation of Stx1. The movement of HRP across butyrate-treated cells was not diminished, and like in the case of inulin, there was a trend towards increased movement of HRP with the higher butyrate dose, especially at 24 h (Table 6).

To determine if it was possible to overcome the butyrate-associated reduction in toxin translocation, we added increasing amounts of Stx1 to the upper chamber (Table 7) and assayed for toxin in the lower chamber. Increasing the concentration of toxin in the upper chamber resulted in increased absolute amounts of translocated toxin but a reduced percentage of the dose added in both the butyrate-treated and untreated monolayers. At higher doses of toxin, the presence of butyrate did not appear to affect toxin movement. There was no alteration of the percentage of inulin transported with different Stx1 concentrations (Table 7).

DISCUSSION

Stx are produced from *E. coli* strains colonizing the gastrointestinal tract, and free toxin is readily detected in the feces of patients infected with these organisms (1, 14, 32). Despite the lack of documentation of the presence of Stx1 or Stx2 in tissues or blood of patients infected with Stx-producing bacteria, the

TABLE 5. Effect of temperature on the translocation of Stx1 and tritiated inulin across CaCo2A monolayers

Expt[a]	Time (h)	Temp (°C)	% Translocation to lower chamber of[b]:	
			Inulin	Stx1
A	2	4	2.1 (0.04)	0.005 (0.0005)
	4	4	3.3 (0.3)	0.01 (0.003)
	24	37	10.2 (0.9)	2.0 (0.2)
B	2	37	6.7 (0.9)	0.1 (0.04)
	4	37	7.7 (0.2)	0.7 (0.07)
	24	37	10.3 (0.03)	2.3 (0.2)

[a] Two experimental conditions were used. In condition A the cells were kept at 4°C to 4 h and then kept at 37°C for the remainder of the experiment. In condition B the cells were kept at 37°C throughout the experiment.

[b] Each datum point represents the mean (SEM) from two experiments.

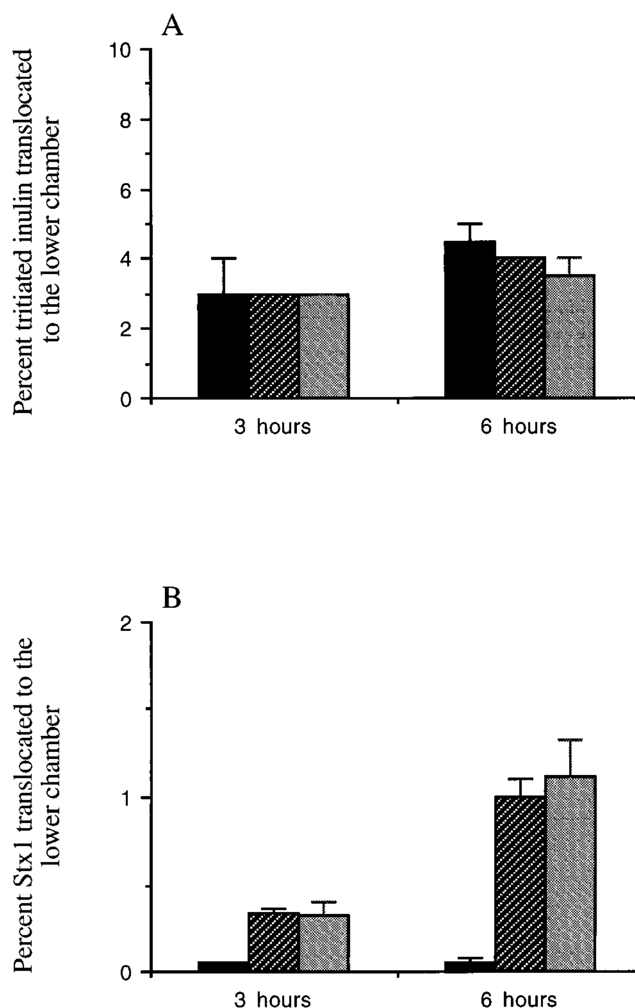

FIG. 2. Tritiated inulin and Stx1 were added to CaCo2A monolayers grown on polycarbonate filters in the presence of either 2,4-DNP (black bars), a comparable level of ethanol (hatched bars), or plain medium (stippled bars). (A) Percent translocation of tritiated inulin; (B) percent translocation of Stx1. Samples were assayed at 3 and 6 h after addition of the compounds to the upper chamber. Bars indicate SEM of duplicate experiments.

