Binding and Transcytosis of Botulinum Neurotoxin by Polarized Human Colon Carcinoma Cells*

(Received for publication, January 13, 1998, and in revised form, May 18, 1998)

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T-84 and Caco-2 human colon carcinoma cells and Madin-Darby canine kidney (MDCK) cells were used to study binding and transcytosis of iodinated *Clostridium botulinum* neurotoxin serotypes A, B, and C, as well as tetanus toxin. Specific binding and transcytosis were demonstrated for serotypes A and B in intestinal cells. Using serotype A as an example, the rate of transcytosis by T-84 cells was determined in both apical to basolateral (11.34 fmol/h/cm²) as well as basolateral to apical (8.98 fmol/h/cm²) directions, and by Caco-2 cells in the apical to basolateral (8.42 fmol/h/cm²) direction. Serotype A retained intact di-chain structure during transit through T-84 or Caco-2 cells, and when released on the basolateral side was toxic in vivo to mice and in vitro on mouse phrenic nerve-hemidiaphragm preparations. Serotype C and tetanus toxin did not bind effectively to T-84 cells, nor were they efficiently transcytosed (8–10% of serotype A). MDCK cells did not bind or efficiently transcytose (0.32 fmol/h/cm²) botulinum toxin. Further characterization demonstrated that the rate of transcytosis for serotype A in T-84 cells was increased 66% when vesicle sorting was disrupted by 5 μM brefeldin A, decreased 42% when microtubules were disrupted by 10 μM nocodazole, and decreased 74% at 18 °C. Drugs that antagonize toxin action at the nerve terminal, such as bafilomycin A₁ (which prevents acidification of endosomes) and methylamine HCl (which neutralizes acidification of endosomes), produced only a modest inhibitory effect on the rate of transcytosis (17–22%). These results may provide an explanation for the mechanism by which botulinum toxin escapes the human gastrointestinal tract, and they may also explain why specific serotypes cause human disease and others do not.

Botulinum neurotoxin (BoNT);¹ which is the etiologic agent responsible for the disease botulism, can enter the body by several different routes, but the most common of these is the gastrointestinal system (1–4). Thus, most cases of intoxication are due to ingestion of food contaminated with preformed toxin or ingestion of food contaminated with bacteria that can produce toxin in the gut. In either case, BoNT escapes the gastrointestinal system to reach the general circulation (lymph and blood). Toxin in the blood is then distributed to peripheral cholinergic nerve endings, which are the target cells for toxin action.

There is now a substantial literature describing the cellular, subcellular, and even molecular aspects of toxin action on cholinergic cells (5–9). This literature derives from a model that envisions the toxin proceeding through three major steps (2, 10–13). During the first step, the toxin binds to receptors that are highly localized in the junctional region of cholinergic cells. The toxin molecule, which is composed of a heavy chain (M₉~100,000) and a light chain (M₉~50,000) linked by a disulfide bond, is thought to rely on the carboxyl terminus of the heavy chain to associate with cells. During the second step, the toxin is productively internalized by vulnerable cells. This process is actually a sequence of two events: 1) penetration of the plasma membrane by receptor-mediated endocytosis, and 2) penetration of the endosome membrane by pH-induced translocation. Although there is much that remains to be determined about productive internalization, there is suggestive evidence that the amino terminus of the heavy chain plays an important role in this process. During the third step in toxin action, the light chain that reaches the cytosol acts as a zinc-dependent endoprotease to cleave polypeptides that are essential for exocytosis (6, 8, 9). Blockade of exocytosis leads to the failure of neuromuscular transmission that is characteristic of botulism.

