Shiga Toxins 1 and 2 Translocate Differently across Polarized Intestinal Epithelial Cells

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Shiga toxin-producing Escherichia coli (STEC) is an important food-borne pathogen that causes hemolytic-uremic syndrome. Following ingestion, STEC cells colonize the intestine and produce Shiga toxins (Stx), which appear to translocate across the intestinal epithelium and subsequently reach sensitive endothelial cell beds. STEC cells produce one or both of two major toxins, Stx1 and Stx2. Stx2-producing STEC is more often associated with disease for reasons as yet undetermined. In this study, we used polarized intestinal epithelial cells grown on permeable filters as a model to compare Stx1 and Stx2 movement across the intestinal epithelium. We have previously shown that biologically active Stx1 is able to translocate across cell monolayers in an energy-dependent, saturable manner. This study demonstrates that biologically active Stx2 is also capable of movement across the epithelium without affecting barrier function, but significantly less Stx2 crossed monolayers than Stx1. Chilling the monolayers to 4°C reduced the amount of Stx1 and Stx2 movement by 200-fold and 20-fold respectively. Stx1 movement was clearly directional, favoring an apical-to-basolateral translocation, whereas Stx2 movement was not. Colchicine reduced Stx1, but not Stx2, translocation. Monensin reduced the translocation of both toxins, but the effect was more pronounced with Stx1. Brefeldin A had no effect on either toxin. Excess unlabeled Stx1 blocks the movement of 125I-Stx1. Excess Stx2 failed to have any effect on Stx1 movement. Our data suggests that, despite the many common physical and biochemical properties of the two toxins, they appear to be crossing the epithelial cell barrier by different pathways.

There are several different types of Escherichia coli that cause disease in humans: enteroinvasive, enterohemorrhagic, enterotoxigenic, and enteroinvasive E. coli, as well as Shiga toxin-producing E. coli (STEC). STEC is also known as vero-toxic E. coli or enterohemorrhagic E. coli (24). Of the various types of pathogenic E. coli, STEC causes the greatest degree of morbidity (24, 28). STEC infection has been associated with hemorrhagic colitis (HC), where severe damage to intestinal tissue results in grossly bloody diarrhea, and with hemolytic-uremic syndrome (HUS), characterized by acute renal failure, thrombocytopenia, and hemolytic anemia (13, 28, 30). Most individuals infected with STEC who develop diarrhea and/or bloody diarrhea will recover from the infection without further complication. However, 5 to 10% of patients, primarily children and the elderly, develop potentially life-threatening systemic complications such as HUS or thrombotic thrombocytopenic purpura (13, 18, 28, 30). One of the most important differences between STEC and the other diarrheagenic types of E. coli is the production of cytotoxins known as Shiga toxins (24).

Shiga toxins are generally thought to be responsible for the thrombocytopenic microangiopathy seen in multiple sites, which is the histopathological hallmark of STEC-related disease (28). The precise sequence of events that leads to HC and/or HUS is unclear. STEC cells are usually either ingested from a contaminated food or water source or transmitted from an infected individual. They colonize the colon and produce Shiga toxins in the intestinal lumen. It is thought that Shiga toxins are then able to cross the epithelial barrier and presumably enter the bloodstream, targeting the endothelia of susceptible tissues in multiple sites resulting in intestinal as well as systemic dysfunction (28).

Members of the family of Shiga toxins produced by E. coli are structurally and functionally related to the Shiga toxin from Shigella dysenteriae type 1 (2). There are two major members of the Shiga toxin family, Shiga toxin 1 (Stx1), which is essentially identical to the Shiga toxin produced by Shigella, and Shiga toxin 2 (Stx2), which has only 56% homology with Stx1 and which is immunologically distinct (2). Variants of Stx2 termed Stx2c, Stx2d, and Stx2e are produced by certain STEC strains, but no variants of Stx1 have been described (22). All Shiga toxin family members are made up of one A subunit exhibiting an RNA-glycohydrolase activity and five B subunits responsible for toxin binding (2). Stx1, Stx2, Stx2c, and Stx2d bind to the well-characterized glycolipid receptor Gb3 present on certain eukaryotic cells (2). The toxins are endocytosed and transported in a retrograde manner to the Golgi apparatus, followed by passage into the endoplasmic reticulum (ER). The toxins then gain access to the cytoplasm, where they interact with the ribosomes acting as a glycohydrolase, cleaving a specific adenine, the result being inhibition of protein synthesis (10, 31).

