Interaction of Escherichia coli Heat-Stable Enterotoxin B with Cultured Human Intestinal Epithelial Cells

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Binding of Escherichia coli heat-stable enterotoxin B (STb) to the human intestinal epithelial cell lines T84 and HT29 and to polarized T84 cells was studied to define the initial interaction of this peptide toxin with target cells. Equilibrium and competitive binding isotherms showed that 125I-STb bound specifically to T84 and HT29 cells; however, the toxin epithelial cell interactions could be characterized by low-affinity binding (≤10^5 M^-1) to a high number of binding sites (≥10^6 per cell). STb binding to T84 and HT29 cells as a function of 125I-STb concentration did not approach saturation at levels well above the effective biological concentration of STb for fluid secretion. Treatment of the 125I-STb-bound T84 and HT29 cells with an acidic saline solution to remove surface-bound toxin revealed that only ~55% ± 10% of 125I-STb could be removed by this treatment at 4°C, suggesting that approximately half of the bound STb was stably associated with the plasma membrane and/or internalized into the cytoplasm. Similar results were obtained when binding and internalization experiments were conducted at 22 and 37°C. Immunofluorescence studies demonstrated that the strongest signal for STb appeared in the plasma membrane even after acid treatment. Toxin-treated cells also displayed diffuse cytoplasmic staining, indicating that once cell bound, STb did not appear to preferentially associate with membrane vesicles or cellular organelles. Binding and subsequent internalization of 125I-STb were not affected by treatment of the cells with trypsin, endoglycosidase F/peptide N-glycosidase F, Vibrio cholerae neuraminidase, tunicamycin, or 5 mM sodium chloride, which blocks sulfation of surface proteoglycans. In addition, the internalization process was not altered by preincubation of the cells with the cytoskeleton inhibitors cytochalasin D and colchicine or cellular perturbants (i.e., 0.45 M sucrose and 5 mM sodium azide), indicating that cell surface proteins or carbohydrates did not function as STb receptors. The binding of 125I-STb to polarized T84 cells was also examined, and the total and nonspecific binding isotherms were found to overlap, indicating that the apical surface of polarized T84 cells did not contain a specific receptor for STb. In comparison to undifferentiated cells, twice the amount of bound STb (~80% ± 10%) was removable from polarized T84 cells after treatment with acidic solution. The percentage of surface-bound STb to polarized T84 cells did not vary significantly with the transepithelial electrical resistance of the cells or when STb was applied basolaterally. Together, our results indicate that STb binds with relatively low affinity to the plasma membrane of cultured intestinal epithelial cells and polarized T84 cells, probably to membrane lipids, and becomes stably associated with the lipid bilayer. The fact that a significant portion of the bound STb becomes free in the cytoplasm, even at a low temperature, suggests that the bound toxin may directly traverse the membrane bilayer.

Enterotoxigenic Escherichia coli strains produce heat-labile and heat-stable enterotoxins that cause intestinal fluid secretion and diarrhea in animals and humans. The heat-labile enterotoxins (LTI and LTII) are oligomeric proteins possessing structural and functional similarities to cholera toxin. By contrast, the heat-stable enterotoxins (STa and STb) are peptides containing multiple disulfide bonds. STa is an 18- or 19-amino-acid acidic peptide with three intramolecular disulfide bonds. STa acts by binding to a high-affinity, specific protein receptor (guanylyl cyclase C) located in the brush border membrane of intestinal epithelial cells, and the molecular mechanism by which STa elicits intestinal fluid secretion is known in detail (5, 20, 21). This elevated intracellular Ca²⁺ concentration has been implicated in possible stimulation of arachidonic acid release from membrane phospholipids, possibly through phospholipase C activity (13). In vivo, STb treatment results in a dose-dependent rise in the level of arachidonic acid and subsequent formation of prostaglandin E₂, an intestinal secretagog (13, 20, 37, 38). STb action also mediates the dose-dependent release of another intestinal secretagog, 5-hydroxytryptamine (5-HT), presumably from intestinal enterochromaffin cells, implicating involvement of the enteric nervous system in the signal transduction process mediated by STb (20, 23, 24). In vitro studies using rat basophilic leukemia (RBL-2H3) cells as a model for 5-HT release indicate that G-protein activation is involved in the STb-mediated release of 5-HT (21). The in
vivo release of 5-HT and the amount of secreted intestinal fluid are also significantly reduced in rat intestines pretreated with pertussis toxin (21a).