In contrast to our knowledge of events at the nerve ending, very little is known about the cellular and subcellular events that account for the ability of the toxin to cross membranes in the gastrointestinal system (14–16). Those data that are available suggest that the mouth is the least effective site of absorption, the stomach is intermediate, and the intestine is the most effective site of absorption. There are no studies that actually demonstrate a mechanism for botulinum toxin penetration of gut cells. However, there is one self-evident matter that can be deduced. The series of steps that underlie toxin movement at the level of individual gut cells cannot be identical to the steps that underlie toxin movement in individual nerve cells. If BoNT were to enter and remain inside cells in the gastrointestinal system, this would prevent it from reaching peripheral cholinergic nerve endings. Thus, there must be fundamental differences that distinguish toxin movement across gastrointestinal membranes and movement across neuronal membranes.

There are two broad categories of toxin transport that could account for movement from the gastrointestinal system to the general circulation. As proposed by Bonventre (16), transport could involve a specific, receptor-mediated mechanism or a nonspecific mechanism. In the present study, work has been done to test the hypothesis that BoNT crosses the gut wall by the process of specific binding and transcytosis. According to this hypothesis, there should be little or no pH-induced translocation of toxin molecules across intracellular membranes.
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Instead, this hypothesis requires that toxin bound on the luminal side of cells must be delivered to the serosal side of cells, and furthermore the transcytosed toxin must be in a conformation that can act upon cholinergic nerves. This hypothesis has been tested by studying the binding, movement, and residual toxicity of BoNT type A (BoNT/A) added to a human epithelial cell line of gut origin (T-84). The resulting data provide the first insights into the cellular and subcellular events that account for toxin movement from the gastrointestinal system to the general circulation.

EXPERIMENTAL PROCEDURES

Materials—T-84 human colon carcinoma cells, Caco-2 human colon carcinoma cells, and Madin-Darby canine kidney cells were obtained from The American Type Culture Collection (Rockville, MD). Tissue culture media and sera were purchased from Life Technologies, Inc. [3H]Inulin and 125I-Bolton-Hunter reagent were purchased from NEN Life Science Products. Reagents were purchased from Sigma, and tissue culture supplies were obtained from Fisher. Sephadex G-25 gel filtration columns were obtained from Amersham Pharmacia Biotech. Tetanus toxin was purchased from Calbiochem (Behring Diagnostics, La Jolla, CA). A sample of BoNT/A-associated proteins was kindly provided by Dr. Roberta Tota (University of Wisconsin-Madison, Madison, WI). Samples of BoNT/C were kindly provided by Dr. Y. Kamata (University of Osaka Prefecture, Osaka, Japan). BoNT/A and BoNT/B were purified according to procedures described in the literature (17–19).

Animals—Swiss-Webster mice (female, 20–25 g), which were purchased from Ace Animals (Boyertown, PA), were housed in an American Association for Accreditation of Laboratory Animal Care-accredited animal colony and allowed unrestricted access to food and water. All procedures involving animals were reviewed and approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Cell Culture—T-84 cells were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (1 g/liter D-glucose) and Ham’s F-12 nutrient medium supplemented with 5% newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 8 μg/ml ampicillin, and 15 mM Hepes. Cultures were maintained at 37 °C in 6% CO2. Caco-2 cells were fed every 2 days. MDCK cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter D-glucose) supplemented with 15% newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 mM Hepes. Cultures were maintained at 37 °C in 6% CO2. Caco-2 cells were fed every 2 days and passed (1:2 split ratio) when 95% confluent, approximately every 6 days. Passages 55 through 76 were used for experiments described in this report. Caco-2 cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter D-glucose) supplemented with 15% newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 mM Hepes. Cultures were maintained at 37 °C in 6% CO2. Caco-2 cells were fed every 2 days and passed (1:2 split ratio) when 95% confluent, approximately every 6 days. Passages 20 through 29 were used for experiments described in this report. MDCK cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter D-glucose) supplemented with 10% newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM Hepes. Cultures were maintained at 37 °C in 6% CO2. MDCK cells were fed every 3 days and passed (1:5) when 95% confluent, approximately every 3 days. Passages 56 through 75 were used for experiments described in this report.

