Visualization of Binding and Transcytosis of Botulinum Toxin
by Human Intestinal Epithelial Cells

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Abbreviations: BoNT, botulinum neurotoxin; DTT, dithiothreitol; GST-SNAP-25, glutathione-s-transferase, synaptosomal associated protein, 25 kDa; PBS-BSA, phosphate buffered saline-bovine serum albumin.
ABSTRACT

Botulinum toxin is an unusually potent oral poison, which means that the toxin must have an efficient mechanism for escaping the lumen of the gut to reach the general circulation. Previous work involving iodination of toxin and analysis of its movement demonstrated a specific process of transepithelial transport. In the present study, botulinum toxin labeled with Alexa Fluor™ 488 was used to visualize the discrete steps of binding, internalization, transcytosis and release. The data revealed that binding sites for the toxin were distributed across the apical surface of epithelial cells, and there was no evidence of significant clustering. The amount of toxin bound to receptors at saturation was too large to be accommodated in a single wave of endocytosis. Toxin that entered epithelial cells did not remain in the vicinity of the endocytosing membrane, which is in striking contrast to events in neuronal cells. Instead, the toxin began to spread across the length of cells, eventually being released on the basolateral surface. Migration of toxin through epithelial cells required redistribution to the cell periphery. This migration pattern could be attributed to the large and centrally located nucleus, which physically displaced transport vesicles. Transcytosed toxin began to reach the contralateral surface within ca. 5 min, and transcytosis was essentially complete within 20 to 30 min.
INTRODUCTION

Botulinum toxin is the etiologic agent that causes the disease botulism. Botulinum toxin acts mainly on peripheral cholinergic nerve cells, where its principal effect is to block exocytosis (Simpson, 2004). Thus, all signs and symptoms of botulism can be explained on the basis of peripheral blockade of acetylcholine release. For example, flaccid paralysis and possible respiratory arrest can be attributed to blockade of exocytosis at neuromuscular junctions, and phenomena such as loss of salivation, gastrointestinal stasis and urinary retention are due to blockade of exocytosis at various autonomic sites.

The fact that botulinum toxin acts at exquisitely low concentrations to paralyze transmission has prompted efforts to decipher its actions on nerve endings and transmitter release. This effort has culminated in a relatively complete understanding of the basis for its toxicity (Herreros et al., 1999; Humeau et al., 2000; Simpson, 2004). The data indicate that toxin action on nerve cells can be envisioned as a sequence of steps, including: 1) binding to receptors on the membranes of nerve endings, 2) productive internalization via receptor-mediated endocytosis, 3) pH-induced translocation to the cytosol, and 4) zinc-dependent endoprotease activity to cleave polypeptides that are essential for exocytosis. There is an abundance of biochemical and pharmacologic data to support this model (Schiavo et al., 2000; Simpson et al., 1999; Simpson, 2004), as well as a small but emerging body of morphologic data (Black and Dolly, 1986a; Black and Dolly, 1986b; Lalli, et al., 1999).

In contrast to the substantial literature describing toxin action on nerve cells, there is only a modest literature describing mechanisms for toxin entry into the body. Most cases of botulism are oral in nature, and therefore the toxin must have the ability to escape the lumen of the gut to reach the general circulation (Bonventre, 1979; Sakaguchi 1983). In the recent past, a number of kinetic experiments were performed in an effort to deduce the mechanism of transepithelial transport (Maksymowych and Simpson, 1998; 2004; Maksymowych et al., 1999). This work
culminated in a model for toxin action that involves binding to receptors on the apical (mucosal) surface of gut epithelial cells, followed by transcytosis and delivery to the basolateral (serosal) side of these cells. Transcytosis is energy dependent, and it delivers toxin to the trans side of cells in the same conformation that binds to the cis side.

In the work that follows, an attempt has been made to visualize the process of binding, transcytosis and release of botulinum toxin by a human gut epithelial cell line (T-84). This represents the first attempt to visualize the sequence of events from toxin association on one side of polarized cells to eventual release on the other side of cells. Indeed, this is the first attempt to visualize the ability of any bacterial toxin to completely traverse an epithelial barrier. The resulting data provide essential insights about toxin transport across epithelial barriers that could not have been obtained using the earlier technique of kinetic analysis (Maksymowych and Simpson, 1998; 2004). However, when taken in the aggregate, the kinetic data and the visualization data provide a reasonably clear picture of the process by which botulinum toxin enters the body to exert its pharmacologic effects.
METHODS

**Materials.** An immortalized human gut epithelial cell line (T-84), all tissue culture media, and serum were obtained from the American Type Culture Collection (Manassas, VA). Reagents and chemicals were purchased from Sigma Chemical Co. (St Louis, MO) and tissue culture supplies were obtained from Fisher Scientific (Pittsburgh, PA). Alexa Fluor™ 488 Protein Labeling Kit was purchased from Molecular Probes (Eugene, OR).

