Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium

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1Research Institute, The Hospital for Sick Children, Toronto M5G 1X8; Departments of 2Molecular and Medical Genetics and 3Pediatrics, University of Toronto, Toronto M5S 1A8; and 4The Intestinal Diseases Research Programme, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Philpott, Dana J., Cameron A. Ackerley, Amanda J. Kiliaan, Mohamed A. Karmali, Mary H. Perdue, and Philip M. Sherman. Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1349–G1358, 1997.—Verotoxin-producing Escherichia coli (VTEC) are pathogenic bacteria associated with diarrheal, hemorrhagic colitis, and hemolytic uremic syndrome. Verotoxins (VTs) elaborated by these organisms produce cytopathic effects on a restricted number of cell types, including endothelial cells lining the microvasculature of the bowel and the kidney. Because human intestinal epithelial cells lack the globotriaosylceramide receptor for VT binding, it is unclear how the toxin moves across the intestinal mucosa to the systemic circulation. The aims of this study were to determine the effects of VT-1 on intestinal epithelial cell function and to characterize VT-1 translocation across monolayers of T84 cells, an intestinal epithelial cell line. VT-1 at concentrations up to 1 µg/ml had no effect on the barrier function of T84 monolayers as assessed by measuring transmonolayer electrical resistance (102 ± 8% of control monolayers). In contrast, both VT-positive and VT-negative VTEC bacterial strains lowered T84 transmonolayer resistance (45 ± 7 and 38 ± 6% of controls, respectively). Comparable amounts of toxin moved across monolayers of T84 cells, exhibiting high-resistance values, as monolayers with VTEC-induced decreases in barrier function, suggesting a transcellular mode of transport. Translocation of VT-1 across T84 monolayers paralleled the movement of a comparably sized protein, horseradish peroxidase. Immunoelectron microscopy confirmed transcellular transport of VT-1, since the toxin was observed within endosomes and associated with specific intracellular targets, including the Golgi network and endoplasmic reticulum. These data present a model of VT-1 uptake by toxin-insensitive cells and suggest a general mechanism by which bacterial toxins lacking specific intestinal receptors can penetrate the intestinal epithelial barrier.

Escherichia coli; Shiga toxin; T84 intestinal epithelial cells

VEROTOXIN-PRODUCING Escherichia coli (VTEC) infection of humans is associated with diarrhea and can lead to systemic complications including hemorrhagic colitis and hemolytic ureemic syndrome (21). These pathogens produce verotoxins (VTs; also referred to as Shiga toxins), which are cytopathic to sensitive cells. At least two bacteriophage-encoded VTs, VT-1 and VT-2, have been identified in VTEC strains associated with human disease (20, 21). VT-1 is essentially identical to Shiga toxin from Shigella dysenteriae type 1, differing only in a single amino acid (8, 35). VTs are subunit toxins comprised of an A (active) subunit and five B (binding) subunits (21). The A subunits of VT-1 and VT-2 bind specifically to the glycolipid, globotriaosylceramide (Gb3), on target cells (30). VT-1 is internalized via receptor-mediated endocytosis, and the A subunit inhibits protein synthesis in the eukaryotic cell by inactivating the 60S ribosomal subunit (42).

VTs do not appear to be involved in the induction of diarrheal illness after VTEC infection. Toxin-negative variants of VTEC still cause diarrhea in animal models (26, 49). The role of VTs in VTEC-associated human diseases may therefore be limited to vascular complications, such as those relating to hemorrhagic colitis and hemolytic uremic syndrome (13, 40). The lack of Gb3 expression in human intestine (4, 18, 24) is consistent with the resistance of human intestinal epithelium to the effects of the toxin. In contrast, the age-related expression of Gb3 in renal glomeruli is in keeping with the glomerular endothelial damage seen in hemolytic uremic syndrome (27, 36).

The mechanisms by which VTs cross the intestinal mucosal barrier to enter the systemic circulation remain to be elucidated (28). We recently demonstrated that VTEC infection decreases monolayer resistance and increases paracellular permeability in a model intestinal cell line, T84, that lacks Gb3 (38). In the present study, we sought to determine whether VTEC-induced decreases in transmonolayer resistance could provide a possible mechanism for VTs crossing the intestinal epithelial barrier.