In this study we systematically examined the binding of E. coli STb to two colonic carcinoma cells, T84 and HT29, and polarized T84 cells (i) to characterize the initial step in toxin action prior to the induction of the above-noted signaling mechanisms and (ii) to identify an STb-specific cell surface receptor. Our results indicate that STb binds to the intestinal epithelial cells in general through low-affinity cell surface receptors that lead to plasma membrane association and internalization of STb into the cytoplasm. The processes by which STb membrane association may mediate fluid secretion in the small intestine through G-protein activation are discussed.

**MATERIALS AND METHODS**

**Preparation of STb.** E. coli STb was purified as described by Harville and Dreyfus (20). Purity of toxin preparations was routinely verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gels containing 6% bisacrylamide and the Tris-Tricine buffer system of Schagger and von Jagow (41). Biological activity of STb was assessed by the ligated intestinal loop assay (51).

**Radiolabeling of STb.** STb (10 μg) in 20 μl of 0.2 M sodium phosphate (pH 7.1) was incubated with 0.5 to 1 mCi of Na-125I (NEN-Dupont, Boston, Mass.) at 22°C in a glass tube coated with 1 μg of Iodo-Gen (Pierce, Rockford, Ill.). After 5 min the reaction was terminated by transferring the mixture to an ice-cold tube. The first tube was then rinsed with 0.4 ml of 10 mM ammonium acetate (pH 5.8), and the combined reaction mixture was incubated for an additional 15 min. Free 125I was removed by filtering the STb-STb sample through a 0.45-μm pore-size filter on a Millipore A/A cartridge. After the filter was washed with water and then with 20% (vol/vol) acetonitrile. Acetonitrile solutions contained 0.05% (vol/vol) trifluoroacetic acid. 125I-labeled toxin was dried in a vacuum concentrator before being dissolved in phosphate-buffered saline (PBS; pH 7.4) - 3 mg of bovine serum albumin (BSA) per ml to a final concentration of ~10 μM. The recovery of 125I-STb from the SepPak cartridge was ~95% ± 5% as determined by SDS-PAGE with known STb concentration standards. In addition, 125I-STb ran as a single, homogeneous species on the Tris-Tricine buffer system (41), and its electrophoretic mobility was indistinguishable from that of unlabeled STb. The amount of free 125I remaining in the 125I-STb preparation was determined by precipitating the purified 125I-STb with 10% (wt/vol) cold trichloroacetic acid (TCA), and the amount of nonprecipitable 125I in the preparations was ~8% ± 3%. Specific activities of 125I-STb preparations ranged between ~25 and 90 Ci/mmol, and 125I-STb retained a full toxicity as determined by the rat ligated intestinal loop assay.

**Equilibrium binding of 125I-STb to human intestinal epithelial cells.**

The human intestinal epithelial cell lines were from the American Type Culture Collection and the human colon carcinoma cell line HT29 (HT18) were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Washington, D.C.)-10% (vol/vol) fetal bovine serum (FBS; JRH Biosciences, Lenexa, Kan.)-penicillin (50 U/ml)-streptomycin (50 μg/ml) in an atmosphere of 10% CO2 and 90% air. Polarized T84 cells were obtained by seeding T84 cells in Transwells (polycarbonate membrane, 12-mm diameter, 0.4-μm pore size; Corning Costar Corp., Cambridge, Mass.) at ~10 cells/ml and allowed to grow to confluency in DMEM-10% FBS. The transcellular electrical resistance of differentiated T84 cells was measured with a Millicell-ERS resistance monitor (Millipore Corp., Bedford, Mass.). The transepithelial resistance of polarized T84 cells used in the binding experiments ranged from ~350 to 1,100 Ω·cm2. In preliminary experiments, T84 cells grown in this manner were examined by phase-contrast and transmission electron microscopy for the presence of brush border membranes as evidence for polarization. Brush border membranes could be detected by phase-contrast microscopy and clearly seen by transmission electron microscopy (47a). During this study, T84 cells grown on membrane inserts were routinely examined by phase-contrast microscopy to confirm that high transepithelial resistance was coincident with the appearance of brush border membranes. For equilibrium binding experiments, T84 and HT29 cells were seeded in 24-well plates (Falcon), and binding experiments were conducted when the cell cultures reached ~80 to 95% confluency (1 to 3 x 106 cells) in ~3 to 4 days. After the cell monolayers were washed three times with PBS and twice with PBS-0.1% (wt/vol) BSA, 125I-STb (0.05 to 7.0 μM) was added to the cells, which were then incubated for the indicated times and at various temperatures. For the polarized T84 cells 1 μl of 0.15 to 0.5 mM sodium chlorate for 3 days at 37°C, a condition that inhibits proteoglycan sulfation, was subsequently used in the toxin binding assay of cells (17, 25, 27). The role of surface proteins in STb binding was assessed by incubating the cells with 0.05 to 0.2 mg of trypsin (Meditrach) per ml for 30 min at 37°C in PBS. Trypsin proteolysis was terminated by the addition of PBS to the reaction mixture to a final concentration of 10% (vol/vol). The cells were then washed twice with 0.2 ml of soybean trypsin inhibitor (0.5 mg/ml; Sigma) in PBS and three times with 1 ml of PBS-0.1% BSA, prior to 125I-STb binding since the biological activity of STb is highly sensitive to trypsin-like protease in the gut of animals (51). These washes were sufficient to inactivate trypsin, since both the unbound 125I-STb in the sample and the cell culture bound 125I-STb were precipitable by TCA to the same extent as the control 125I-STb, which did not come in contact with trypsin. Trypsin proteolysis resulted in STb fragments which were not efficiently precipitated with TCA (4a, 6). Binding experiments were performed at least three times with triplicate samples. Each figure represents a typical experiment, with data points indicating the mean ± standard deviation of triplicate determinations.