**Animals.** Swiss-Webster mice (female, 20-25 g), which were purchased from Ace Animals (Boyertown, PA), were housed in the animal care facility of Jefferson Medical College and allowed unrestricted access to food and water. All procedures involving animals were reviewed and approved by the 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 and Ham's Nutrient Mixture F-12 (D-MEM/F-12), supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 8 µg/ml ampicillin. Cultures were maintained at 37°C in 5% CO₂. Cells were fed every 2-3 days and passaged (1:2) when 95% confluent, approximately every 6-8 days. Passages 63-90 were used for experiments described in this paper.

**Botulinum toxin purification.** Botulinum neurotoxin type A was purified to homogeneity following the method described by DasGupta and Sathyamoorthy (1984). The concentration of the pure neurotoxin was determined spectrophotometrically using the relationship $1.63A_{278} = 1$ mg/ml, where $A_{278}$ is the absorbance at 278 nm (DasGupta and Sathyamoorthy, 1984). Homogeneity of the purified neurotoxin was determined by SDS-PAGE, in which the toxin sample was separated in a 10% polyacrylamide gel by the method of Laemmli (1970). Gels were run under both reducing and non-reducing conditions. Bands corresponding to the light chain (ca. 50 kDa), heavy chain (ca. 100 kDa), or holotoxin (ca. 150 kDa) were almost completely free of contamination, as indicated by coomassie blue staining.
Labeling of botulinum neurotoxin. Purified toxin was labeled with Alexa Fluor™ 488 Protein Labeling Kit (Molecular Probes, Oregon), essentially according to manufacturer’s instructions. The only notable modification was that the toxin was allowed to mix with the reactive dye for 30 min (instead of 60 min) at room temperature. Labeled toxin was separated from free dye by column chromatography, and the degree of labeling was determined from the following calculations:

\[
\frac{\left[A_{280} - (A_{494} \times 0.11) \right]}{244,499} \times \text{dilution factor}
\]

Protein concentration (M) = \( \frac{\left[A_{280} - (A_{494} \times 0.11) \right] \times \text{dilution factor}}{244,499} \) (eq. 1)

where 244,499 is the molar extinction coefficient of pure botulinum neurotoxin, and 0.11 is a correction factor to account for absorption of the Alexa 488 dye at 280 nm.

\[
\frac{A_{494} \times \text{dilution factor}}{71,000 \times \text{protein concentration (M)}}
\]

Moles dye per mole protein = \( \frac{A_{494} \times \text{dilution factor}}{71,000 \times \text{protein concentration (M)}} \) (eq. 2)

where 71,000 is the approximate molar extinction coefficient of the Alexa™ Fluor 488 dye at 494 nm. The moles of dye per mole of labeled toxin averaged 7.5.

In vivo and in vitro toxicity testing of the labeled toxin. The toxicity of the labeled material was bioassayed either by intraperitoneal injection into mice or by addition to mouse phrenic nerve-hemidiaphragm preparations (Simpson et al., 2001). For the in vivo bioassay, 1 \( \mu \)g of labeled or native botulinum toxin was administered in a 100 \( \mu \)l aliquot of phosphate buffered saline (PBS). Animals (group n=8) were monitored for survival for a period of 6 hr. For the in vitro bioassay, tissues were excised and suspended in physiological buffer that was aerated with 95% O\(_2\), 5% CO\(_2\) and maintained at 35°C (Simpson et al., 2001). Phrenic nerves were stimulated continuously (1.0 Hz; 0.1-0.3 ms duration), and muscle twitch was recorded.
Labeled or native toxin (1x10^{-11} M) was added to tissues (group n=4 each), and paralysis was measured as a 50% reduction in muscle twitch response to neurogenic stimulation.