Although Stx1 and Stx2 are structurally and functionally similar, several clinical studies have reported that STEC producing only Stx2 or Stx2 plus Stx1 is more frequently associated with disease than is STEC producing only Stx1 (6, 23, 26, 28, 32, 35). One comprehensive study of an international collection of STEC, representing numerous serotypes, concluded that STEC cells containing the Stx2 gene were five times more likely to be associated with severe disease than STEC cells of the same serotype that did not have the Stx2 gene (6). Exper-
iments with mice and gnotobiotic pigs also suggest that Stx2 may be more pathogenically relevant (11, 34, 37). Stx2 has been shown to be 400-fold more toxic to mice than Stx1 (34). Despite the evidence suggesting that the risk of developing systemic complications is more frequently associated with Stx2-producing STEC infections, very little is known about differences between Stx1 and Stx2 that provides insight into this observed clinical phenomenon. It is speculated that STEC strains may be capable of producing more Stx2 than Stx1 in the gut lumen and that carriage of the Stx2 gene may be accompanied by some yet-undiscovered pathogenic factor that is lacking in strains that produce Stx1 only (28).

Unlike Shigella spp., STEC strains are not invasive and are thought to be restricted to the lumen of the gut, where Shiga toxins are produced, from which they can be recovered in the stools of infected patients (1, 4, 28). Our laboratory is interested in understanding how Shiga toxins penetrate the intestinal epithelial cell barrier and gain access to the underlying tissue. We have previously described a tissue culture model to investigate the movement of Stx1 across polarized intestinal epithelial cells and have shown that biologically active Stx1 was able to cross both T84 and CaCo2A intestinal epithelial cell lines without destroying the cells or the tight junctions (4). Stx1 translocation appeared to be both energy dependent and saturable (4). The aim of the present study was to investigate whether the observed translocation of disease with Stx2-producing strains might be explained by differences in the movement across intestinal epithelial cell monolayers between Stx2 and Stx1 (6, 23, 26). We examined the movement of both toxins under several conditions, including the use of drugs that affect various cellular processes. The results from these studies indicate that there are indeed significant differences in the movement of the two toxins.

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MATERIALS AND METHODS

Tissue culture. Cells used in this study include the human intestinal epithelial cell lines CaCo2A and HCT-8, as well as the African green monkey kidney epithelial Vero cell line. All cell lines were grown at 37°C in 5% CO2 with 100 U of penicillin and streptomycin per ml and 10% heat-inactivated fetal bovine serum (Gibco/BRL). CaCo2A cells were grown in Dulbecco modified Eagle medium (high glucose)-25 mM HEPES; HCT-8 cells were grown in RPMI 1640-10 mM HEPES; Vero cells were grown in modified McCoy's 5a media. Vero and HCT-8 cells were obtained from the American Type Culture Collection (Rockville, Md.), and CaCo2A cells were obtained from the GRASP Tissue Culture Core, New England Medical Center.

Polycarbonate filter inserts (0.33-cm2, 5-μm-pore-size Transwell membranes; Costar, Cambridge, Mass.) were placed in 24-well or 12-well plates and were precoated with rat tail collagen prior to being seeded with either CaCo2A or HCT-8 cells. The cells were fed every 2 days, and experiments were conducted 8 to 10 days postseeding. For inverted monolayers, where cells are grown on the underside of the filter, cells were added to filters placed upside down in a 12-well plate and allowed to attach overnight. The next day, the filter membranes were flipped back upright in a 24-well plate. This serves to reverse the polarity of the cells, with the basolateral surface facing up and the apical surface facing down (27).

Experimental procedures. Purified Stx1 and Stx2 (5) were used at 1 μg/ml unless otherwise indicated. [3H]Hinulin (New England Nuclear, Boston, Mass.) was used at 2.5 μCi/ml. Horseradish peroxidase (HRP; Sigma Chemical Co., St. Louis, Mo.) was used at 500 μg/ml. These molecules were diluted in the appropriate culture medium and added to either the upper chamber (100 μl) or the lower chamber (600 μl) of the Transwell membrane. These volumes were used in accordance with the manufacturer’s instructions to avoid the effects of hydrostatic pressure. Following the addition of the various agents to the upper or lower chamber according to the study protocol, the plates were incubated at 37°C in 5% CO2 for 24 h unless otherwise noted. Samples were then collected from the lower or upper chamber and frozen at −70°C prior to further processing (4).