*Binding of 125I-STb to T84 and HT29 cells.* Immunocytochemical and indirect immunofluorescence analysis was performed to visualize STb bound to the T84 and HT29 cells (19). Anti-STb antibody was obtained by immunizing New Zealand White female rabbit with purified STb covalently coupled to itself by disuccinimidyl suberate (DSS; Pierce; Rockford, Ill.). A standard course of immunizations was followed (47a), and the immunoglobulin G (IgG) fractions from the preimmune and high-titer anti-STb antibody-containing sera were prepared by protein A-Sepharose fast protein liquid chromatography. For indirect immunofluorescence, STb (0.5 to 6 μM) was incubated for 30 min at 37°C with T84 or HT29 cells grown on circular glass coverslips (Fisher) in 24-well tissue culture plates. Cells were washed before and after STb binding as described for the equilibrium binding assay. STb-bound cells were fixed for 15 min with freshly prepared 4% (vol/vol) paraformaldehyde (Sigma) and washed twice with PBS-0.2% (vol/vol) Triton X-100 (Sigma). The cells were then incubated with a 1:25 dilution of anti-STb antibody or preimmune serum in PBS-3% (wt/vol) BSA for 1 h at 22°C. The STb-bound cells were then incubated with a 1:100 dilution of goat anti-rabbit IgG fluorescein isothiocyanate (Calbiochem-Novabiochem) in PBS-3% BSA for 30 min. After being washed, STb-bound cells were visualized and photographed with a camera-equipped, incident fluorescence (Olympus BX50) microscope. Samples were illuminated with a 100-W mercury lamp, using a 20-nm bandpass excitation filter centered at 552 nm and a 570-nm emission filter.

**RESULTS**

*Binding of 125I-STb to T84 and HT29 cells.* The interaction of STb with intestinal epithelial cells is poorly understood. Based on cross-linking experiments, Hitotsubashi et al. reported that STb binds to a 25-kDa protein found in the deter-
gent extracts of mouse intestinal epithelial cells (22). However, little else is known about this 25-kDa STb-binding protein or its specific interaction with STb. We therefore examined the binding of STb to two well-characterized human intestinal epithelial cell lines, T84 and HT29, in order to identify the putative STb receptor as well as to characterize the primary interaction with toxin. Both cell lines in undifferentiated forms had been previously shown to undergo signal transduction in response to STb treatment (reference 5 and unpublished results). Competitive binding experiments were conducted to determine if STb binds specifically to the cultured intestinal epithelial cells. The binding of 125I-STb to T84 cells was reduced by ~50% in the presence of 5-fold excess unlabeled STb, but the binding did not decrease further in the presence of 100- to 1,000-fold excess STb (Fig. 1A), indicating that unlabeled STb could specifically compete with 125I-STb bound to a receptor on T84 and HT29 cells. Competitive binding isotherms of typical high-affinity receptor-ligand interactions, however, are characterized by a more dramatic decrease in the amount of signal in the presence of excess unlabeled ligand (12, 15).

The binding of 125I-STb to T84 and HT29 cells as a function of 125I-STb concentration (50 to 700 pmol) was performed to obtain an equilibrium binding constant and determine the number of binding sites on these cell types. Both total and specific binding curves leveled off slightly at higher ligand concentrations (~200 pmol of 125I-STb) (Fig. 1B); however, the binding was generally linear through the STb concentration range tested. The binding of 125I-STb to the T84 and HT29 cells was indicative of a process involving a low-affinity receptor. Assuming a one-ligand–one-site model, the binding constant and the number of binding sites per cell were estimated to be $10^7$ M$^{-1}$ and $10^9$, respectively, using the binding data fitting program LIGAND (32). The binding isotherms could not be fitted by LIGAND if a two-binding-site model was considered.