**SNAP-25 cleavage assay.** Construction and expression of recombinant GST-SNAP-25 (~50 kDa), as well as its use as a substrate for botulinum toxin, have been described previously (Simpson et al., 2001). Briefly, reactions were initiated by reducing the labeled or native botulinum toxin (100-200 nM) with DTT (8 mM) at room temperature for 30 min in cleavage buffer (50 mM HEPES, pH 7.1; and 20 µM ZnCl₂). Approximately 150 ng of GST-SNAP-25 was added to the reduced toxin and the reaction was allowed to proceed for 3 hr at 37°C. Enzymatic cleavage reactions were terminated by adding an equal volume of 2x sample buffer (125 mM Tris-HCl, pH 6.8; 1% SDS; 20% glycerol; 700 mM β-mercaptoethanol; 0.01% bromophenol blue). Samples were denatured by incubating at 95°C with subsequent cooling, then separated in 10% polyacrylamide gels (Laemmli, 1970). SNAP-25 was transferred from gels to nitrocellulose membranes (0.45 µm, Immobilon-NC, Fisher Scientific) and probed with rabbit anti-SNAP-25 C-terminal antibody (StressGen Biotechnologies Corp, Victoria BC, Canada) at 1:5,000 dilution. Donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ) was used as secondary antibody at a dilution of 1:10,000. The antibody reaction was visualized by chemiluminescence (ECL Plus, Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ) using X-ray film (Kodak Bio Max, Fisher Scientific).

**Conditions for fluorescence imaging.** T-84 cells were grown on rat tail collagen-coated 8-well culture slides (Beckton Dickinson) for 18-24 h at 37°C, followed by slow cooling to 3°C (~1 hr). The cells were rinsed with ice cold PBS containing 1% BSA and incubated at 3°C for 2 hr with different dilutions of the labeled toxin (10^{-9} - 10^{-6} M). After three washes with ice cold PBS-BSA, the cells were fixed with freshly prepared 4% paraformaldehyde for 15 min. The paraformaldehyde was decanted off, followed by washing with PBS only. Cover glasses were
mounted on slides in Slowfade (Molecular Probes, Eugene, OR). Cells were examined with an Olympus BX60 System Microscope, and the images were formatted in Adobe Photoshop (version 3.0) by SPOT RT Software v 3.0 (Diagnostic Instruments, Inc., Sterling Heights, MI) on an Apple Macintosh computer. For fluorescence observation, dye was excited by a 100 W Hg arc lamp operated by a stabilized power supply. A filter that was appropriate for Alexa™ Fluor 488 dye was used (absorption and emission maxima of approximately 494 nm and 519 nm, respectively). Cells on glass slides were observed with a 40x objective.

Fluorescence images were recorded for each cell field with an integrating CCD array camera (RT Color, Diagnostic Instruments, Inc., Sterling Heights, MI) connected to the microscope. The cells were exposed for 4 seconds utilizing clear filter at pixel bit depth of 12 (mono) bits per pixel. The image generated was subjected to fluorescence brightness values and quantified on a monochromatic scale as Fluorescence Arbitrary Units. Fluorescence brightness values from 20 different locations on each cell were averaged, and at least 25 cells were included for each experimental condition. A background image obtained from a control, toxin-free cell field was subtracted from each fluorescence image.

Transcytosis of toxin by polarized T-84 cells. Transwell® Clear Inserts (Corning Incorporated, NY) were coated with rat tail collagen type I as previously described (Maksymowych and Simpson, 1998). T-84 cells were plated at confluent density (~1x10⁵ cells) on inserts. Medium in the upper chamber (0.5 ml) bathed the apical (or mucosal) surfaces of cells, and medium in the lower chamber (1.0 ml) bathed the basolateral (or serosal) surfaces of cells. Cells were incubated at 37°C, and medium was changed every 2 days. The cultures were allowed to differentiate for a minimum of 10 days, and experiments were typically performed on days 10 to 15. Formation of tight junctions between cells in the monolayer was confirmed by measuring transepithelial resistance.

Cells grown on culture inserts were cooled to 3°C and rinsed with ice cold PBS-BSA. Labeled toxin (1x10⁻⁷ M) was added to the upper wells of the Transwell apparatus, at 3°C for 90
min (time for asymptote to binding). Unbound labeled toxin was removed by washing cells with ice cold PBS-BSA. Cells were then incubated at 37°C with pre-warmed culture medium in the upper chamber and Hanks Balanced Salt Solution (GIBCO) in the lower chamber for different time intervals, ranging from 0 min to 20 min. The contents of lower wells were collected to monitor the transport of labeled toxin from the apical to basolateral side, and the labeled material was detected in a luminescence spectrometer (Perkin-Elmer, Model LS50B) using FL WinLab software (Perkin-Elmer, Norwalk, CT).