Electrical resistance measurements across CaCo2A and HCT-8 monolayers grown on filter membranes for 8 to 10 days were taken prior to each experiment with a Millipore ERS resistance reader (Millipore Corporation, Bedford, Mass.) to insure that the monolayers demonstrated intact tight junctions and thus formed a functional barrier (27). After 8 days, measurements were 750 to 950 Ω/cm2 for CaCo2A monolayers and 1,000 to 1,200 Ω/cm2 for HCT-8 cells.

Toxin, [3H]Hinulin, and HRP measurement. Two assay systems were used for the measurement of Stx1 and Stx2. First, biological activity was measured by quantifying the inhibition of incorporation of [3H]leucine into the protein as previously described (8). Bioactivity was converted to amount of toxin by comparison with a control dose-response curve run concurrently. In circumstances where other reagents interfered with the bioassay, Stx1 and Stx2 were quantified as amounts of antigen by a monoclonal antibody capture enzyme-linked immunosorbent assay (ELISA) (3). [3H]Hinulin concentration was determined by adding 10 μl of sample to 4 ml of liquid scintillation fluid and measuring the counts per minute in a scintillation counter. HRP was measured in terms of activity as described by Hecht et al. (14).

The amounts of toxin, [3H]Hinulin, and HRP in the starting material as well as in the medium recovered from the opposite chamber following translocation were measured. Values are reported as the amount of translocated as a percentage of the amount added.

Effect of drugs. All drugs (colchicine, monensin, and brefeldin A) were purchased from Sigma Chemical Co.). To initiate the experiment, medium in both chambers was replaced with medium containing various concentrations of colchicine (10−8 to 10−3 M), monensin (10−8 to 10−3 M), or brefeldin (0.1 to 500 ng/ml) and the chambers were held for 1 h at 37°C in 5% CO2. Then the medium in the bottom chamber was replaced with medium containing various concentrations of drug with Stx1 or Stx2 and [3H]Hinulin. Samples were collected from the bottom chamber at 24 h as described above. The ELISA rather than the bioactivity assay was used to quantify toxin in these experiments because the drugs interfered with the bioactivity assay.

Toxin binding. CaCo2A monolayers on 96-well plates or Transwell membranes were cooled to 4°C and then exposed to 125I-Stx1 or 125I-Stx2 for 1 h followed by washing as described previously (17). 125I-Stx1 and 125I-Stx2 were labeled with Bolton-Hunter reagent in accordance with the manufacturer’s instructions (ICN Pharmaceuticals Inc. Irvine, Calif.). Alternatively, 125I-Stx1 was labeled with chloramine-T (17). Data was subjected to Scatchard analysis to determine the binding affinity and the number of binding sites per cell for each toxin.

Competitive toxin movement experiments. 125I-Stx1 (0.5 μg/ml) was added to the apical chambers of inverted CaCo2A monolayers with a 100-fold excess (50 μg/ml) of, or without, unlabeled Stx1 or Stx2. After 24 h at 37°C, counts per minute in the basolateral chamber were determined with a gamma counter and compared to the toxin, thus facilitating toxin visualization on a gel.

Statistics. Statistics were performed with the Instat statistics program for Macintosh computers. Unpaired Student’s t tests were used to compare differences in sets of data; P < 0.05 was considered significant.

RESULTS

Stx1 and Stx2 translocation. Intestinal cell monolayers demonstrated stable electrical resistance after having grown for 8 to 10 days on collagen-coated filters. To insure that each monolayer remained intact and represented an epithelial barrier during the course of the experiment, the widely used para-cellular marker [3H]Hinulin was added with toxin in each experiment (21). Only a small amount of [3H]Hinulin (3 to 10% over age of the amount added.

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Stx1 and Stx2 at doses of 1 μg/ml added to the apical compartment of intestinal epithelial cell monolayers did not compromise the integrity of monolayers in a 24-h period. Resistance and [3H]Hinulin movement were not significantly altered in the presence of either toxin (data not shown). CaCo2A cells
were also shown to be highly insensitive to both Stx1 and Stx2. Concentrations as high as 10 μg of toxin/ml did not affect protein synthesis of CaCo2A cells (unpublished observations).