The rates of 125I-STb (0.05 μM) binding to T84 cells in the absence (total binding) and presence (nonspecific binding) of 200-fold STb (10 μM) at 4, 22, and 37°C were determined. The binding interaction was rapid, and specific binding reached an equilibrium by 5 to 10 min at all temperatures tested (not shown). Increasing the incubation period for the STb-intestinal epithelial cell interaction up to 90 min did not increase the amount of 125I-STb bound (not shown). The effect of temperature on the equilibrium binding of 125I-STb to T84 and HT29 cells was determined, and the total binding of 125I-STb to the intestinal epithelial cells at 22 and 4°C was ~50% lower than that at 37°C (not shown). However, the extent of binding at 22 and 4°C were the same within experimental error. That is, 125I-STb binding to receptors on the intestinal epithelial cells was not affected by a temperature below 22°C.

**Fate of 125I-STb binding to T84 and HT29 cells.** Once formed, a ligand-receptor complex could remain on the cell surface or be internalized by various endocytotic processes. Treatment of the ligand-bound cells with isotonic acidic solutions at different temperatures is routinely used to assess the fate of ligand-receptor complexes and to characterize receptor-mediated endocytosis (18, 29, 40, 45). The fate of cell-bound 125I-STb was determined by washing toxin-treated cells to remove free 125I-STb, followed by incubation with dilute acetic acid solution to remove surface-bound toxin. The radiolabel recovered in this washing represented the fraction of STb that was cell surface bound at the time of incubation with the acidic solution. The 125I-STb which remained with the cells following acid treatment was either associated with the membrane lipid bilayer and/or internalized by the cell. We observed that ~55% ± 10% of the total bound 125I-STb was removed from T84 cells by acid treatment at both 37 and 4°C, indicating that nearly half of the 125I-STb was stably associated with the cell membrane and/or present in cytoplasm (Fig. 2A and B). The amount of surface-bound 125I-STb on HT29 cells was slightly higher than that on T84 cells (~65% ± 12%). For both cell types, the fraction of surface-bound 125I-STb remained constant at all 125I-STb concentrations tested (0.05 to 1.6 μM) (not shown). Increasing the incubation time of the 125I-STb-bound cells with acidic saline solution (up to 1 h) did not significantly increase the amount of 125I-STb removed from the cells (not shown). Approximately 70% ± 10% of radiolabel removed from the cell surface by acid treatment was precipitable by TCA, indicating that cell surface-bound radiolabel was intact STb. The majority of cell-associated radiolabel was also intact 125I-STb, based on SDS-PAGE analysis of the cell-bound 125I-STb following acetic acid treatment. Treatment of the 125I-STb bound to cells with other acidic solutions (0.1 M glycine [pH 2.5]–0.15 M NaCl or 2.0 M guanidine·HCl [pH 2.5]–0.15 M NaCl) gave results similar to those shown for acetic acid treat-

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**FIG. 1.** (A) Competitive binding of 125I-STb and STb to intestinal epithelial cells. 125I-STb (0.05 μM) was incubated with T84 cells in the absence and presence of 0.05 to 50 μM STb for 60 min at 37°C. (B) Specific binding of 125I-STb to T84 cells as a function of STb concentration. 125I-STb (15 to 700 pmol) was incubated with T84 cells in the absence (total; ■) and presence (nonspecific) of 200-fold STb for 30 min at 37°C. Specific binding (●) was calculated by subtracting nonspecific binding from total binding.
incubating the cells with trypsin prior to 125I-STb binding. The binding studies. The role of surface proteins was assessed by various enzymes and chemical perturbants prior to or during to its receptor on T84 and HT29 cells by treating the cells with V. cholerae neuraminidase (0.1 U/ml), which hydrolyzes α-2,3-, α-2,6-, and α-2,8 linkages of terminal N-acetyleneuraminic acid (30). 125I-STb bound similarly to both treated and untreated cells (Table 1), indicating that sialic acid residues were not involved in STb binding to its receptor. Glycosaminoglycans are the negatively charged polysaccharide components of extracellular matrix-associated proteoglycans. Disaccharide units contain various amounts of sulfate groups on the carbohydrate backbone and extensive sulfation of glycosaminoglycans results in a polyanionic cell surface structure (24). In cultured cells, chlorate prevents the formation of adenosine 3’-phosphate-5’-phosphosulfate, thus leading to a reduction in N-sulfation, and results in a stoichiometric increase in unmodified chondroitin sulfate (N-acetylgalactosamine) and heparan sulfate (N-acetylgalactosamine) residues (3, 4, 17, 25, 27). To assess the role of sulfated proteoglycans in STb binding, T84 and HT29 cells were grown in complete DMEM containing 5 mM sodium chlorate for 3 days. T84 cells grown in chlorate-containing medium bound 125I-STb as well as untreated cells (Table 1), indicating that the degree of proteoglycan sulfation did not affect the capacity of intestinal epithelial cell receptors to bind 125I-STb. Since sialic acid and sulfated glycosaminoglycans are only two of a number of potential carbohydrate-associated residues which might interact with STb, we also examined the general requirement of surface carbohydrates in STb binding to T84 and HT29 cells. In one experiment, cells were treated with F. meningosepticum endo F/PNGase F (1 U/ml) to release all N-linked oligosaccharides from the polypeptide backbone (10). In addition, cells were grown in DMEM containing tunicamycin-