**Visualization of binding and transcytosis.** For visualization experiments, cells were treated as just described. At various times after initiation of internalization (i.e., raising temperature to 37°C), cells were fixed with freshly prepared 4% paraformaldehyde for 20 min followed by washing with PBS only. Membranes from the culture inserts containing the fixed cells were carefully excised and placed on glass slides. Cover glasses were mounted on slides in Slowfade (Molecular Probes, Eugene, OR), and cells were examined using a BIO-RAD MRC 1024 confocal imaging system (Krypton-Argon laser [488 nm excitation] fitted to an Olympus IX70 inverted microscope). Images were captured using a 60x PlanApo objective, and the pixel size was 0.11 µm. For a uniform comparison, cells of similar heights (16 µm - 20 µm) were randomly selected to monitor internalization and transcytosis of toxin, and serial x-y confocal sections were recorded in 2 µm increments. Images were formatted using a proprietary, customized software.

**Data analysis.** In all figures, the data points represent the mean ± S.D. The number of observations for each experiment is stated in the text or figures. Significance and differences were determined using Student's t-test.
RESULTS

Purification, labeling and bioassay of toxin preparations. Botulinum toxin type A was isolated by conventional techniques (DasGupta and Sathyamoorthy, 1984; Simpson et al., 1988). As judged by scanning densitometry of SDS-PAGE gels of the final product, the toxin was greater than 95% homogeneous. This material was labeled with Alexa Fluor™ 488 according to manufacturers instructions. However, this produced a higher than necessary ratio of moles of dye to mole of toxin, which in turn appeared to cause an unacceptable loss of pharmacologic activity. Therefore, reaction conditions were slightly modified (i.e., shorter incubation time; 30 min), resulting in a final product with an average ratio of moles of dye per mole of toxin of ca 7.5.

The fluorescent dye has a succinimidyl ester moeity that reacted with primary amines of proteins to form a dye~toxin conjugate. This chimeric molecule was stable and usable for at least three months.

Both in vivo and in vitro experiments were done to assess residual toxicity of the dye~toxin complex. For in vivo experiments, animals (group n=3) were challenged intraperitoneally with 1 µg of toxin (or toxin equivalent for dye~complex). The mean survival time of animals receiving untreated toxin was 75 ± 5 min; that of animals receiving dye~toxin complex, 90 ± 19 min (p > 0.05). For in vitro experiments, a concentration of $1 \times 10^{-11}$ M toxin was added to isolated phrenic nerve-hemidiaphragm preparations (group n=3), and paralysis times were monitored. The results for native toxin and for complex, respectively, were 46 ± 3 min and 53 ± 6 min (p > 0.05). These results make clear that limited modification of primary amines did not abolish the biological activity of botulinum toxin.

SNAP-25 cleavage assay. As a further test of residual activity, the dye~toxin complex was assayed for its ability to cleave SNAP-25. As shown in Figure 1, the chimeric molecule retained enzymatic activity, and its ability to cleave SNAP-25 was comparable to that of native toxin.
Concentration of labeled toxin. Several preliminary experiments were performed to determine the optimum concentration of dye-toxin complex for visualization of binding to cells. Various concentrations of the complex (10^{-9} to 10^{-6} M) were incubated with cultures of T-84 cells for 2 hr at 3°C. The duration of incubation was sufficient to ensure that binding had reached equilibrium (see below). Cells were then washed with iced medium to remove any loosely associated material.

Examination of cells for amount and distribution of binding provided three major findings. First, auto-fluorescence from T-84 cells, under conditions that pertain to detection of the Alexa dye, was very low and did not impede efforts to detect the dye-toxin complex. Second, cell association of the dye-toxin complex with the surface of T-84 cells could be measured over the entire concentration range (Fig. 2). However, a maximum fluorescence was attained in the vicinity of 1 \times 10^{-7} M. Unless otherwise stated, this is the concentration that was employed in subsequent studies. Third, binding sites for the toxin were not localized in any particular region. To the contrary, binding appeared to be distributed across the cell surface. This was true, regardless of toxin concentration, which indicates that there was no concentration-dependent binding to any specific domain on the cell surface.

Rate of toxin association with cells. The prospects for visualizing toxin internalization and transport across cells can be maximized in two ways: 1.) ensuring that there is saturation or near-saturation of binding sites on the cell surface, while 2.) ensuring that bound toxin remains at the cell surface until the internalization process is initiated. The latter is especially important, because it allows one to synchronize a single wave of toxin movement through all cells in a monolayer (see below).