This study demonstrates that purified VT-1 does not alter T84 epithelial function as assessed by examining changes in transepithelial resistance, a measure of the barrier function of epithelia. In addition, a VT-negative variant of VTEC, strain 85–170 (49), decreased T84 monolayer resistance despite the lack of cytotoxin production. Using a number of complimentary techniques, we show that VT-1 translocates across T84 cell monolayers of high electrical resistance. In monolayers with VTEC-induced decreases in resistance, VT-1 moved across the epithelial barrier to the same extent as in uninfected T84 cells.

A comparison of VT-1 translocation with horseradish peroxidase (HRP) showed equivalent transmonolayer movement of these two large proteins, indicating nonspecific and probable pinocytotic uptake in T84 cells. Transmission electron microscopy (TEM) revealed HRP within endosomes in T84 cells. By immunoelectron microscopy, VT-1 was also found within endosomes of toxin-treated T84 cells. In contrast to the intracellular localization of HRP, VT-1 was associated with specific intracellular targets, including the Golgi network, endoplasmic reticulum, and nuclear membrane. These find-
ings present a mechanism for VT-1 penetration of the intestinal epithelial barrier and may impact on the general mode of bacterial toxin translocation across the human intestine.

**MATERIALS AND METHODS**

**Bacterial Strains and Toxin**

The bacterial strains used in this study are listed in Table 1. Organisms were grown for 18 h in static, nonaerated cultures of Penassay broth (DIFCO Laboratories, Detroit, MI) at 37°C. Bacteria were centrifuged at 2,500 g for 15 min, and the pelleted organisms were resuspended in sterile phosphate-buffered saline (PBS, pH 7.4) to a concentration of 1 × 10^8 colony-forming units (CFU)/ml. Viable counts of bacteria were obtained by serial, 10-fold dilutions that were plated onto MacConkey agar plates. VT-1 was purified by methods described previously (37).

**Cell Culture**

T84 epithelial cells were grown on tissue culture-treated Transwell filter support units (0.4-µm pore size; Costar, Cambridge, MA). The culture medium employed was a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Canserna International, Rexdale, ON, Canada) and 2% (vol/vol) penicillin-streptomycin (GIBCO) and grown at 37°C in 5% CO₂ (11). Antibiotic-free medium contained all the ingredients listed above except for penicillin-streptomycin. The monolayers were grown for 7 days postseeding to attain polarized epithelial cells with transepithelial resistance measurements of >1,000 Ω-cm².

Vero cells were grown in flasks and 96-well plates with 2% (vol/vol) penicillin-streptomycin and 5% FCS. HEp-2 epithelial cells (ATCC, Rockville, MD) were grown in flasks and cultured in MEM supplemented with 2% (vol/vol) penicillin-streptomycin with 15% FCS.

**Thin-Layer Chromatography and VT-1 Overlay Binding Assay**

Lipids were extracted from ∼10⁸ T84 cells and 2 × 10⁷ HEp-2 cells with chloroform-methanol (2:1 vol/vol) by the method of Folch et al. (12). Extracted lipid fractions and glycolipid standards (Matreya, Pleasant Gap, PA) were then applied to thin-layer chromatography (TLC) plates (Polygram Sil G, Macherly-Nagel, Germany) and separated in chloroform-methanol-water (60:40:9) (29, 47). The TLC plates were then dried, and lipid separation was monitored with iodine for lipids and orcinol spray (0.5% in 3MH₂SO₄) for glycolipids (32).

For the VT-1 overlay binding assay (30), residual binding sites on the TLC plates were blocked with 1% gelatin in PBS overnight at 37°C. After three washes in tris(hydroxymethyl)-aminomethane (Tris)-buffered saline (TBS, pH 7.4), the plates were incubated with 1 µg of VT-1 in TBS for 1 h at room temperature with shaking. The plates were washed three times to remove unbound toxin and then treated with Ph1, a murine monoclonal antibody to VT-1 (6), for 1 h at room temperature. The plates were washed and the secondary antibody conjugated to HRP was added for 1 h at room temperature. Bound peroxidase was then visualized with 4-chloro-1-naphthol substrate (30).