![Graph 1](michael.png)  
**FIG. 2.** (A and B) Internalization of 125I-STb bound to T84 cells as a function of toxin concentration (20 to 40 pmol) at 37 and 4°C. (C and D) Time course of 125I-STb internalization at 22 and 4°C. 125I-STb (10 pmol) was incubated with T84 cells for the indicated times. The amounts of 125I-STb and surface-bound 125I-STb from the trypsin-treated cells were determined as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[125I-STb] (mM)</th>
<th>Untreated cells</th>
<th>Treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.05</td>
<td>43 ± 17</td>
<td>37 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>42 ± 12</td>
<td>49 ± 17</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>0.15</td>
<td>44 ± 5</td>
<td>53 ± 15</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>49 ± 11</td>
<td>49 ± 15</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.15</td>
<td>36 ± 2</td>
<td>37 ± 7</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>36 ± 9</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Endo F/PNGase F</td>
<td>0.10</td>
<td>35 ± 12</td>
<td>37 ± 15</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>38 ± 6</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>0.05</td>
<td>55 ± 9</td>
<td>49 ± 8</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>62 ± 8</td>
<td>61 ± 13</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>0.10</td>
<td>42 ± 1</td>
<td>49 ± 8</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>38 ± 2</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.10</td>
<td>33 ± 3</td>
<td>32 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>33 ± 1</td>
<td>34 ± 5</td>
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<td>Sodium azide</td>
<td>0.10</td>
<td>42 ± 1</td>
<td>46 ± 2</td>
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<td>42 ± 5</td>
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<td>Sucrose</td>
<td>0.10</td>
<td>46 ± 7</td>
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<tr>
<td></td>
<td>0.20</td>
<td>44 ± 3</td>
<td>37 ± 7</td>
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</table>

*Percentage of acid-resistant 125I-STb that associated with untreated and treated cells (mean ± standard deviation of three to five binding experiments with triplicate samples). All experiments used T84 cells except the endo F/PNGase F and colchicine experiments, which used HT29 cells. Reaction conditions were as described in Materials and Methods.
cin (10 μg/ml) for 16 h to metabolically block N-linked glycosylation of proteins (10, 28). Neither treatment reduced the binding of 125I-STb to the HT29 cells (Table 1), indicating that the oligosaccharides moieties of surface glycoproteins are not likely to function as STb receptors.

**Immunocytochemistry of STb-bound cells.** Indirect immunofluorescence was used to assess the fate of cell-bound STb. Following binding to T84 and HT29 cells, the resulting fluorescence was primarily concentrated at the plasma membrane of both intestinal epithelial cell types (Fig. 3C to F). Toxic-treated cells, subsequently washed with acidic saline solution to remove surface-bound STb, displayed a significant fluorescence signal at the periphery of T84 (Fig. 3D) and HT29 cells (Fig. 3F); however, the fluorescence intensities were lower than those for untreated cells (Fig. 3C and E). Although diffuse cytoplasmic staining was observed in toxin-treated cells, staining of vesicles or cellular organelles was negligible (Fig. 3D and F). It should be noted that in this experiment the fluorescence signal at the plasma membrane is possibly accentuated by varied thickness of the cells. In control experiments, STb-bound cells incubated with goat anti-rabbit IgG-rhodamine conjugate alone (no primary antibody) failed to yield fluorescent signal (not shown). STb-bound cells incubated with preimmune IgG as the primary antibody yielded a weak fluorescent signal (Fig. 3A). This background fluorescence observed with preimmune IgG (Fig. 3A) was reduced by acid treatment, indicating the presence of low-level cross-reactive anti-STb antibodies in this IgG fraction (Fig. 3B). We also examined STb binding to Chinese hamster ovary (CHO) and mouse 3T3 fibroblast cells by indirect immunofluorescence to determine if STb binding and internalization were cell-type specific events. STb bound to CHO and mouse 3T3 fibroblast cells with low affinity, as detected by weak (above background) fluorescence signals (not shown), while the T84 and HT29 cells always fluoresced uniformly (Fig. 3C to F). In addition, the intensities of immunostained CHO and mouse 3T3 fibroblast cells varied significantly within and between experiments compared to T84 and HT29 cells.