To determine the timecourse for dye-toxin association with cell surface receptors, T-84 cells grown on culture plates were cooled to 3°C (to prevent receptor-mediated endocytosis), then exposed to the labeled toxin (1 \times 10^{-7} M) for varying lengths of time (0 to 120 min). After exposure, cells were gently washed (3x) with iced PBS-BSA, fixed with 4% paraformaldehyde,
then submitted to a second series of washings. Under these conditions, the amount of dye-toxin complex that bound to cells increased over a period of approximately 90 min, and remained stable thereafter (Fig 3). The rate of approach to asymptote indicated a half-time for association of 30 to 40 min.

**Rate of toxin dissociation from cells.** Toxin dissociation from neuronal receptors on intact cells is known to be slow (Schiavo *et al.*, 2000; Habermann and Dreyer, 1986; Wellhöner, 1992; Halpern and Neale, 1995). The phenomenon of toxin dissociation from epithelial cells has not been previously described.

The dye-toxin complex was allowed to associate with cells under conditions that maximize binding (1 x 10^{-7} M; 90 min; 3°C), then washed gently with iced PBS-BSA and resuspended in medium with unlabeled toxin (1 x 10^{-7} M). The extent of binding after washing was monitored for an additional 90 min (3°C). The results (Fig. 4) indicated that toxin dissociation was slow relative to the rate of association. Only approximately 16% of the cell surface labeling was lost over the 90 min of observation. This finding is important, because it means that the majority of toxin molecules that associate with cells during a binding paradigm remain on the cell surface and therefore are potentially available for receptor-mediated endocytosis.

**Transcytosis of labeled toxin.** T-84 cells were grown on Transwell inserts to form a monolayer with tight junctions (10 to 15 days). The existence of tight junctions that impede paracellular diffusion in these preparations was confirmed by measurement of transepithelial resistance, which averaged ca. 300 Ω/cm^2.

Dye-toxin complex was added to cells under conditions that maximize binding (see above). After being washed, cells were rapidly warmed to 37°C to trigger internalization and transcytosis of the chimeric molecule. The contents of the lower wells were then assayed for labeled toxin. As shown in Figure 5, traces of fluorescent material began to appear on the
contralateral side of monolayers within as little as 5 min, and delivery of this material continued for at least 20 min.

**Absence of toxicity associated with the binding paradigm.** Experiments were done to ensure that the paradigm of lowering temperature during the binding paradigm did not adversely affect the transcytosis process. For this purpose, cells were grown and maintained as described earlier. Half of the Transwell inserts with monolayers were then cooled to 3°C for 90 min, whereas the others were maintained at 37°C. At the end of this process, all cells were washed and transferred to medium at 37°C. Transcytosis was initiated by adding dye–toxin complex, and the rate of appearance on the contralateral side of monolayers was monitored. The data revealed that labeled toxin began to appear in all of the lower wells within the same amount of time (ca. 5 min). There was no evidence for a slower rate of transcytosis in cells that had been transiently cooled.

**Visualization of binding and transcytosis.** T-84 cells were grown on culture inserts, then exposed to toxin under conditions that maximize binding (as above). After washing, cells were exposed to medium at physiological temperature to trigger internalization. At various times after warming, cells were submitted to serial confocal sectioning at 2 µm increments.

As shown in Figure 6, toxin association with cells at time 0 min was concentrated at the apical cell surface. Serial sections through cells revealed progressively lesser amounts of dye–toxin with progressively greater depth. Optical slicing always revealed small patches of labeling outside the field of the cell of interest, which at least in part was due to neighboring cells that were not the same height.

Within 5 min of initiating internalization, a substantial fraction of the dye complex at the cell surface had entered cells and made progress in transcytosis from the apical to the basolateral surface. There were two major observations that emerged from these images. First, although the only toxin that was available for internalization was that already bound to the cell surface, this toxin was not internalized as a single pool. In as little as 5 min, fractions of the
total pool were distributed throughout most of the cell. One implication of this finding is that at saturation of binding, the number of toxin–receptor complexes is greater than can be accommodated, or more disperse than can be accommodated, by a single simultaneous wave of receptor-mediated endocytosis.