**Measurements of T84 Transepithelial Resistance**

Tissue culture medium was changed to antibiotic-free medium before bacterial infection. Bacterial strains were then introduced into the apical compartment of the Transwell unit at a concentration of 5 × 10⁷ CFU/ml; this inoculum of bacteria was shown in our previous studies to result in maximal decreases in monolayer resistance (39). After incubation for 15 h 37°C, T84 monolayers were mounted in modified Ussing chambers and bathed in oxygenated Krebs buffer (pH 7.3, at 37°C). Krebs buffer bathing the serosal surface contained 115 mM NaCl, 8 mM KCl, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 2.0 mM KH₂PO₄, 25 mM NaHCO₃, and 10 mM glucose. In the luminal compartment of the Ussing chamber, 10 mM mannitol was substituted for glucose (23). Resistance values were determined from the potential difference and the imposed current by applying Ohm's law. T84 monolayer resistance was also monitored using a Millicell-ERS system (Millipore, Bedford, MA). Transepithelial resistance provides an indication of the barrier properties of the T84 monolayer to the paracellular movement of ions and molecules (41).

**T84 Cell Viability**

To assess the impact of VT-1 treatment on T84 cell viability, lactate dehydrogenase (LDH) release assays were performed, as described previously (15, 39). After incubation for 24 h, the medium from the T84 cell monolayers was collected and analyzed for LDH activity (39). Total intracellular LDH concentration was determined by adding 1.5 ml of 0.1% (vol/vol) Triton X-100 in PBS to each filter for 25 min and vigorously pipetting the contents of each well to ensure complete lysis of cells. The homogenate was then analyzed for LDH activity. Results are expressed as the amount of LDH released into the medium as a percentage of total cellular LDH activity.

**Transmonolayer Movement of VT-1 and HRP**

The movement of VT-1 and HRP across uninfected and VTEC-infected T84 monolayers was assessed by two complementary methods, described below.

**Method 1. Movement of intact VT-1 and HRP across T84 cell monolayers.** In separate experiments, VT-1 and HRP movement across both uninfected and VTEC-infected T84 monolayers by a VT-1 bioassay. After 3–15 h of incubation with the toxin (0.20–1.0 µg), basal medium was sampled and tested in a Vero cell cytotoxicity assay to detect cytotoxic activity, as described previously (22). Briefly, doubling dilutions of basal media to be tested were prepared and added to monolayers of Vero cells grown in 96-well microtiter plates and incubated at 37°C and 5% CO₂ for 3 days. The end point was determined as the titer of VT-1-producing cytotoxic effects in 50% of cells in the monolayer as visualized by bright-field microscopic examination of crystal violet-stained cells (22).

**Table 1. Strains of Escherichia coli employed in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Toxin</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL56</td>
<td>O157:H7</td>
<td>VT-1, VT-2</td>
<td>VTEC</td>
<td>HC, HUS Toronto (45)</td>
</tr>
<tr>
<td>84-289</td>
<td>O157:H7</td>
<td>VT-1, VT-2</td>
<td>VTEC</td>
<td>HUS Ottawa (49)</td>
</tr>
<tr>
<td>85-170</td>
<td>O157:H7</td>
<td>Negative</td>
<td>VTEC</td>
<td>Laboratory variant of 84-289 (49)</td>
</tr>
</tbody>
</table>

HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; VT, verotoxin; VTEC, verotoxin-producing E. coli.
In a subset of studies, 0.5–1 µg of VT-1 was added to the apical compartment of uninfected and 15 h VTEC-infected monolayers and then incubated for 2 h either at 37°C or 4°C. T84 monolayers to be incubated with toxin at 4°C were precocultured for 1 h. Basal medium was then collected and assayed as described above.

HRP movement across uninfected and VTEC-infected monolayers was also examined, since this molecule is frequently used as a marker to assess fluid-phase uptake in both cultured cells (7, 17) and intestinal tissues (16). HRP (0.5 mg/ml, type VI, Sigma, St. Louis, MO) was added to the apical compartment of uninfected T84 monolayers and T84 monolayers infected with VTEC for 15 h, and the basal medium was then sampled after 2 h of incubation. The amount of HRP translocating the monolayer was determined by the Worthington method (51). Briefly, 0.15 ml of sample was added to 0.8 ml of reaction buffer (0.009% o-phenididine in 0.1 M phosphate buffer with 0.003% H2O2). The rate of increase in optical density at 460 nm was determined over 2–5 min to generate linear correlations between enzyme activity and enzyme concentration.