A comparison of the movement of Stx1 with that of Stx2 across CaCo2A monolayers showed that there was approximately 10-fold less biologically active Stx2 translocating across CaCo2A cells in a 24-h period than Stx1 (P = 0.008) (Table 1). The same trend was observed in HCT-8 cells, although it was less pronounced and failed to reach statistical significance (P = 0.051) (Table 1). There was no difference in [3H]inulin movement between cells treated with Stx1 and cells treated with Stx2 (Table 1).

To address the question of whether this observed difference in toxin movement resulted from a loss of Stx2 biological activity or an increase in Stx1 biological activity during the translocation process, the amounts of translocated toxin in several samples were measured by both biological activity determinations and ELISA. Table 2 demonstrates that the amount of translocated toxin measured as bioactivity equals the amount measured by ELISA for both Stx1 and Stx2. Thus toxin bioactivity is proportional to the quantity of toxin protein.

In this set of experiments, there was again a significant (P < 0.0001) reduction in Stx2 movement, as measured by ELISA, compared with Stx1 movement (Table 3), with no difference in [3H]inulin movement (Table 3). In addition, we measured the movement of HRP (44 kDa), which more closely approximates the molecular masses of toxin, 72 kDa; inulin, 5 kDa; HRP, 44 kDa.

To determine whether the observed differences in toxin movement are a result of the differences in molecular mass of the toxins, [3H]inulin translocation was not affected by intestinal cell polarity (Table 5). [3H]inulin translocation was not affected by intestinal cell polarity (Table 5). Stx1 showed a preference for apical-to-basolateral movement irrespective of whether Stx1 was moving from the top to the bottom chamber (P = 0.028) or from the bottom to the top chamber (P < 0.0001) (Table 5). Stx2 translocation exhibited a preference for apical-to-basolateral movement when moving from the top to the bottom chamber (P = 0.029); however, there was no significant polarity preference for Stx2 movement from the bottom to the top chamber (P = 0.311) (Table 5).

Binding experiments. Using 125I-labeled Stx1 and Stx2, we performed a Scatchard analysis on the binding of Stx1 and Stx2 to CaCo2A cells seeded on 96-well plates in order to determine whether there were any differences in binding that might explain the observed differences in translocation. Experiments were performed at 4°C to prevent toxin internalization. The

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### TABLE 1. Comparison of Stx1 and Stx2 movement across two different intestinal epithelial lines grown on polycarbonate filters as measured by biological activity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>n</th>
<th>Mean % movement (SD) of:</th>
<th>Stx1</th>
<th>[3H]inulin</th>
<th>Stx2</th>
<th>[3H]inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCo2A</td>
<td>5</td>
<td>5.2 (3.1)a</td>
<td>9.0  (3.2)</td>
<td>0.6 (0.4)b</td>
<td>8.4  (2.9)</td>
<td></td>
</tr>
<tr>
<td>HCT-8</td>
<td>4</td>
<td>1.0 (0.5)b</td>
<td>4.5  (0.3)</td>
<td>0.3 (0.3)b</td>
<td>4.5  (0.4)</td>
<td></td>
</tr>
</tbody>
</table>

a P = 0.008.
b P = 0.051.
c Movement across cells treated with Stx1.d Movement across cells treated with Stx2.

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### TABLE 2. Comparison of biological activity and ELISA measurements of translocated Stx1 and Stx2 across CaCo2A cells

<table>
<thead>
<tr>
<th>Toxin</th>
<th>n</th>
<th>Mean % movement (SD) by:</th>
<th>Bioactivity</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx1</td>
<td>6</td>
<td>2.0 (1.0)a</td>
<td>1.9 (1.0)b</td>
<td></td>
</tr>
<tr>
<td>Stx2</td>
<td>4</td>
<td>0.3 (0.2)c</td>
<td>0.2 (0.01)b</td>
<td>6.3  (4.4)</td>
</tr>
</tbody>
</table>

a P = 0.01.
b P = 0.01.
c Movement across cells treated with toxin indicated in the left column.

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### TABLE 3. Comparison of Stx1 and Stx2 translocation across CaCo2A cells

<table>
<thead>
<tr>
<th>Toxin</th>
<th>n</th>
<th>Mean % movement (SD) of:</th>
<th>[3H]inulin</th>
<th>HRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx1</td>
<td>4</td>
<td>4.2 (1.0)d</td>
<td>6.1 (0.2)</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>Stx2</td>
<td>6</td>
<td>0.1 (0.1)d</td>
<td>6.2 (0.5)</td>
<td>0.08 (0.09)</td>
</tr>
</tbody>
</table>

a P < 0.0001.
d Movement across cells treated with toxin indicated in the left column.