The second observation pertains to the morphology of human gut epithelial cells. As shown in Figure 6, toxin that had left the surface and approached the middle of the cell assumed a "halo-like" appearance. This outcome was a by-product of cell anatomy. Human gut epithelial cells possess unusually large, centrally located nuclei, and this of necessity displaces transcytotic vesicles to the periphery (Madara et al., 1987). The unusually large size of these nuclei is illustrated in the phase contrast images (Fig. 6).

Within 10 min of initiating internalization, all or nearly all of the toxin had left the surface and was localized at the midline or lower. Clearly, some fraction of the molecules had already reached the basal surface and was available for release. As before, toxin in the midsection of the cell assumed a halo-like appearance, but toxin toward the bottom of the cell - which presumably had progressed beyond the nucleus - was distributed across the cell width.

The pattern of labeling at 20 min continued the pattern seen at earlier timepoints. In a progressive manner, dye–toxin complex that was initially bound on one side of the monolayer was all moving toward the opposite side, presumably for release. It is encouraging that the timecourse of this morphologic analysis (e.g., Fig. 6) correlates well with the earlier analysis of timecourse based on capture and measurement of transcytosed material in the lower wells (e.g., Fig 5).
DISCUSSION

Botulinum toxin is an oral poison, which means that the molecule must have an efficient mechanism to escape the lumen of the gut and reach the general circulation. A number of kinetic studies utilizing iodinated toxin have been performed in an effort to clarify the mechanism by which the toxin crosses epithelial barriers (Maksymowych and Simpson, 1998; 2004; Maksymowych et al., submitted for publication). The results of this work indicate that the toxin penetrates cellular barriers by a specific process of binding, transcytosis, and eventual release.

The work with iodinated toxin has contributed to our understanding of epithelial transport, yet it leaves many important questions unanswered. Most obviously, the earlier studies do not localize the sites of toxin binding on the cell surface, they do not identify the paths of toxin movement through cells, and they do not localize the sites of toxin release on the contralateral surface of the cells. Therefore, the present study was undertaken to address these issues. More precisely, work was done: a) to develop a technique for better characterizing the interaction between botulinum toxin and epithelial cells, b) to visualize the processes of binding, transcytosis, and release of toxin by a human gut epithelial cell line, and c) to compare the handling of botulinum toxin by transport cells (absorptive enterocytes) and by target cells (peripheral cholinergic neurons).

Visualizing the process. Botulinum toxin was labeled with a fluorescent dye (Alexa Fluor™ 488) that reacts preferentially with surface lysine residues. Both the heavy chain and the light chain of botulinum toxin type A have large numbers of exposed lysines (Fig. 7). As indicated earlier (Methods), labeling was done under conditions that resulted in only 7-to-8 moles of dye per mole of toxin. Therefore, it was not surprising that the toxin-fluor complex retained the biological activity needed to cross epithelial barriers as well as to block neuromuscular transmission.

In relation to epithelial binding and transcytosis, a protocol was used that imposed synchrony on toxin action. The labeled molecule was incubated with cells under conditions that
allowed cell surface binding to go to completion, but that retarded the process of internalization. The observations that resulted from this procedure allow one to conclude that: a) binding sites are distributed across the apical surface of gut epithelial cells, and b) there was no clustering of binding, as is sometimes associated with lipid rafts. The latter observation does not necessarily rule out an involvement of lipid rafts. It does, however, mean that any clustering, should it occur, must happen on a microscale.

The internalization process was triggered by warming monolayers. The manner in which the labeled toxin moved into the cells permits one to draw two more conclusions. Firstly, the layer of toxin bound to the cell surface did not enter cells, nor did it move through cells, as a tightly discrete band. To the contrary, within approximately 5 min the internalized toxin was dispersed throughout a substantial portion of the cell length. As indicated earlier, this suggests that a single wave of receptor-mediated endocytosis cannot accommodate the number of toxin–receptor complexes that are occupied at saturation.

The second conclusion relates to events that occur after all observable toxin has left the cell surface. Interestingly, none of this toxin appears to be retained locally in the vicinity of endocytosis sites. This outcome is strikingly different from that seen at cholinergic nerve endings (see below).

Visualization experiments demonstrated that endocytosed toxin moved from one cell surface to the opposite cell surface, but the path followed by the toxin was certainly not direct. In the midsection of the cell, all transported toxin was displaced to the periphery. This was an inescapable byproduct of cell anatomy. Human gut epithelial cells have unusually large and centrally located nuclei. The transport process had to navigate around this anatomical obstacle.