Method 2. Movement of VT-1 and HRP across T84 monolayers in Ussing chambers. The movement of VT-1 and HRP across T84 monolayers was also assessed in Ussing chambers by measuring unidirectional mucosal-to-serosal movement of the two molecules. Uninfected T84 cell monolayers and monolayers infected with VTEC strains for 15 h were mounted into Ussing chambers, and experiments were performed under short-circuited conditions. VT-1 and HRP were added to the mucosal reservoir (final concn of 0.05 µg/ml and 10 µg/ml, respectively), and after a 30-min equilibration period, samples were taken from the serosal compartment for three 30-min periods. Samples were subjected to either VT-1 bioassay or Worthington HRP assay, as described above, to determine the amount of VT-1 and HRP, respectively, present in the serosal chamber.

Electron Microscopy

To examine the intracellular localization of HRP, T84 monolayers treated with 10–3 M HRP were subjected to TEM. Briefly, T84 monolayers were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (vol/vol, pH 7.4) and then processed for 3,3'-diaminobenzidine tetrahydrochloride (DAB) cytochemistry using 0.5 mg/ml DAB (Sigma) and 0.01% H2O2 (vol/vol) in Tris buffer (3). After washes in TBS, samples were dehydrated through a series of graded acetone washes and embedded in Epon, and ultrathin sections were placed onto 300-mesh copper grids (46). Grids were then counterstained with uranyl acetate and lead salts, as described previously (46).

For intracellular visualization of VT-1 by immunoelectron microscopy, VT-1-treated (0.5 µg/ml for 18 h) and untreated T84 cells were fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h and then rinsed thoroughly in phosphate buffer. Samples were then infused with sucrose for 3 h. Specimens were frozen in liquid N2 for 72 h, warmed to −20°C, and infiltrated with Lowicryl HM20 (B E Electron Microscopy Services, Dorval, PQ, Canada). After a change in Lowicryl, samples were embedded in gelatin capsules and cold polymerized under an ultraviolet lamp. Ultrathin sections were then cut and mounted on Formvar-coated nickel grids. The sections were incubated with a 1:100 dilution of Ph1 antibody to VT-1 for 1 h at room temperature. Sections were rinsed thoroughly with PBS and incubated for 1 h with goat anti-mouse immunoglobulin G conjugated to 10 nm gold particles. Grids were then stained with aqueous uranyl acetate and lead citrate before examination by TEM.

Statistical Analyses

Results are presented as means ± SE. VT-1 titers represent the reciprocal of the sample dilution resulting in 50% Vero cell monolayer death and are expressed as a geometric mean titer (GMT; Ref. 22) of at least three separate samples. To test statistical significance among multiple groups, a one-way analysis of variance was used followed by post hoc comparisons with the Newman-Keuls test. The Student's t-test was used where indicated.

RESULTS

T84 Cells Lack Gb3 Receptor for VT-1

To determine whether T84 cells possess the Gb3 receptor for VT-1, extracted lipids from T84 cells were separated by TLC and overlaid with VT-1. Bound toxin was detected with an anti-VT-1 antibody followed by HRP-conjugated secondary antibodies. As shown in Fig. 1A, VT-1 bound to the Gb3 standard as well as to Gb3 in a glycolipid extract of Hep-2 cells employed as a positive control (48). VT-1 binding was not detected in the lipid extract of T84 cells (arrow). In addition, TLC plates with T84-separated lipids were stained with orcinol to visualize glycolipids. Glycolipids in the region of the Gb3 standard were not present in the T84 lipid extracts (Fig. 1B).

Alterations in T84 Barrier Function by VTEC Are Independent of Toxin

We have shown previously that infection of T84 cells with VTEC, strain CL56, results in a time-dependent decrease in transepithelial resistance (38). In the present study, T84 cell monolayers were incubated for 24 h with concentrations of VT-1 up to 1 µg. As shown in Fig. 2A, no changes in baseline resistance values were observed at any concentration of VT-1 employed. In addition, spontaneous LDH release, expressed as a percentage of the total cellular LDH content, did not differ in VT-1 treated (4.7 ± 0.6%) compared with uninfected T84 cells (6.0 ± 0.8%). These findings confirm that VT-1 had no effect on T84 cell viability after 18 h of treatment.

To confirm the lack of an effect of VT-1 on T84 resistance responses, barrier function of T84 monolayers was examined after infection with VTEC, strain 84–289, and the toxin-negative variant of VTEC, strain 85–170. A decrease in monolayer resistance was measured after infection with both of the strains compared with uninfected T84 cells (6.0 ± 0.8%). These findings confirm that VT-1 had no effect on T84 cell viability after 18 h of treatment.