Inulin Flux—To control for membrane integrity and to estimate diffusion between cells, the rate of passage of [3H]Inulin from the upper to the lower reservoir was determined essentially as described previously (20). According to this procedure, the medium in both reservoirs contained unlabeled 1 mM inulin. The assay was initiated by addition of [3H]Inulin to the upper reservoir. Rates for inulin flux from the upper to the lower wells were determined in a γ-counter. Nonspecific counts were estimated by measuring the rate of [3H]inulin movement from the upper reservoir into the lower chamber (see above). Experiments were performed on cultures that were between 10 and 15 days old.

The transcytosis assay was initiated by adding 1 × 10−8 to 10−6 M 125I-labeled botulinum neurotoxin either to the upper or lower chamber. Transport of radiolabeled toxin was monitored in two ways. Initially, the entire contents of the lower chamber were aspirated and placed on ice. Five μl of a 20 mg/ml stock of bovine serum albumin was added per ml of sample as a carrier (final concentration of 100 μg/ml) followed by trichloroacetic acid to a final concentration of 10%. Proteins in the samples were precipitated on ice overnight. The precipitates were washed three times with 10% trichloroacetic acid, and the radioactivity of pellets was measured in a γ-counter. Nonspecific counts were estimated by identifying identical experiments at 10 °C.

Subsequently, transport was monitored by collecting all of the medium from the appropriate chamber. An aliquot (0.5 ml) from each sample was filtered through a Sephadex G-25 column, and 0.5-ml fractions were collected. The amount of radioactivity in the fractions was determined in a γ-counter. Labeled toxin eluted at void volume, and the radioactivity contained in the void volume fractions was summed to determine the total amount present.

Of the two methods, the latter was preferable due to better reproducibility among replicates. A minimum of two replicates per condition was included in each experiment.

Cell Fractionation—Cell fractions for assay of intracellular toxin were prepared by standard methods. Briefly, BoNT/A (1 × 10−6 M) was added to the upper reservoir at 37 °C for 1 h. The wells were subse-
quently cooled to 18 °C over a 45-min time period, after which the cells were washed five times (upper and lower surface) with cold Dulbecco’s phosphate-buffered saline without Ca$^{2+}$ or Mg$^{2+}$. Cells were trypsinized at 37 °C for 10 min, washed once with medium, then twice with homogenization buffer (255 mM sucrose, 1 mM EDTA, 20 mM Heps, pH 7.4) containing protease inhibitors (5 μM antipain, 1 μM aprotinin, 10 μM leupeptin, 5 μM pepstatin A, 100 μM phenylmethylsulfonyl fluoride). Harvested cells were homogenized at 3 °C (on ice) in a glass-Teflon homogenizer (2 × 20 strokes). The homogenate was fractionated using an SS-34 rotor in a Sorvall RC5B centrifuge (Sorvall, Inc., Newtown, CT). Homogenates were spun at 1000 × g for 5 min. The resulting supernatant (S-1) was recentrifuged at 10,000 × g for 10 min. The second supernatant (S-2) was used for determination of cytosolic toxin.

Electrophoresis—Samples for autoradiographic analysis were separated on 7.5% polyacrylamide gels according to Laemmli (24), using reducing or non-reducing conditions. Gels were fixed for 30 min in destaining buffer, dried on blotter paper, and exposed to film (Hyperfilm™ MP, Amersham Pharmacia Biotech).

In Vivo and in Vitro Toxicity Testing—The toxicity of transcytosed BoNT/A that was collected from the lower reservoir was bioassayed either by intraperitoneal injection into mice or by addition to mouse physiological buffer that was aerated with 95% O$_2$, 5% CO$_2$ and maintained at 35 °C. The physiological solution had the following composition (millimolar): NaCl, 137; KCl, 5; CaCl$_2$, 1.8; MgSO$_4$, 1.0; NaHCO$_3$, 24; Na$_2$HPO$_4$, 1.0; d-glucose, 11; and gelatin, 0.01%. Phrenic nerves were stimulated continuously (1.0 Hz; 0.1–0.3-ms duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a percentage reduction in muscle twitch response to neurogenic stimulation.