Perhaps of greater interest, toxin that had made its way past the nucleus did not remain on a direct path to the basal surface. Somewhat surprisingly, at least a portion of the routes for transport moved back toward the cell midline before delivering the toxin to the basal membrane for subsequent release. These results suggest that it would be instructive to test microtubule
and microfilament disruptive agents to determine the underlying mechanisms that govern the routes of transport.

**Cell handling of toxin.** Poisoning due to botulinum toxin involves major interactions with at least two cell types. Epithelial cells ferry the molecule from the lumen of the gut to the general circulation, and thus these can be labeled “transport cells”. Peripheral cholinergic nerve endings are the principal site of toxin action, and thus they can be called “target cells”. It is instructive to compare the handling of botulinum toxin by transport cells and target cells.

Black and Dolly performed the first - and thus far the only - detailed examination of botulinum toxin binding to motoneurons (Black and Dolly, 1986a; Black and Dolly, 1986b). This elegant study demonstrated specificity and saturability of binding, and it also showed the distribution of binding sites. On the one hand, binding was largely confined to those portions of the nerve cell in close apposition to muscle; but on the other hand, binding was rather evenly distributed in these regions. At least in the latter respect, nerve cells and epithelial cells are similar. Both display binding sites across the cell surface.

There are many studies that have reported the kinetics of toxin association and dissociation with intact nerve cells or isolated membrane preparations of CNS origin (Habermann and Dreyer, 1986; Wellhöner, 1992, Halpern and Neale, 1995). The time constants reported in the present study are apparently in keeping with this earlier literature on neuronal receptors. However, there is one aspect of epithelial binding that differs from that of neuronal binding. The experiments that quantified amount of dye~toxin binding as a function of dye~toxin concentration suggest that affinity for epithelial sites is less than that associated with neuronal sites. This possibility will require further study to confirm, but it is worth noting that there could be a plausible explanation for any differences in affinity. The principal site of toxin absorption is the upper small intestine, although binding can also occur in the stomach and distal portions of the intestine (Maksymowych et al., 1999). This means that the potential surface area that mediates binding is enormous, and thus binding sites associated with a $K_d$ in
the range of $10^9$ to $10^7$ would be highly efficient in achieving absorption. By contrast, the principal site of toxin binding in the periphery is the cholinergic nerve ending. The surface area of all peripheral motor nerve endings is miniscule compared to the surface area for toxin absorption in the gut. Therefore, the need for a lower $K_d$ to achieve a high level of affinity and a high level of toxicity is obvious.

For both neuronal cells and epithelial cells, the binding step is followed by an internalization process. It is at this point that cell handling of the toxin begins to differ dramatically. In peripheral cholinergic nerve endings, internalization involves two major events: receptor-mediated endocytosis and pH-induced translocation to the cytosol. In addition, the structure of the molecule is altered. The interchain disulfide bond is reduced, leading to release of the catalytically active light chain. The release of active polypeptide in the immediate vicinity of the endocytosing membrane is a prelude to eventual blockade of exocytosis (Simpson, 2004). By contrast, internalization in epithelial cells does not lead either to pH-induced translocation or to local accumulation of toxin. The visualization experiments reveal that the bulk of endocytosed toxin rapidly disappears from the vicinity of the endocytosing membrane en route to the opposite side of the cell. Furthermore, toxin released from the contralateral side of epithelial cells has no obvious structural changes. To the contrary, it must be modified experimentally (viz., disulfide bond reduction) before it will cleave isolated substrate.

The differences in toxin handling by the two cell types are a reflection of the differences in their function in systemic poisoning (Simpson, 2004). As discussed earlier, epithelial cells can be envisioned as transport cells. The toxin binds exploitatively to them and is carried from the lumen of the gut to the general circulation. Motor neurons can be envisioned as target cells. The toxin once again binds exploitatively, but in this instance it gains access to the cytosol and blocks exocytosis.