VT-1 also did not alter baseline secretion of T84 cells as assessed by measuring short-circuit current (Isc) in Ussing chambers. T84 monolayers treated with 1 µg/ml of VT-1 for 15 h at 37°C demonstrated similar baseline Isc compared with untreated cells (Isc of VT-1 treated cells was 97 ± 6% of controls, n = 6).
Our previous studies have shown that pathogenic E. coli decrease T84 cell monolayer resistance by altering ZO-1 protein in the tight junction complex (38, 39). We therefore hypothesized that VT-1 could move across the epithelial barrier by passing paracellularly through VTEC-damaged tight junctions. To test this theory, VT-1 (0.2 µg) was added to the apical compartment of uninfected T84 cells and to cells infected simultaneously with the VT-negative VTEC, strain 85–170. Both epithelial monolayer resistance and VT-1 titer in the basal compartment were monitored over 15 h. Resistance of 85–170-infected T84 cells was decreased at 12 h postinfection (1,350 ± 297 Ω·cm²) compared with uninfected cells (2,509 ± 403 Ω·cm², P < 0.05) and reached a maximal decrease after 15 h of infection (45 ± 21% of uninfected values, P < 0.05; Fig. 3). Biologically active VT-1 was first detected in the basal compartment of both uninfected and VTEC-infected T84 monolayers at 6 h and reached a maximum at 12 h (Fig. 3). Fifteen hours postinfection, VT-1 titers reached 8 GMT for both uninfected and 85–170-infected cells. This amount of toxin translocating across T84 monolayers represents ~64 pg or 0.04% of apically added VT-1 based on an 8 pg CD50 for Vero cells in the cytotoxicity assay (22). These findings indicate that comparable amounts of VT-1 moved across both uninfected and VTEC-infected T84 monolayers despite the measurable differences in monolayer resistance.

Fig. 1. A: verotoxin-1 (VT-1) overlay binding assay onto lipids separated by thin-layer chromatography (TLC). Lanes 1–4 contain the following lipid standards: 1, phosphatidylethanolamine; 2, globotriaosylceramide (Gb3); 3, gangliotriaosylceramide + globotetraosylceramide; 4, galactosylceramide. Lanes 5–8 contain lipid extracts from HEp-2 and T84 cells: 5, HEp-2 glycolipid fraction; 6, HEp-2 phospholipid extract; 7, T84 glycolipid fraction; 8, T84 phospholipid extract. VT-1 bound to Gb3 standard (lane 2) and Gb3 present in glycolipid fraction of HEp-2 cells (lane 5). No binding was detected in glycolipid fraction of T84 cells (arrow). B: orcinol-stained lipids from TLC-separated lipid standards and T84-extracted lipids. Lanes 1–9: 1, gangliotriaosylceramide; 2, gangliotetraosylceramide; 3, globo series lipid mixture containing (from top to bottom) galactosylceramide, Gb3, and globotetraosylceramide; 4, phosphatidylethanolamine; 5, T84 cholesterol extract; 6, T84 glycolipid fraction; 7, T84 phospholipid fraction; 8, Folch upper fraction; 9, galactosylceramide + ganglioside. Arrows indicate Gb3 in globo series lipid mixture (lane 3) and lack of corresponding band in T84 glycolipid fraction (lane 6).

Fig. 2. T84 transepithelial resistance responses to VT-1 and VT-producing Escherichia coli (VTEC) infection. A: increasing concentrations of VT-1 incubated with cells for 24 h had no effect on monolayer resistance, an indicator of barrier function of monolayer (n = 3). B: uninfected and VT-1-treated (1 µg/ml) T84 cells displayed similar monolayer resistance. T84 cells infected for 15 h with VT-1- and VT-2-positive VTEC, strain 84–289, and VT-negative VTEC variant, strain 85–170, both exhibited decreased monolayer resistance values that were 45 ± 7 and 38 ± 6%, respectively, of uninfected values (n = 3 for each group). *P < 0.05.
VT-1 translocation of T84 monolayers

Similar Amounts of VT-1 and HRP Move Across Uninfected and VTEC-Infected T84 Monolayers

The movement of VT-1 and HRP across both uninfected and VTEC-infected T84 monolayers was then examined in Ussing chambers. For each of the 30-min time periods, the amount of VT-1 translocating uninfected and VTEC-infected monolayers was comparable (Table 2). This titer of VT-1 corresponds to ~0.06–0.08% of the VT-1 added to the mucosal reservoir. Similarly, the amount of HRP moving across the monolayer was comparable for uninfected and VTEC-infected cells at each of the three time periods (Table 2). Relating HRP crossing the monolayer to HRP activity, the amount of activity assayed from the serosal chamber was ~0.17% of the HRP activity on the mucosal side. This relative proportion of HRP translocating the monolayer is similar to the proportion of VT-1 titered from the serosal chamber.