For both in vivo bioassay, tissues were excised and suspended in physiological buffer that was aerated with 95% O$_2$, 5% CO$_2$ and maintained at 37 °C. The physiological solution had the following composition (millimolar): NaCl, 137; KCl, 5; CaCl$_2$, 1.8; MgSO$_4$, 1.0; NaHCO$_3$, 24; Na$_2$HPO$_4$, 1.0; d-glucose, 11; and gelatin, 0.01%. Phrenic nerves were stimulated continuously (1.0 Hz; 0.1–0.3-ms duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a percentage reduction in muscle twitch response to neurogenic stimulation.

RESULTS

In Vivo and in Vitro Toxicity Testing—For BoNT/A binding to T-84 cells, three separate experiments were done with three replicates for each. For BoNT/B, BoNT/C, and tetanus toxin binding to T-84 cells, as well as BoNT/A binding to MDCK cells, two separate experiments were done with three replicates each. Data for BoNT/A binding to MDCK cells are presented separately (inset) due to the low amount of specific binding.
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Transcytosis of T-84 or MDCK cells. T-84 (●) or MDCK (○) cells were grown on culture inserts and incubated with $1 \times 10^{-8}$ M $^{125}$I-BoNT/A added to the upper chamber. Cultures were incubated for the times indicated, at which point the medium in the lower well was collected. Proteins in the collected samples were precipitated overnight with 10% trichloroacetic acid on ice. The precipitates were washed three times and the pellets were counted in a γ-counter to determine radioactivity. Each data point represents an average ± S.E. of four separate experiments with three replicates each. Values determined at 3 °C were subtracted from each data point to correct for nonspecific transcytosis. The average nonspecific counts represented 35 ± 4% of total radioactivity.

well as tetanus toxin. Similar experiments were done on MDCK cells, which have the ability to differentiate, polarize, and transcytose, but which have not been implicated in clostridial toxin action. Fig. 2, which illustrates data obtained for iodinated, homogeneous serotype A, clearly shows that T-84 cells are capable of uptake, transcytosis, and release of toxin. In contrast, MDCK cells did not transcytose serotype A at a significant rate.

T-84 cells were also assessed for their ability to transcytose other serotypes of botulinum toxin and tetanus toxin. Interestingly, the cells showed a substantial ability to discriminate among the toxins. Botulinum toxin type B was efficiently transcytosed (83% of A; n = 5), whereas neither serotype C nor tetanus toxin (8–10% of A) was efficiently transcytosed.

Effects of Concentration on the Rate of BoNT/A Transcytosis—Data presented thus far indicate that T-84 cells transcytose BoNT/A. The experiments illustrated in Fig. 3 show that increases in toxin concentration were associated with increases in transcytosis in T-84 cells. The rates of apical → basolateral transcytosis increased 600-fold from $1 \times 10^{-10}$ M to 168.50 fmol/cm$^2$/h at $10^{-7}$ M toxin. Conversely, the fractional amount of toxin added to the top well (Fig. 3, numbers in parentheses) that was transported across cells decreased 2-fold from 12% at $10^{-10}$ M to 6% at $10^{-7}$ M in T-84 cultures. When equivalent experiments were done on MDCK cells, there was much less indication of a dose-dependent capacity for transcytosis.

Further Characterization of BoNT/A Transcytosis—Data presented thus far demonstrate that polarized human epithelial cells transcytose certain clostridial toxins. Therefore, serotype A was selected as a prototype, and further work was done to establish rate constants. In addition, an effort was made to compare apical → basolateral transcytosis with basolateral → apical transcytosis. The data on rate constants are presented in Table I.