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### TABLE 4. Effect of temperature on the translocation of Stx1, Stx2, and [3H]inulin across CaCo2A monolayers

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>n</th>
<th>Mean % movement (SD) of:</th>
<th>Stx1</th>
<th>[3H]inulin</th>
<th>Stx2</th>
<th>[3H]inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>0.001 (0.002)</td>
<td>1.0  (0.1)</td>
<td>0.0003 (0.0002)b</td>
<td>1.0  (0.1)</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>3</td>
<td>0.2 (0.3)c</td>
<td>2.0  (0.1)</td>
<td>0.006 (0.003)b</td>
<td>2.1  (0.4)</td>
<td></td>
</tr>
</tbody>
</table>

a P = 0.0014.
b P = 0.019.
c Movement across cells treated with Stx1.d Movement across cells treated with Stx2.
association constant ($K_a$) of Stx1 binding was $2.04 \times 10^9 \pm 1.02 \times 10^9 \text{ M}^{-1}$, whereas the $K_a$ for Stx2 binding was $0.48 \times 10^9 \pm 0.13 \times 10^9 \text{ M}^{-1}$. This represents a significant fourfold difference ($P = 0.008$). The numbers of toxin binding sites per cell were similar for the two toxins ($6.68 \times 10^8 \pm 1.87 \times 10^8 \text{ M}^{-1}$ for Stx1 and $5.26 \times 10^8 \pm 1.64 \times 10^8 \text{ M}^{-1}$ for Stx2) ($P = 0.548$).

Given that these cells are grown on a solid surface rather than a Transwell filter, we cannot confirm that the monolayer represents a polarized barrier by measuring electrical resistance. Therefore, we wanted to confirm that the fourfold difference in the binding of Stx1 versus that of Stx2 observed in cells grown on 96-well plates was consistent with cells grown on Transwell filters, where resistance can be tested. We chose a single concentration of $125\text{I}$-Stx1 and $125\text{I}$-Stx2 and corrected for specific activity. The counts associated with the monolayers were $3,905 (\pm 3,568)$ for Stx1 and $5,260 (\pm 3,290)$ for Stx2 ($P = 0.03$ ($P = 0.06$) for Stx1 and $P = 0.008$ ($P = 0.006$) for Stx2) ($P = 0.548$).

### Effect of a high concentration of Stx2 on Stx1 movement.

Based on the above data, we speculated that Stx1 and Stx2 use separate pathways across intestinal epithelial monolayers. Previous data showed that the translocation of Stx1 across the CaCo2A monolayer was a saturable process; as the amount of Stx1 added to the apical surface increased, the overall percentage of translocated Stx1 decreased (4). If Stx1 and Stx2 were following different pathways, we hypothesized that excess Stx2 would not reduce the amount of Stx1 translocation. As shown in Table 6, increasing amounts of Stx2 added to the apical surface (up to 1,000-fold in excess of Stx1) had no effect on

### Table 5. Directional movement of Stx1, Stx2, and [3H]inulin

<table>
<thead>
<tr>
<th>Direction of movement</th>
<th>Mean % movement (SD) of indicated molecule added froma:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stx1-treated cellsb</td>
</tr>
<tr>
<td>Top chamberb</td>
<td>Sx1</td>
</tr>
<tr>
<td>Stx1</td>
<td>4.3 (5.2)</td>
</tr>
<tr>
<td>[3H]inulin</td>
<td>0.4 (0.4)</td>
</tr>
<tr>
<td>Bottom chamberb</td>
<td>Stx1</td>
</tr>
<tr>
<td>Stx1</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>[3H]inulin</td>
<td>0.00 (0.001)</td>
</tr>
</tbody>
</table>

a [3H]inulin and toxin concentrations were kept constant when added to either the upper (100 μl) or lower chamber (600 μl), and values are percentages of the amount of starting material.

b For measurement of apical-to-basolateral movement, cells were grown on the upright sides of permeable filters, with toxin and [3H]inulin added to the bottom chamber (basolateral) and recovered from the top chamber (apical). For measurement of basolateral-to-apical movement, cells were grown on the undersides of permeable filters, with toxin and [3H]inulin added to the bottom chamber (basolateral) and recovered from the top chamber (apical).

c Statistical significance is indicated by $P$ values or NS (not significant) following the slashes in the basolateral-to-apical row of values.