To further investigate the cellular mechanisms involved in the transmonolayer movement of VT-1, experiments were then performed at low ambient temperatures. As measured in Ussing chambers at room temperature, less total toxin was detected in the serosal chamber after 90 min of incubation compared with the amount of toxin present when experiments were carried out at 37°C (Table 3). The amount of VT-1 passing across T84 monolayers incubated with VT-1 at 4°C for 2 h was also less compared with the amount translocating monolayers incubated at 37°C for the same time period (Table 3). Similar results were observed for the translocation of HRP at 4°C (Table 3). This treatment, although providing general metabolic inhibition (9, 25), had no effect on transepithelial resistance values during the course of the experiment (99 ± 6% of baseline resistance values).

Intracellular Detection of HRP and VT-1

Electron microscopic examination of T84 cells treated with HRP and cytochemically stained for peroxidase activity showed presence of the reaction product in membrane-bound vesicles in the apical aspect of the cell (Fig. 4). Less frequently, reaction product was also detected at the basal pole of cells (Fig. 4A, inset).

Table 2. Unidirectional mucosal-to-serosal movement of VT-1 and HRP across uninfected and VTEC-infected T84 monolayers in Ussing chambers

<table>
<thead>
<tr>
<th>VT-1, GMT</th>
<th>HRP, units</th>
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<tbody>
<tr>
<td>Uninfected</td>
<td>VTEC infected</td>
</tr>
<tr>
<td>Uninfected</td>
<td>VTEC infected</td>
</tr>
<tr>
<td>VTEC infected</td>
<td></td>
</tr>
<tr>
<td>VT-1, GMT</td>
<td>HRP, units</td>
</tr>
<tr>
<td>2 (2–8)</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>3 (2–8)</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>3 (2–8)</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>4 (2–16)</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>4 (2–8)</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>5 (2–32)</td>
<td>3.5 ± 0.5</td>
</tr>
</tbody>
</table>

VT-1 and horseradish peroxidase (HRP) were added to mucosal compartment of Ussing chambers (final concn 0.5 μg/ml and 10^-5 M of VT-1 and HRP, respectively) containing either uninfected or 15-h VTEC-infected T84 monolayers. Samples were taken 30, 60, and 90 min after addition from serosal chamber, as described in MATERIALS AND METHODS. VT-1 was assessed by VT cytotoxicity bioassay, whereas HRP activity was measured by enzymatic Worthington method and is given as means ± SE. Values are not statistically different by analysis of variance. GMT is geometric mean titer of VT-1 detected in serosal chamber from 4 separate experiments. Range of titers for the particular group is given in parentheses. Transmonolayer T84 resistance values were 2,000 ± 234 and 875 ± 63 Ω·cm² for uninfected and VTEC-infected T84 monolayers, respectively (n = 4).

Table 3. Movement of VT-1 and HRP across T84 monolayers at varying ambient temperatures

<table>
<thead>
<tr>
<th>VT-1 or HRP Translocated to Basal Compartment</th>
<th>Total Flux Measured in Ussing Chamber</th>
</tr>
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<tbody>
<tr>
<td>Amount of VT-1 or HRP</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>37°C</td>
</tr>
<tr>
<td>22°C</td>
<td>37°C</td>
</tr>
<tr>
<td>VT-1, GMT</td>
<td>HRP, units</td>
</tr>
<tr>
<td>4 (2–8)</td>
<td>15 ± 0.2*</td>
</tr>
<tr>
<td>8 (2–32)</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>4 (2–32)</td>
<td>NT</td>
</tr>
<tr>
<td>7 (2–32)</td>
<td>NT</td>
</tr>
</tbody>
</table>

For VT-1 and HRP measurements at 4 and 37°C, 0.5 μg/ml and 10^-5 M of VT-1 and HRP, respectively, were added to apical compartment of uninfected T84 monolayers. Samples from basal compartment of Transwells were taken after 2 h of incubation at respective temperatures. For VT-1 flux experiments carried out at 22 and 37°C in Ussing chambers, 0.5 μg/ml VT-1 was added to the mucosal reservoir of the chamber containing uninfected T84 cells and sampled from the serosal reservoir after 90 min of incubation. Titer of VT-1 or the amount of HRP was assessed as described in MATERIALS AND METHODS and expressed as GMT or HRP units, respectively. For VT-1 experiments, range of titers for the particular group is given in parentheses (n = 8–12). For HRP analyses, units are expressed as means ± SE (n = 4). NT, not tested. *P < 0.05 compared with flux at 37°C (Student’s t-test).