T-84 cells were shown to transcytose iodinated BoNT/A at a rate of 11.29 fmol/h/cm$^2$ when toxin was added to the upper reservoir. Transcytosis from the basolateral surface to the apical surface was less efficient (8.98 fmol/h/cm$^2$). Cooling cultures to 18 °C reduced the rate of transcytosis to 2.26 fmol/h/cm$^2$ (apical → basolateral). As shown in Table I, transcytosis of BoNT/A in Caco-2 cells (apical → basolateral) was less than that observed in T-84 cells, but still substantial. In contrast, transcytosis of the toxin by MDCK cells was negligible.

Effects of Brefeldin A, Nocodazole, and Temperature on Transcytosis of BoNT/A—Several drugs and procedures were used to further characterize the process of toxin movement across cells. Each drug and procedure was selected on the basis that previous studies have shown them to increase or decrease transcytosis (25–29).

Fig. 4 summarizes the results of the studies with brefeldin A, nocodazole, and temperature on transcytosis of BoNT/A. As shown in Table I, transcytosis of BoNT/A in Caco-2 cells (apical → basolateral) was less than that observed in T-84 cells, but still substantial. In contrast, transcytosis of the toxin by MDCK cells was negligible.

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Table I

| Cell line | Conditions | Transcytosis | Percent of control | p |
|-----------|------------|--------------|--------------------|---|
| T-84      | 37 °C (A→B) | 11.29 ± 0.30 | 100                |   |
|           | 37 °C (B→A) | 8.91 ± 0.20  | 80                 | <0.001 |
|           | 18 °C (A→B) | 2.26 ± 0.46  | 20                 | <0.001 |
| Caco-2    | 37 °C (A→B) | 8.42 ± 0.49  | 75                 | <0.001 |
| MDCK      | 37 °C (A→B) | 0.32 ± 0.07  | 2.8                | <0.001 |

Transcytosis was assayed in T-84, Caco-2, or MDCK cell cultures. Assay was initiated by addition of $1 \times 10^{-8}$ M $^{125}$I-BoNT/A to the upper or lower chamber. Cultures were subsequently incubated for 6–24 h at the temperature indicated. At the end of each experiment aliquots were collected and gel-filtered. Void volume fractions were assayed for radioactivity, and the toxin peak was summed to determine total counts. The amount of transcytosis was calculated based on the specific activity of labeled toxin. The rates reported are mean ± S.E. for T-84: 37 °C (A→B) n = 14; 37 °C (B→A) n = 4; 18 °C n = 7; Caco-2 n = 4; MDCK n = 9. All other details are provided under "Experimental Procedures."
incubation with 10 μM nocardazole. In keeping with expectations, transcytosis of the toxin was significantly decreased (p < 0.001; ~75%) when temperature was lowered from 37 to 18 °C.

Effects of Bafilomycin A₁ and Methylamine HCl on Transcytosis of BoNT/A—Bafilomycin A₁ and methylamine HCl block toxin action in the nerve ending by inhibiting pH-induced translocation of the molecule from endosomes to the cytosol (20, 30, 31). Therefore, these drugs were examined for their effects on toxin movement across T-84 cells. Table II summarizes data from transcytosis experiments performed with bafilomycin A₁ or methylamine HCl. Preincubation and assay in the presence of either of the agents resulted in a small yet significant (p < 0.01) decrease in the rate of transcytosis (Table II). Neither bafilomycin A₁ (22% decrease) nor methylamine HCl (17% decrease) completely inhibited toxin transport across gut cells.

** Autoradiography of Transcytosed Toxin—**The work thus far indicates that polarized intestinal epithelial cells are capable of binding, transcytosis, and release of BoNT; however, this work does not answer two critically important questions. First, is the toxin that is transcytosed by T-84 or Caco-2 cells intact? Second, is the toxin that is transported across cells and delivered on the serosal side in a conformation that can evoke toxicity?