### Table 6. Effect of increasing doses of Stx2 on Stx1 translocation across CaCo2A monolayers

<table>
<thead>
<tr>
<th>Molecule (amt [ng]) added to top (apical) chamber</th>
<th>Mean % movement (SD) of:</th>
<th>Stx1</th>
<th>[3H]inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1 (1.4)</td>
<td>3.3 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Stx2 (100)</td>
<td>2.5 (2.0)</td>
<td>3.3 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Stx2 (1,000)</td>
<td>2.6 (1.7)</td>
<td>3.3 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Stx2 (10,000)</td>
<td>2.7 (0.9)</td>
<td>3.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>HRP (10,000)</td>
<td>2.3 (0.9)</td>
<td>3.4 (0.2)</td>
<td></td>
</tr>
</tbody>
</table>

a Stx1 (10 ng/well) and [3H]inulin were added to the upper chambers with increasing doses of Stx2 or HRP. Samples were collected from the bottom, and Stx1 was measured by ELISA using the Stx1-specific monoclonal antibody (4D3), n = 4 for each condition.
on collagen-coated filters at which point they developed electrical resistance levels of 750 to 900 kΩ/cm². Colchicine, which interferes with microtubule formation, was used at doses ranging from 10⁻⁸ to 10⁻² M. The CaCo2A monolayers were pretreated with various doses of colchicine for 1 to 2 h at 37°C. Stx1 or Stx2 and [³H]Inulin were then added, and samples were taken from the basolateral chamber after 24 h as in previous experiments. There was no significant change in the movement of either [³H]Inulin or Stx1 at colchicine doses less than 10⁻⁷ M. At 10⁻⁶ M colchicine, the amount of Stx1 crossing the monolayer was 67.5% (+/−14.1%) of that crossing untreated control monolayers (Fig. 2A). There was no effect on [³H]Inulin movement at this colchicine dose. As the concentration of colchicine was further increased, the amount of Stx1 across the monolayer decreased. At 10⁻⁵ to 10⁻³ M colchicine there was an increase in [³H]Inulin movement, indicating that the monolayers became leaky at these higher doses, which suggests the loosening of tight junctions or damage to cells (20).

Deomonolayers became leaky at these higher doses, which suggests that did not affect [³H]Inulin movement (Fig. 2B). As both greater than that in the untreated control at doses of colchicine of Stx2 that crossed the CaCo2A monolayer increased and was than the amount translocating in the control.

The effect of colchicine on Stx2 movement was completely different. As the dose of colchicine was increased, the amount of Stx2 that crossed the CaCo2A monolayer increased and was greater than that in the untreated control at doses of colchicine that did not affect [³H]Inulin movement (Fig. 2B). As both toxins were quantified by the ELISA method, we ran control ELISAs in the presence of colchicine to determine whether colchicine had any effect on the assay and found none (data not shown).

Translocation of Stx1, Stx2, and [³H]Inulin in CaCo2A cells treated with monensin was also measured. This drug, which interferes with receptor-mediated endocytosis, was used in concentrations from 10⁻⁶ to 10⁻⁴ M. As the dose was increased, the amount of Stx1 translocation increased across in a dose-dependent fashion with a maximum increase at 10⁻⁴ M monensin of 55.3% (+/−8.9%) of the untreated control. This dose had no effect on [³H]Inulin movement (Fig. 2C). At higher doses of monensin, both toxins and [³H]Inulin began to leak across the monolayer (Fig. 2C). Monensin had similar effects on Stx2, although less pronounced (Fig. 2D). Stx2 movement decreased with increasing concentrations of monensin, reaching the maximal decrease at 10⁻³ M monensin of 65.2% (+/−12.8%) of control. As monensin was further increased, the amounts of [³H]Inulin and Stx2 translocation increased (Fig. 2D).

Brefeldin A, which disrupts the Golgi apparatus, had no apparent effect on Stx1 or Stx2 translocation until doses were high enough to cause [³H]Inulin leaks (Fig. 2E and F). At this point the amounts of both Stx1 and Stx2 movement increased over that of the untreated controls (Fig. 2E and F). When Stx1 and Stx2 ELISAs were performed in the presence and absence of monensin and brefeldin A, no effect on the ELISA results was found.