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Fig. 4. Electron photomicrographs of uninfected and VTEC-infected T84 cells treated with $10^{-5}$ M horseradish peroxidase (HRP) for 18 h and stained for peroxidase activity. A: HRP reaction product (arrow) was observed in vesicles in apical aspect of uninfected T84 cells (original magnification, $\times 25,600$) and in basal pole of cells (inset; original magnification, $\times 48,600$). B: similar amounts of HRP reaction product (arrow) were observed in VTEC-infected T84 cells (original magnification, $\times 35,040$). C: HRP reaction product was excluded from paracellular space by intercellular tight junctions (arrow; original magnification, $\times 35,040$).
Vesicles containing reaction product were identified in a number of cells in the monolayer and were seen as frequently in uninfected T84 cells (Fig. 4A) as in VTEC-infected cells (Fig. 4B). As reported in previous studies (7), HRP was excluded from the paracellular space by intercellular tight junctions (Fig. 4C).

Immunogold detection of VT-1 in T84 cells is shown in Fig. 5. VT-1 was found within endocytic vesicles at the apical portion of T84 cells (Fig. 5A). VT-1 was also found associated with intracellular organelles, including the Golgi apparatus (Fig. 5B) and endoplasmic reticulum (Fig. 5C). VT-1 was also found associated with the nuclear membrane (Fig. 5D). Untreated T84 cells showed no reaction with the antibody after immunoelectron microscopy (data not shown).

**DISCUSSION**

In this report, the effects of VT-1 on the barrier function of T84 cell monolayers were examined. This cell line was also employed to examine the mechanism of VT-1 translocation across the epithelial cell barrier. We found that T84 cells were insensitive to the cytopathic effects of VT-1. Transmonolayer resistance, a
measure of the barrier function of an epithelium (41), was not affected by VT-1 even in increasing concentrations up to 1 µg/ml, a dose corresponding to ~100,000 times the CD50 on Vero cells (22). Consistent with the lack of cytopathic effect of the toxin, T84 cells did not express detectable levels of the VT glycolipid receptor, Gb3. Because human intestinal epithelial cells also lack Gb3 (4, 18, 24), we propose that the T84 intestinal epithelial cell line provides a suitable in vitro model for the investigation of VT-1 translocation across the colonic epithelium. The T84 cell line also provides an advantage over other intestinal cell lines, including Caco-2 and HT-29, which express Gb3 (19).

We have recently shown that VTEC infection of T84 cells results in reduced barrier function of the epithelium. Disruption of intercellular tight junctions was the mechanism underlying the VTEC-induced effect on epithelial function (38). In this study, we extend these findings by demonstrating that the VTEC-induced reduction in T84 monolayer resistance is independent of toxin production. A VT-negative variant of VTEC, strain 85–170, still induced transmonolayer resistance decreases in T84 cells.

The possibility that VTEC-induced changes in barrier function would allow VT-1 to pass between adjacent epithelial cells through disrupted tight junctions was then examined. VT-1 moved across uninfected monolayers with high electrical resistance, similar to the findings of Acheson et al. (2). Comparable amounts of VT-1 also translocated VTEC-infected monolayers with reduced monolayer resistance. In contrast to these findings, VTEC-infected T84 monolayers have increased permeability to small probe molecules, including 51Cr-EDTA, compared with uninfected cells with high transepithelial resistance (38). These findings, demonstrating restricted paracellular permeability of T84 cells to large proteins despite decreases in transmonolayer resistance, support the concept of a finite tight junction permeability in T84 cells (31). Previous studies of tight junction structural development in T84 cells indicate that paracellular permeability is normally limited to molecules with a hydrodynamic diameter of ~30 Å (31). Intestinal permeability studies in humans indicate a limit of ~50 Å (52). In contrast, the hydrodynamic diameter of VT-1 is estimated as ~70 Å (5), well above the limit imposed by tight junctions in both T84 cell monolayers and human intestinal cells. Our findings indicate that, despite bacterial infection, tight junctions of T84 cells maintain their sieving properties and limit the paracellular movement of large molecules.