**FIG. 4.** Effect of brefeldin A, nocodazole, and temperature on transcytosis of 125I-BoNT/A in differentiated, polarized T-84 cells. T-84 cells were grown on culture inserts and transcytosis was assayed at 37 °C (control), in the presence of 5 μM brefeldin A (37 °C) or in the presence of 10 μM nocardazole (37 °C) or at 18 °C. Cultures were incubated for 18 h, at which point the medium in the lower well was collected. Proteins in the collected samples were precipitated overnight with 10% trichloroacetic acid on ice. The precipitates were washed three times, and the pellets were counted in a γ-counter to determine radioactivity. Each bar represents an average ± S.E. for control (n = 8), brefeldin A (n = 3), nocardazole (n = 3), and 18 °C (n = 6) with three replicates per experiment.

**TABLE II**

| Treatment          | Transcytosis | p     |
|--------------------|--------------|-------|
|                   | fmol/h/cm²   |       |
| Control            | 11.94 ± 0.29 |       |
| Bafilomycin A₁     | 9.31 ± 0.58  | <0.01 |
| Control            | 13.61 ± 0.54 |       |
| Methylamine HCl    | 11.27 ± 0.46 | <0.01 |

**FIG. 5.** Comparison of SDS-polyacrylamide gel electrophoresis mobility of native versus cystolic and transcytosed 125I-BoNT/A from T-84 and Caco-2 cultures. Native BoNT/A complex (neurotoxin, hemagglutinin, and non-toxin non-hemagglutinin protein), pure neurotoxin prepared from complex, pure 125I-neurotoxin, and 125I-toxin that had passed through T-84 or Caco-2 cells were compared on SDS-polyacrylamide gel electrophoresis (7.5%). A, lane 1, BoNT/A complex (non-reducing gel); lane 2, pure neurotoxin (non-reducing gel); lane 3, BoNT/A complex (reducing gel); lane 4, pure neurotoxin (reducing gel). Gel stained, B, autoradiogram of pure 125I-neurotoxin (reducing or non-reducing conditions): lane 1, 125I-neurotoxin (reducing gel); lane 2, neurotoxin from the cytosolic fraction of T-84 cells (reducing gel); lane 3, neurotoxin transcytosed by T-84 cells (nonreducing gel); lane 4, neurotoxin transcytosed by T-84 cells (reducing gel); lane 5, neurotoxin transcytosed by Caco-2 cells (reducing gel). HC—ss—LC, neurotoxin heavy chain (~100 kDa) linked via a sulfhydryl bond to the neurotoxin light chain (~50 kDa); HC, neurotoxin heavy chain (~100 kDa); LC, neurotoxin light chain (~50 kDa).

**In Vivo and in Vitro Toxicity of Transcytosed Toxin—**To ensure that toxin delivered to the basolateral side of cells retained the ability to poison neuromuscular transmission, two types of bioassay were performed: *in vivo* toxicity experiments with mice, and *in vitro* toxicity experiments with isolated mouse phrenic nerve-hemidiaphragm preparations. For *in vivo* bioassay, animals were injected with an aliquot of medium collected from the lower well of T-84 cultures that were exposed to BoNT/A and monitored for toxicity for 12 h. This aliquot of medium (equivalent to ~50 ng of neurotoxin/animal, as calculated on the basis of experimental rates reported above; also see “Experimental Procedures”) was diluted in phosphate-buffered saline-bovine serum albumin and administered intraperi-
toneally. All of the animals in this group died in an average of 255 ± 20 min (−4 h). This time frame is equivalent to that obtained when 50 ng of native, nontranscytosed toxin was used (data not shown). An equivalent volume of medium (see above) collected from the bottom of T-84 cells exposed to BoNT/C was not toxic to animals (none dead at 12 h), nor was an equivalent volume of medium collected from the lower well of MDCK cultures exposed to BoNT/A (none dead at 12 h). The results obtained in the animal bioassay are in line with expectations based on binding and transcytosis data presented earlier.