**DISCUSSION**

Shiga toxins are generally thought to be responsible for the development of both HC and the life-threatening condition HUS, which occur in patients infected with STEC (28). Strong evidence suggests that these complications arise as a result of Shiga toxins directly targeting microvascular endothelial cells in the kidney and other sites (25). Human microvascular endothelial cells may be exquisitely sensitive to Shiga toxin (25). Since STEC infection is localized to the luminal side of the epithelial cell barrier in the intestine, it is important to understand how Shiga toxins gain access to the endothelial cell beds of the colon, kidney, and elsewhere.

An in vitro model developed to address this question demonstrated that high doses of Stx1 are not cytotoxic to the intestinal epithelial cell lines CaCo2A and T84 (4). Stx1 has no effect on barrier function; however significant amounts of biologically active Stx1 are capable of crossing the intact epithelial monolayer (4). Stx1 translocation is drastically reduced both when cells are chilled to 4°C and when cells are treated with the metabolic inhibitor 2,4-dinitrophenol, indicating that Stx1 translocation is an energy-requiring process (4). Since the epithelial monolayers maintain high resistance in the presence of Stx1 and since Stx1 movement across the monolayer is an active process, we hypothesized that Stx1 translocates across the CaCo2A monolayer via a transcellular rather than a paracellular route (4). This idea is supported by the study of Philpott et al. (29). Using T84 cells as a model, they proposed that Stx1 translocation was transcellular and demonstrated immunogold localization of Stx1 within endosomes and exclusion from the paracellular space (29).

The most striking observation in the present work is that the translocation of Stx2 across polarized intestinal epithelia in vitro differs significantly from that of Stx1. While neither toxin affects the barrier function of CaCo2A cells, we consistently observed in numerous experiments that less Stx2 than Stx1 translocates across CaCo2A cells in a 24-h period. Data from experiments in which levels of translocation of both Stx1 and Stx2 in the same set of CaCo2A monolayers were compared showed that there was, on average, a 40-fold difference in movement. Both toxins retain biological activity after translocation. A comparison of biological activity with antigen amount showed that both Stx1 and Stx2 were neither activated nor inactivated. Temperature had less of an inhibiting effect on Stx2 movement than it had on Stx1 movement. Stx1 movement was clearly directional, with a preference for apical-to-basolateral translocation, whereas the directional preference of Stx2 movement was not clear. Stx2 appeared to also prefer apical-to-basolateral translocation, but this preference was not maintained when Stx2 moved across the monolayer against gravity. The reasons for this difference are unknown. Using iodinated toxins we found that Stx1 had a binding affinity to the apical surface of CaCo2A higher than that of Stx2; however the difference was only fourfold, insufficient to explain the difference in toxin movement.

We hypothesize that while both toxins are capable of crossing the intestinal epithelial monolayer, they do so via different pathways. This model was supported by our competition experiments in which a 100-fold excess of Stx1 blocked the translocation of 125I-Stx1, whereas a 100-fold excess of Stx2 had no blocking effect. As much as a 1,000-fold excess of Stx2 had no effect on Stx1 translocation.

Several intracellular vesicle pathways rely upon microtubule assembly and disassembly for the shuttling of vesicles throughout the cell (5, 7, 36). Transcytosis of proteins within vesicles from the apical to the basolateral surface or from the basolateral to the apical surface is thought to be microtubule dependent. Agents that disrupt microtubule function can cause a significant decrease in the amount of protein that is transcytosed (5, 7). Colchicine binds to tubulin dimers and inhibits polymerization, preventing microtubule assembly (20). Our study demonstrates that high doses of colchicine reduce the amount of Stx1 translocation across CaCo2A cells in a 24-h period by about 60 to 70%. This reduction in Stx1 movement was maintained even at doses of colchicine that caused leakage of [³H]Inulin. [³H]Inulin (5 kDa) is a much smaller molecule than Stx1 (72 kDa); thus small breaches in tight-junction in-
FIG. 2. Effects of increasing doses of colchicine, monensin, and brefeldin A on the translocation of [3H]inulin (solid circles) and toxin (open circles) across CaCo2A monolayers. The x axis represents the dose of drug added to the cell monolayer, with control representing the absence of drug. The percent translocation of [3H]inulin is shown on the left y axis. The control value for the translocation of toxin was set at 100%, and values within the same experiment are reported as percentages of the control value (shown on the right y axis). Each data point represents the mean (±SD) of pooled data (n = 2 to 9) from multiple experiments.
tegrity may result in differential molecular movement across paracellular space depending on the size of the molecule. We interpret the large decrease in Stx1 movement when cells are exposed to colchicine as further evidence for the transcellular movement of Stx1. In contrast, colchicine did not inhibit Stx2 movement. In fact, as colchicine concentrations increased, Stx2 movement also increased at doses that did not compromise the monolayer. This further implies that the toxins are moving across by different pathways, suggesting a microtubule-dependent vesicle pathway for Stx1 translocation and a microtubule-independent pathway for Stx2.