The amounts of 125I-STb bound to either the insoluble plasma membrane or soluble cytoplasmic fractions of T84 and HT29 cells were determined to ensure that the fluorescent staining detected at plasma membrane was not solely due to the varied thickness of the cells as mentioned above. Fractionation of 125I-STb-bound cells treated with acetic acid indicated that 14.3% ± 5.7% (37°C) and 16.8% ± 6.2% (4°C) of the acid-resistant 125I-STb was associated with the particulate fraction intestinal epithelial cells after the acid treatment. For the untreated cells, slightly higher percentages of total 125I-STb bound was found to associate with the cell membrane fraction at 37°C (20.1% ± 7.8%) and at 4°C (23.7% ± 6.5%). The majority of the cytotoxic 125I-STb of both untreated and acid-treated cells was an intact toxin, since ~65% ± 10% of radiolabel in the supernatant following cell fractionation could be precipitated with 10% TCA. Although immunofluorescence analysis of STb-bound cells suggested that the toxin was predominantly plasma membrane associated, subcellular fractionation results indicated that a greater fraction of cell-bound 125I-STb was in the soluble cytoplasmic fraction. However, it was not possible to account for the amount of membrane-bound 125I-STb that may be dislodged from the membrane fraction during the homogenization process.

**Effects of inhibitors on the internalization of 125I-STb.** The association of STb with the cell plasma membrane and subsequent internalization of toxin into the cytoplasm could occur through several endocytic pathways involving clathrin-coated pits, coated vesicles, and caveolae or plasma laminal vesicles. We tested the effect of cytoskeleton-disrupting agents, which inhibit endocytic processes, on 125I-STb binding and internalization by T84 cells. Pretreatment of T84 cells with cytochalasin D (10 μg/ml) or HT29 cells with colchicine (40 μg/ml) did not significantly lower the extent of STb binding to these cell types (Table 1). These data indicated that actin filaments and microtubules were not involved in the membrane association or subsequent internalization of STb. To determine if STb internalization occurs through an energy-dependent process, T84 and HT29 cells were metabolically blocked by treatment with 5 mM sodium azide and then assessed for toxin internalization. Azide treatment had no effect on the equilibrium binding or internalization of 125I-STb to the intestinal epithelial cells (Table 1). Finally, abrogation of endocytosis by treatment of the cells with 0.45 M sucrose to induce a hypertonic condition (39) did not greatly affect the fate of surface-bound STb (Table 1). Although the total 125I-STb bound to T84 cells decreased by ~30% in sucrose containing buffer, the ratio of surface bound to the internalized 125I-STb was the same for sucrose-treated and control cells (Table 1).

**Binding of STb to polarized T84 cells.** Our experimental results with undifferentiated T84 and HT29 cells under various experimental conditions strongly indicated that STb is binding to a nonproteinaceous receptor(s) on the cell surface with low affinity. It is likely that STb bound to these same receptors is responsible for the influx of Ca²⁺ into Madin-Darby canine kidney epithelial, human HT29 intestinal epithelial, and rat primary pituitary cells (5) and the release of 5-HT in rat basophilic leukemic (RBL-2H3) cells (21). However, in vivo, STb initially comes in contact with the apical surface of the polarized intestinal epithelial cells in the lumen. It is possible that an STb-specific receptor exists exclusively on the apical surface of polarized intestinal epithelial cells but not on the surface of undifferentiated intestinal epithelial cells. We used polarized T84 cells to determine if the apical surface contains a specific receptor for STb. Results for equilibrium binding of 125I-STb (7.5 to 90 pmol), applied apically, in the presence (nonspecific) and absence (total) of 100-fold STb to polarized T84 cells are shown in Fig. 4. Unlike STb binding to undifferentiated T84 and HT29 cells (Fig. 1B), the total and nonspecific binding isotherms for STb-polarized T84 cells overlapped, indicating that the apical surface of polarized T84 cells did not have a high-affinity, specific receptor for STb. We then asked whether STb binding to polarized T84 cells is stably associated with the cells. We found that ~80% ± 10% of cell-bound STb was removable by treatment with acidic saline solution (Fig. 5). This value for polarized T84 cells (~80% ± 10%) is higher than those for T84 cells (55% ± 10%) and HT29 cells (~65% ± 12%). However, the percentage of surface-bound 125I-STb was the same within experimental errors for samples containing excess STb and for samples in which the toxin was applied basolaterally (not shown). We also observed that the percent of surface-bound 125I-STb did not vary as a function of transepithelial resistance of polarized T84 cells (Fig. 5).