pathogenesis of the systemic complications of EHEC is nonetheless generally believed to be due to the effects of toxin. The failure to find toxin in tissue is probably a reflection of the small amounts of toxin needed to induce microangiopathic lesions and/or to the lack of sufficiently sensitive detection systems. Although there are no data concerning the amount of absorbed Stx required to cause disease in humans, experiments with pigs have demonstrated that as little as 3 ng of Stx per kg of body weight will kill 50% of the animals (21). If one estimates that a 20- to 30-kg child will need to absorb only about 100 ng of Stx to become sick (50% lethal dose based on the porcine model) and if one takes into consideration the fecal concentration of Stx, which is greater than 100 pg/ml as determined by enzyme immunoassay (1), then about 15 pg of toxin will be absorbed per cm^2 of intestinal surface on the basis of the model we have described. Given the villus nature of the mucosal surface of the intestine compared with the surface area of a flat monolayer (0.33 cm^2), the absolute quantities of Stx that we observed translocating across epithelial cell lines are relevant in terms of disease pathogenesis. There is com-

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TABLE 6. Effect of sodium butyrate concentration on the translocation of tritiated inulin and Stx1 across polarized CaCo2A cells at 6 and 24 h

Sodium butyrate (mM)	*n*	% translocated to the lower chamber[a]					
		Tritiated inulin		Stx1		HRP	
		6 h	24 h	6 h	24 h	6 h	24 h
0	4	3.5 (0.5)	4.6 (0.4)	2.3 (1.2)	4.8 (2.0)	0.005 (0.003)	0.03 (0.01)
0.25	2	4.7 (0.3)	5.5 (0.4)	1.5 (0.4)	5.0 (0.03)	NT	NT
0.5	4	3.7 (0.4)	5.9 (0.3)	0.4 (0.2)	1.7 (1.1)	0.007 (0.003)	0.08 (0.02)
1.0	2	4.3 (1.6)	7.7 (1.0)	0.1 (0.05)	0.5 (0.4)	0.005	0.10
2.0	2	2.6 (0.5)	10.4 (0.4)	0.1 (0.02)	0.9 (0.2)	0.006 (0.002)	0.38 (0.035)

[a] Each datum point for the translocation of tritiated inulin and Stx1 represents the mean (SEM) from *n* experiments as shown. The HRP data show the means (SEM) from duplicate experiments, except for the 1.0 mM butyrate experiment because one of the wells had become leaky (24-h inulin translocation was 25.2%); therefore, those data were excluded from analysis. NT, not tested.

pelling evidence that endothelial cells are one of the most critical targets for Stx (20, 26, 31). However, since infection with toxin-producing *E. coli* is restricted to the apical surface of enterocytes, whether the damaged endothelial cells are in the kidney (27), brain (37), or even the colon (15), it is likely that the toxins reach the final site of action by first traversing the intestinal epithelial barrier to gain access to the systemic circulation. How does this happen?

Stx-producing *E. coli* strains are not generally considered to be invasive in vivo, although they have been shown to invade some tissue culture cell lines (28). In the absence of mucosal invasion, as in other diseases associated with Stx-mediated pathology, such as shigellosis due to *S. dysenteriae*, there are a limited number of possible routes that toxin may take to move from the gut lumen to underlying tissues. First, toxin could pass through holes created by destruction of individual mucosal cells. Second, toxin could move between the cells along an altered paracellular pathway. A third route would be translocation through intact epithelial cells. EHEC strains are capable of affecting cellular physiology and have, for example, been shown to cause elevations of intracellular inositol 1,4,5-triphosphate and intracellular free calcium in HEp-2 cells, even in the absence of the formation of attaching and effacing lesions (11). Recent studies with the gnotobiotic piglet model of Stx-related disease have shown that the formation of attaching and effacing lesion is not a prerequisite for the systemic effects of Stx (38). This is actually not a surprising observation in view of the ever-increasing number of clinical EHEC isolates which cause human disease but do not produce attacing and effacing lesions (36). The data from the present study suggest that a transcellular route may be responsible, at least in the in vitro model we have employed. It is also possible that toxin breeches the mucosa in vivo across other cells abutting the intestinal lumen, such as M cells.

TABLE 7. Effect of varying the concentration of Stx1 on percent toxin translocation across polarized CaCo2A cells[a]

Amt of Stx1 in upper wells (ng)	% translocated to the lower chamber:			
	Without sodium butyrate		With sodium butyrate	
	^{3}H-I	Stx1	^{3}H-I	Stx1
10	8.0 (0.4)	3.8 (0.4)	7.3 (0.1)	2.0 (0.2)
1,000	7.1 (0.3)	0.5 (0.2)	7.9 (0.3)	0.8 (0.2)
10,000	7.5 (0.6)	0.1 (0.04)	8.4 (0.1)	0.14 (0.06)

[a] Three different quantities of Stx1 were added to the upper chamber of CaCo2A cells grown on Transwell filters in the presence or absence of 0.5 mM sodium butyrate. The amount of [^{3}H]inulin (^{3}H-I) was the same in each experiment. [^{3}H]inulin and Stx1 in the lower chamber were measured at 6 h. Each datum point represents the mean (SEM) from two experiments.