Monensin is a ionophore that interferes with vesicular Na+/H+ exchange. At low doses (10⁻⁷ to 10⁻⁵ M), it is reported to prevent the acidification of intracellular compartments, including ER, Golgi apparatus, and endosomes (33). Monensin is capable of disrupting transcytosis in epithelial cells (7, 15); more specifically, it is thought to inhibit receptor-mediated endocytosis because the rise in endosomal pH prevents receptor-ligand disassociation (7, 33). Monensin inhibits movement of Stx1 by 40 to 50% at 10⁻⁶ M, further suggesting a transcellular route for Stx1 translocation and also indicating that receptor-mediated endocytosis may be involved. Monensin resulted in a decrease in Stx2 movement, but one not as large as the decrease in Stx1 movement seen.

Brefeldin A disrupts Golgi stacks by interfering with vesicle trafficking. It is not clear what the role of Gb3 is in this pathway, even though it is reported to prevent the acidification of intracellular compartments, including ER, Golgi apparatus, and endosomes (33). Monensin inhibits movement of Stx1 by 40 to 50% at 10⁻⁶ M, further suggesting a transcellular route for Stx1 translocation and also indicating that receptor-mediated endocytosis may be involved. Monensin resulted in a decrease in Stx2 movement, but one not as large as the decrease in Stx1 movement seen.

From this work it remains unclear whether transcytosing Stx1 and Stx2 bind to an apical receptor and enter the cell via receptor-mediated endocytosis or via nonspecific fluid phase endocytosis. However, the monensin data suggests that receptor-mediated endocytosis may be more involved in Stx1 translocation than in Stx2 translocation. Monensin does not inhibit the movement of HRP, which has been reported to transcytose by a fluid phase nonreceptor mechanism (15). The only well-established receptor for Stx1 and Stx2 is Gb3, which is involved in the delivery of Shiga toxins to the cytoplasm of cells for intoxication. Gb3 is expressed at significantly lower levels on CaCo2A cells than on cells that are sensitive to Shiga toxins such as Vero cells (4, 17). Sodium butyrate increases Gb3 receptor expression and sensitivity to Stx1 in CaCo2A cells. When numbers of receptors are increased by this mechanism, Stx1 translocation is significantly decreased (4). Although the evidence suggests that Stx1 appears to be internalized via receptor-mediated endocytosis and translocates via transcytotic vesicles, it is not clear what the role of Gb3 is in this pathway, if any.

Transcytosis of numerous proteins across various epithelia have been described previously (5, 7, 15, 36, 38); these include cholera toxin, a bacterial toxin produced in the intestines of individuals infected with *Vibrio cholerae* (19). While Stx1 and, to a lesser extent, Stx2 can move across epithelial layers in vitro, the significance of Shiga toxin translocation in vivo is unknown. Given the apparent greater pathogenicity of Stx2-producing STEC in clinical situations, it was surprising to discover that Stx2 crossed human intestinal epithelial cells significantly less efficiently than did Stx1. However, it has been reported that intestinal endothelial cells are 10-fold more sensitive to Stx2 than to Stx1 and that glomerular endothelial cells are 1,000-fold more sensitive to Stx2 (16, 25). If these various in vitro observations represent the in vivo situation, the increase in sensitivity of the endothelium to Stx2 over Stx1 may compensate for the diminished Stx2 translocation. We acknowledge that the situation in the human intestine is far more complex, involving many different cell types, including both intestinal and inflammatory cells. Thus a better understanding of how toxins interact with the epithelium in vivo will require further study. Developing a further understanding of how these very similar toxins differ from one another will help explain the epidemiological association of Stx2 in human STEC disease. It will also facilitate the development of therapies that are geared toward preventing toxins from crossing the epithelium at the early stages of the disease, which may aid in preventing systemic sequelae.

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**REFERENCES**


TRANSLLOCATION OF Stx1 AND Stx2 ACROSS EPITHELIUM