**DISCUSSION**

Like most extracellular molecules that elicit specific cell and tissue responses, the *E. coli* heat-stable enterotoxin STb must initially interact at the surface of target cells. Due to the lack of information on the interaction of STb with columnar epithelial cells of the mammalian small intestine, we investigated the interaction of STb with cultured intestinal epithelial cells and polarized T84 cells by using a combination of 125I-STb binding and indirect immunofluorescence techniques designed to address basic questions regarding STb action. Our results...

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**VOL. 65, 1997 BINDING OF STb TO CULTURED INTESTINAL EPITHELIAL CELLS 3213**
FIG. 3. Immunofluorescence analysis of STb binding to T84 and HT29 cells at 37°C. STb (2 and 1 µM) was incubated with T84 (A to D) and HT29 (E and F) cells, respectively, for 30 min at 37°C. Visualization of STb bound T84 and HT29 cells by using goat anti-rabbit IgG-rhodamine conjugate was as described in Materials and Methods. (A and B) Indirect immunofluorescence of untreated (A) and acidic saline solution-treated (B) STb-bound T84 cells incubated with normal rabbit serum and goat anti-rabbit IgG-rhodamine conjugate. (C and D) Indirect immunofluorescence of untreated (C) and acidic saline solution-treated (D) STb-bound T84 cells incubated with anti-STb polyclonal antibody and goat anti-rabbit IgG-rhodamine conjugate. (E and F) Indirect immunofluorescence of untreated (E) and acidic saline solution-treated (F) STb-bound HT29 cells incubated with anti-STb polyclonal antibody and goat anti-rabbit IgG rhodamine conjugate.
distribution following specific receptor binding events. After undifferentiated intestinal epithelial cells, respectively. Our stably integrated into the plasma membrane of polarized and bound, nearly one-quarter and one-half of the STb became intestinal epithelial cells.

That is, the apical surface of polarized T84 cells did not contain selection with membrane lipids or membrane bilayer, instead of a specific surface proteins all failed to reduce STb bind-

indicate that STb readily associates with intestinal epithelial cells, reaching equilibrium within 5 to 10 min after addition to cells, and that temperature had no effect on STb binding to T84 and HT29 cells at 22°C and below (4°C). In addition, competition for 125I-STb binding by unlabeled STb was limited and gradual, suggesting that STb interacts with multiple receptors. Both total and specific binding isotherms for undifferentiated T84 cells appeared to plateau at concentrations of STb (≥700 pmol of 125I-STb and 70 nmol of STb) that far exceed the dose of STb (~100 pmol) required for cellular signaling and interaction with plasma membranes, since STb binds to polarized T84 and HT29 cells. We then examined the STb binding to polarized T84 cells to see if we could identify an STb-specific receptor on the apical surface of intestinal epithelial cell, since the distribution of proteins and lipids differs for the apical and basolateral membranes, and the protein and lipid compositions of basolateral membrane of polarized epithelial cells resemble those of the plasma membrane of undifferentiated cells (44). The results for polarized T84 cells are in agreement with those for STb binding to undifferentiated intestinal epithelial cells. That is, the apical surface of polarized T84 cells did not contain a specific receptor for STb, and ~20% ± 10% of bound STb is stably associated with the cells when applied basolaterally or apically. The lack of some proteins and lipids on the basolateral surface of polarized T84 cells did not appear to inhibit the amount of stably bound STb. Together, these data suggest that STb binds to cultured intestinal epithelial cells (both undifferentiated and polarized) in manner probably involving interaction with plasma membranes or membrane bilayer, instead of a specific protein receptor. However, STb appears to have selectivity in interaction with plasma membranes, since STb binds with lower affinity to CHO cells and fibroblasts than to intestinal epithelial cells.