Data obtained for in vitro experiments confirmed that medium collected from the lower wells of T-84 cultures exposed to BoNT/A contained toxin that was able to paralyze an isolated neuromuscular junction. Aliquots of transcytosed toxin equivalent to ~1.0 µg of neurotoxin, calculated on the basis of experimental rates reported earlier (see above), were added to a 40 ml of organ bath. The resulting paralysis time was 56 ± 8.4 min. This time is equivalent to paralysis times recorded when 1.0 µg of native toxin was added to organ baths. Thus, comparison of data obtained with transcytosed toxin to data obtained for equivalent concentrations of native toxin demonstrated that transcytosed toxin retained full biological activity.

**Uptake of Toxin-associated Proteins by Differentiated T-84 and MDCK Cultures**—Botulinum toxin type A ordinarily occurs as part of a complex in which it is noncovalently associated with two other major proteins: a family of hemagglutinins (92, 57, 35, 21.5, and 17 kDa), and a nontoxic, nonhemagglutinating protein that shares some homology with neurotoxin (~120 kDa). Therefore, experiments were done to determine whether these proteins enter cells (viz. bulk-phase endocytosis) or are actively carried across cells (viz. transcytosis).

The associated proteins were iodinated identically to BoNT/A, then added to the upper wells bathing T-84 cells. The concentration of protein (10⁻⁷ M) and experimental protocol were the same as used in earlier experiments with toxin (e.g. Table I).

The data, which are illuminated in Fig. 6, revealed two major findings. First, associated proteins did accumulate in the cytosol of T-84 cells. The majority of this protein appeared to be the intact material that was added to the upper well, although there were some novel bands that may reflect metabolites. Second, there was little delivery of associated proteins to the lower well. The two major components that were recovered from the bottom well were the nontoxic, nonhemagglutinating protein and a trace contamination of toxin that was in the associated protein fraction. These data suggest that the associated proteins undergo bulk-phase endocytosis but little transcytosis.

**DISCUSSION**

Botulinum toxin is an unusually potent substance that produces a type of food poisoning known as botulism. To produce its poisoning effects, the toxin must proceed through two separate and essential sequences of events. Initially, ingested toxin must traverse the gastrointestinal system and be absorbed into the general circulation. Subsequently, toxin must diffuse to peripheral cholinergic nerve endings, where it is internalized to produce blockade of exocytosis. The fact that the toxin is so potent means that both of these sequences of events must occur with a high degree of efficiency.

There is now a significant literature pertaining to binding and endocytosis of BoNT at the cholinergic nerve ending (see Introduction). This is in sharp contrast to the literature on toxin transport in the gut, for which there is almost a complete absence of work on cellular, subcellular, and molecular mechanisms. Therefore, the present study was undertaken with the goal of trying to identify an efficient mechanism by which intact and biologically active toxin could move from the mucosal to the serosal side of gut cells.

Experiments were conducted on two human cell lines of intestinal origin (T-84 and Caco-2), as well as a canine cell line of renal origin (MDCK). The results of this work support three conclusions: 1) human gut cells can bind and transport certain clostridial neurotoxins, 2) the toxins that are transcytosed are the same ones that are associated with human illness due to oral poisoning, and 3) the ability to bind and transcytose BoNT is not a generalized property of all differentiated and polarized cells that form tight junctions.

There are several routes in vivo that BoNT could exploit to enter the general circulation and ultimately reach the peripheral nervous system. First, there could be binding and transcytosis by intestinal epithelial cells. Second, there could be binding to M cells of Peyer’s patches (32), presentation to the lymphatic system, and subsequent escape into the blood. And finally, the toxin could injure cells or disrupt tight junctions (33, 34), which would then permit nonspecific penetration of the gut. The work presented in this study was designed to test the possibility of binding and transcytosis by epithelial cells, but it did not rule out the possibility of involvement by M cells. For those serotypes that were transported (BoNT/A and BoNT/B), there was no evidence for loss of integrity of cellular membranes or tight junctions.