In contrast to CHO and HeLa cells, both T84 cells and CaCo2A cells form polarized monolayers with intact tight junctions, as determined by ultrastructural studies that revealed intact tight junctions and by observations that the movement of tritiated inulin was inversely proportional to the electrical resistance across the monolayers. Tritiated inulin flux was markedly diminished in the polarized cell lines with high resistances (CaCo2A and T84 cells) compared with that in nonpolarized cell lines (CHO and HeLa cells). When the intestinal cell lines were incubated with Stx1 for 24 h, there was no effect on either the electrical resistance or the movement of [^{3}H]inulin, and no morphological alterations were observed by transmission electron microscopy. However, toxin was able to translocate across both intestinal cell lines, and active toxin was recovered from the basal chamber of the Transwell apparatus. While Stx1 was able to move in both directions across intestinal epithelial cell monolayers, movement was greater in the mucosal-to-serosal direction. This may in part be due to differences in the cell surface area in contact with the fluid phase for the basolateral and apical surfaces.

Inhibition of translocation of Stx1 occurred when cells were chilled to 4°C, suggesting that this is an energy-requiring process. This concept is further supported by studies employing the uncoupler of oxidation phosphorylation 2,4-DNP (22), which had no effect on the paracellular movement of tritiated inulin but almost completely blocked the movement of Stx1. The maintenance of electrical resistance in the presence of toxin, the ultrastructural evidence of tight junction integrity, and the molecular size of the toxin (approximately 70 kDa) also do not support the notion of a paracellular route for the transfer of Stx1 from the apical to the basal side of the monolayers.

CaCo2A cells can express receptors for Stx1 and have the potential for upregulating these receptors following treatment with sodium butyrate while at the same time becoming more sensitive to the effects of toxin (12). T84 cells, on the other hand, do not have detectable receptors for Stx1, and butyrate does not induce receptors in this cell line. In addition to butyrate altering transcription of various genes in cells (3, 33), Sandvig et al. (34) recently showed that butyrate treatment also altered the length of the fatty acid chains of the Stx receptor, which may affect efficiency of binding or uptake and intracellular release of the toxin. We speculated that increasing the capacity of Stx1 receptors on CaCo2A cells would concomitantly increase the translocation of toxin from the upper to the lower chamber as well. In fact, the opposite was observed, as there was a butyrate concentration-dependent drop in Stx1

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movement. Despite this drop in toxin translocation, butyrate did not affect the movement of either tritiated inulin or HRP. In fact, there was a suggestion that at the highest concentrations of butyrate studied (2 mM), there was actually an increase in translocation of these two molecules. The most obvious explanation for these observations is that the induced glycolipid receptors bind the Stx1 in a manner that reduces the amount of Stx1 available for translocation to the lower chamber. Because sodium butyrate treatment caused no reduction in the movement of [^{3}H]inulin or HRP, the butyrate-induced drop in Stx transport is unlikely to be a nonspecific decline in the translocation rate of all molecules. In addition, a decreasing proportion of serially increasing quantities of toxin added to the upper chamber was translocated to the lower chamber. This observation suggests that the operative translocation pathway is saturable, in addition to being energy requiring.

The concept that the number of apical receptors for Stx1 may affect the movement of the toxin across the monolayer is supported by the data from our experiments with HeLa and CHO cells. There was little difference between the nonpolarized HeLa and CHO cells in the movement of tritiated inulin and HRP. However, the amount of toxin translocated from the upper to the lower chamber across HeLa cell monolayers was much reduced compared with that for CHO cells. In this respect, HeLa cells, which express large amounts of Stx1 receptor, resemble CaCo2A cells. In contrast, CHO cells, which neither express the toxin receptor nor form tight junctions, were readily permeable to all three molecules. One explanation for these findings is that the binding of Stx to toxin receptors on the HeLa cells removes toxin from the medium and sequesters the toxin away from the translocation mechanism. Consistent with this hypothesis, increasing the level of Stx1 receptor in CaCo2A cells did not increase (and in fact reduced) the translocation of Stx. This suggests that in CaCo2A cells the amount of Stx which moves across the translocation pathway is not directly related to the binding of Stx to the globotriaosylceramide receptor on the cell surface. Thus, if the receptor-bound Stx1 is being taken up by its usual receptor-mediated pathway, it is not finding its way across the intestinal cell monolayer. We suspect that toxin is being transcytosed by some other, as-yet-undefined pathway, possibly via nonspecific endocytosis (10). Recent work suggests that cholera toxin may also translocate across T84 cells to the serosal surface, possibly via transcytotic vesicles (18). The use of compounds such as monensin or dansylcadaverine, which selectively inhibit receptor-mediated endocytosis and block toxin bioactivity in HeLa cells (4, 16, 17), will be valuable in determining the pathways involved. These questions are the subject of current investigations.

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