A somewhat unexpected finding was that once STb was cell bound, nearly one-quarter and one-half of the STb became stably integrated into the plasma membrane of polarized and undifferentiated intestinal epithelial cells, respectively. Our conclusion is based on standard methods used to assess ligand distribution following specific receptor binding events. After

normal ligand-receptor interactions, the bound ligand could either remain on the cell surface or enter the cells through a receptor-mediated endocytosis involving clathrin-coated or uncoated pits or vesicles. When formed at 4°C, typical ligand-receptor complexes remain on the cell surface as long as cells are maintained at a low temperature, and all bound ligand on the cell surface can be removed by treatment of the cells with acidic saline solutions (18, 29, 40, 45). Acid-resistant ligand represents the fraction which was stably associated with the membrane and/or endocytosed by the cell. Typically, warming the ligand-bound cells from 4 to 37°C results in a time- and temperature-dependent increase in acid-resistant 125I-STb as the ligand becomes internalized by the cell. As reported here, STb was internalized by cultured intestinal epithelial cells by a process which was independent of temperature, cytoskeleton rearrangements, energy, or hypertonic conditions. This observation suggests that processes such as clathrin-dependent and clathrin-independent endocytosis, internalization of caveolae, or micropinocytosis are not involved in the apparent membrane association or internalization of STb (36). Further, the association of 125I-STb with the cells in a temperature-independent fashion suggests that acid-resistant 125I-STb may also reflect the formation of a stable lipid-associated STb complex rather than classical cellular internalization of bound ligand. One hypothesis for the behavior of STb is that the membrane-associated form of toxin may somehow interact with the cell matrix or peripheral membrane proteins of the membrane inner leaflet such as G proteins or other regulatory proteins.

Recently, Hitotsubashi et al. reported that STb binds to a 25-kDa protein in concentrated detergent extracts of mouse intestinal epithelial cells (22). The 25-kDa protein was not a glycoprotein and was not observed in mouse spleen, liver, lung, or kidneys. In an attempt to repeat the findings of Hitotsubashi et al. (22), we cross-linked 125I-STb to HT29 cells, brush border membrane extracts, and primary rat intestinal epithelial cells, using DSS under conditions such that we detected specific binding with cultured intestinal epithelial cells. Although DSS did cross-link 125I-STb to several proteins in the range of
10 to 140 kDa, the same proteins were cross-linked to $^{125}$I-STb in the presence of 1,000-fold unlabeled toxin (not shown). It is unclear whether the 25-kDa protein is located at the cell surface, embedded in the membrane, or free in the cytoplasmic fraction, but it may represent a protein to which STb binds following membrane association and internalization. Based on the experimental data presented in this report, we suggest that the 25-kDa protein does not represent a surface-exposed specific receptor for STb but rather may represent either a cytoplasmic protein or a protein associated with the inner leaflet of the plasma membrane to which STb binds.

The structure of STb, antiparallel $\alpha$ helices joined by disulfide bonds, has been identified in only two other proteins and in a group of related peptides (thionins) (46). Thionins from various plant species are of special interest to us since these amphipathic, cysteine-rich (three to four disulfide bonds), basic (~5,000 Da with high concentrations of lysine and arginine residues) peptides also induce cellular uptake of Ca$^{2+}$, cause K$^+$ efflux, release arachidonic acid, and activate phospholipase A$_2$ (8, 26, 33, 47). As for STb, the initial binding of thionins to mammalian erythrocytes and phospholipid vesicles proceeds through an electrostatic interaction of the positively charged thionins and negatively charged cell membrane (33, 34, 48, 49) to exert hemolytic, neurotoxic, and cytotoxic effects on bacteria, fungi, and mammalian cells (9, 11, 31). Most importantly, results of experiments designed to detect a thionin-specific protein receptor on the erythrocytes have all been negative (49).

The molecular details by which G$_s$ activation by STb occurred from toxin binding to the plasma membrane to initiate the signal transduction cascade are unknown. However, since the amphipathic $\alpha$-helical domain of STb has a strong membrane association potential (43, 46) and our experimental results suggest STb interaction with plasma membrane of the intestinal epithelial cells, it is possible that bound STb penetrates the membrane bilayer or simply disrupts the bilayer sufficiently to directly activate G$_s$. Several other naturally occurring and synthetic peptides and nonpeptide compounds including the wasp venom mastoparan, neuropeptide subunits and synthetic peptides and nonpeptide compounds, are thought to be primary to the intestinal secretory action of STb (20, 21, 39).

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REFERENCES

a thionin isolated from nuts of *Pyrularia pubera* by human erythrocytes.