To determine whether the route of uptake of VT-1 across T84 monolayers was transcellular, VT-1 translocation across T84 monolayers was compared with another large protein, HRP. Movement of VT-1 across T84 cell monolayers paralleled the movement of HRP. Lowered ambient temperature reduced VT-1 translocation across T84 monolayers, suggesting that metabolic processes affect toxin translocation (25). Passage of HRP through T84 monolayers was similarly inhibited by incubation at low temperatures. The amount of inhibition, however, was not as great as that reported in some other studies of protein translocation conducted at lowered ambient temperatures (7, 9, 16). Therefore the possibility that a small amount of toxin can leak through the filter support cannot be ruled out entirely.

The mechanisms of uptake of large proteins like HRP and bovine serum albumin have been studied extensively. HRP has been used to elucidate fluid-phase uptake and transport both in animal intestine and in cell lines such as Caco-2 (17) and T84 (7). Bovine serum albumin has also been employed in a number of studies to assess intestinal macromolecular permeability (9). Both macromolecules pass intact in small amounts through the epithelium by a transcellular route involving endocytic uptake at the apical membrane, transcellular passage, and exocytosis through the basolateral membrane (33). Findings from this study and others (1) also suggest a transcellular route for VT-1 translocation across intact epithelial monolayers in vitro.

To further investigate the mechanism of VT-1 uptake and transcytosis in T84 cells, immunoelectron microscopic studies were performed. As in previous findings with T84 cells (7), HRP reaction product was located within endocytic vesicles throughout T84 cells. Both uninfected T84 monolayers and monolayers infected with VTEC displaying reduced monolayer resistance showed HRP staining up to the level of the tight junction. However, staining was excluded from the paracellular space. These results confirmed the quantitative HRP analyses, which suggested a transcellular mode of HRP translocation even in T84 cells with VTEC-induced decreases in barrier function.

Immunogold localization of VT-1 in T84 cells showed the presence of toxin within endosomes of T84 epithelial cells. Immunogold-labeled VT-1 was associated with intracellular organelles involved in the retrograde transport of toxin, including the endoplasmic reticulum, the Golgi network and the nuclear membrane. Previous studies with Shiga toxin of S. dysenteriae type 1 showed that retrograde transport and association of toxin with the endoplasmic reticulum and nuclear membrane are specific to toxin-sensitive cells and dependent on the fatty acid composition of the Gb3 receptor on the cell surface (44). Therefore the explanation for these observations in Gb3-negative and toxin-resistant T84 cells is not known. The possibility that Gb3 is present on T84 cells at levels that are undetectable by biochemical means cannot be ruled out. Nevertheless, detailed analyses of intestinal glycosphingolipids consistently report a lack of Gb3 in human epithelial cells isolated from the large intestine (18, 24). Alternatively, a low-affinity VT-1 receptor distinct from Gb3 might be present on T84 cells. A preliminary report has suggested the possibility of a protein receptor for VT-1 in addition to Gb3 (10). Taken together, these findings indicate the need for additional studies to search for alternate VT-1 receptors in T84 cells and on surface epithelial cells in the human intestinal tract.

The lack of cytotoxic effects of VT-1 on T84 cells despite toxin entry and association with intracellular
targets is of interest. A preliminary report also showed a lack of cytotoxic effects of VT-1 on Gb3-positive human mesangial cells even though protein synthesis is inhibited by the toxin (50).

These results impact on current concepts of how luminal bacterial toxins penetrate the intestinal epithelial barrier to enter the systemic circulation of the infected host. The passage of large toxins, such as Clostridium botulinum toxins and Staphylococcus aureus enterotoxins, may well proceed in a similar fashion. The T84 cell line provides a model system to further elucidate the mechanisms of intestinal uptake of these and other bacterial toxins causing systemic illnesses in humans.

In summary, these studies provide evidence that a large protein toxin, VT-1, translocates monolayers of T84 cells by a transcellular route rather than passing through tight junctions between adjacent epithelial cells. Although the amount of toxin transported in this fashion is small, the level of toxin in the bloodstream may well be biologically and clinically relevant (34).

Further studies are required to elucidate the receptor for VT-1 binding in Gb3-deficient intestinal epithelial cells including T84 and to further define the intracellular trafficking of this toxin and its role in disease pathogenesis.

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