Indicated BoNT/A was actively transported across differentiated and polarized T-84 cells. Passive mechanisms such as diffusion between cells did not appear to play a significant role, as judged by the study of inulin flux. In cells that had formed tight junctions, the movement of inulin from one cell surface to the other was greatly impaired. Given that inulin (13 Å radius) is approximately 30-fold smaller in molecular weight and 50-fold smaller in size than BoNT/A (48 Å radius) (35), and given that T-84 junctional strands are essentially impermeable to molecules with radii ≥ 5 Å (20), one can deduce that passive
diffusion of BoNT is likely to be negligible.

There were several observations that support the conclusion that movement of toxin across T-84 cells was an active process. First, labeled BoNT/A was capable of binding to T-84 cells, and this binding was significantly reduced in the presence of a molar excess of unlabeled toxin. This result suggests that the transport process begins with a specific binding step. Next, movement of toxin was greatly retarded when cells were incubated at low temperature, which indicates that the transport process was energy-dependent. Finally, movement of toxin was altered by drugs that have previously been shown to modify transcytosis, such as breflidine A and nocodazole. When taken collectively, these data offer strong support for the belief that human intestinal cells can bind and transcytose BoNT.

Both in vivo and in vitro toxicity experiments showed that toxin delivered to the serosal side of cells was intact and biologically active. Thus, when toxin was added to the upper chamber of the Transwell® apparatus and aliquots of medium were recovered from the lower chamber, subsequent injection of the aliquots produced toxicity in mice that was identical to that produced by native toxin. Similarly, addition of aliquots from the lower chamber to in vitro tissue baths with phrenic nerve-hemidiaphragm preparations led to blockade of neuromuscular transmission. These data from toxicity experiments were in keeping with results from SDS-polyacrylamide gel electrophoresis gels, in which toxin recovered from the basolateral side of cells migrated identically to native toxin. The combined data show that human intestinal cells can bind and transcytose toxin in a form that is structurally and functionally intact.

It is interesting to note that the literature already has a number of studies that demonstrate transcytosis of intact proteins across intestinal epithelial cells (36–40), and in some cases the studies report rates of transcytosis (29, 41–43). The rate of transcytosis of BoNT/A determined in the present study is within the range of that reported for other proteins, but it is at the low end of the range. In this context, some recent preliminary experiments may prove to be important. It has been found that the rate of transcytosis of native unlabeled toxin is greater than that of iodinated toxin. Therefore, work is underway to determine whether iodination hinders binding, transcytosis, or release of BoNT.

There is highly suggestive evidence that the phenomenon of BoNT transcytosis described in this report is relevant to oral poisoning by the toxin. Epidemiology studies on human adults indicate that certain clostridial neurotoxins are associated with human illness caused by ingestion of toxin (e.g. BoNT/A and BoNT/B), whereas others are not (e.g. BoNT/C and tetanus toxin). It is interesting that this is precisely the same pattern one observes in the ability of human intestinal cells to bind and transcytose toxins. BoNT/A and BoNT/B were transported across T-84 and Caco-2 cells, but BoNT/C and tetanus toxin were translocated at much lower rates. This coincidence of patterns suggests that the relative absence of foodborne BoNT/C and tetanus toxin poisoning is due to the relative inability of these clostridial neurotoxins to penetrate from the gut to the general circulation.

The results with BoNT/C are particularly revealing. In a recent study, the actions of several botulinum toxins were studied on surgically excised and viable human neuromuscular junctions (23). This work demonstrated that human tissues are currently underway.

Acknowledgments—We thank Dr. James Madara and Rachel Kerner for sharing information on growth, maintenance, and differentiation of T-84 cells, Dr. Bihbuti DasGupta for providing a sample of toxin-associated hemagglutinins and guidance in purification of BoNT/A, and Dr. Nabil Bakry for assistance during toxin purification.

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