The fact that botulinum toxin interacts so differently with epithelial cells and with neuronal cells helps to explain the mechanisms that underlie its pharmacologic actions.
However, there is a second implication to the data that also warrants comment. Botulinum toxin is one of only a relatively small number of molecules that are handled differently by different cell types. Thus the toxin - or its minimal binding and internalization domain – could be used advantageously as research tools to help identify and characterize the cellular mechanisms for sorting and trafficking of macromolecules. Experiments that are designed to take advantage of this property of the toxin are currently in progress.
REFERENCES


**FOOTNOTES**

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LEGENDS FOR FIGURES

FIG. 1. **SNAP-25 cleavage by labeled toxin**  Botulinum toxin labeled with dye was reduced with DTT (8 mM) at room temperature for 30 min in cleavage buffer (see Methods and Materials). GST-SNAP-25 (150 ng) was added to reduced toxin and the reaction was allowed to proceed for 3 hr at 37°C. Enzymatic cleavage reactions were terminated by adding an equal volume of 2x sample buffer, and the samples were separated in a 10% polyacrylamide gel. The cleavage products were transferred to a nitrocellulose membrane and probed with rabbit anti-SNAP-25 antibody. Anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody, and the reaction was visualized by chemiluminescence using X-ray film. Lane 1 is the control SNAP-25 without any toxin treatment. Lanes 2, 3, 4, and 5 show the cleavage products of SNAP-25 by native toxin (in ascending concentrations of 50, 100, 150, and 200 nM, respectively). Lanes 6, 7, 8, and 9 show the cleavage products of SNAP-25 by labeled toxin (in ascending concentrations of 50, 100, 150, and 200 nM, respectively). A concentration of 200 nM of either native or labeled toxin cleaved the SNAP-25 completely (lanes 5 or 9). Lower concentrations of each preparation had diminished but comparable abilities to cleave substrate (lanes 2 and 3 or 6 and 7).

FIG. 2. **Determination of optimum fluorescence values.**  T-84 cells were incubated with different concentrations of the Alexa Fluor™ 488 labeled toxin (1x10⁻⁹ - 1x10⁻⁶ M) at 3°C for 2 hr. After three washes with iced PBS-BSA, cells were fixed with 4% paraformaldehyde, mounted and observed under a fluorescence microscope. The resulting fluorescence brightness was quantified on a monochromatic scale as Fluorescence Arbitrary Units.
FIG. 3. **Time course for asymptote to binding.** T-84 cells were incubated at 3°C with 1x10^{-7} M labeled toxin for different time intervals (0 - 120 min), followed by washing and fixation. Toxin binding to the T-84 cell surface was found to increase with time up to 90 min, as revealed by the increase in fluorescence. The asymptote to binding curve indicates that the half-time of botulinum toxin binding to T-84 cells is 30 to 40 min.

FIG. 4. **Dissociation of toxin binding.** T-84 cells were pre-incubated with the labeled toxin (1x10^{-7} M) for different time intervals (0, 30, 60, and 90 min) at 3°C. All of the wells that were incubated for 0, 30, and 60 min, as well as half the wells incubated for 90 min, were used to quantify the amount of toxin association. The remaining wells, which had been incubated for 90 min, were washed and then incubated in the absence of labeled toxin and in the presence of unlabeled toxin (1x10^{-7} M). The amount of toxin dissociation was only ~16% when observed for 90 min after washing.

FIG. 5. **Transcytosis of toxin across polarized T-84 cells.** T-84 cells grown on culture inserts were incubated with labeled toxin (1x10^{-7} M) at 3°C for 90 min. Unbound toxin was removed by washing, and the cells were incubated at 37°C for different time intervals, ranging from 0 min to 20 min. Labeled toxin was collected in the lower wells and detected by fluorescence spectrometry. Control (culture medium only) or 0 min incubated cells (C and 0, respectively) showed little or no dye~toxin fluorescence in the lower wells. Cells incubated for 5 min showed a small initial amount of transcytosis of dye~toxin, and cells incubated for 10 or 20 min showed substantial amounts of transcytosis.

FIG. 6. **Internalization and transcytosis of toxin in polarized T-84 cells.** T-84 cells grown on culture inserts were incubated with the labeled toxin (1x10^{-7} M) at 3°C for 90 min. Unbound toxin was removed by washing, and the cells were incubated at 37°C for different time intervals,
ranging from 0 min to 20 min. The cells were washed with cold PBS-BSA and fixed with 4% paraformaldehyde. Membranes containing the fixed cells were carefully excised, placed on glass slides and mounted for confocal microscopy. Serial x-y confocal sections were obtained in 2 µm increments. The first lane (0 min) shows bound toxin on the apical side of the cell. After 5 min of incubation at 37°C, the toxin entered cells, and moved to the midline or slightly beyond (5 min). As explained in the text, the "halo-like" appearance was due to migration of toxin around the nucleus (see phase contrast images in the right column). At later timepoints (10 min; 20 min) the toxin continued to move through the cell - and past the nucleus - to reach the basal surface.

Fig 7. Structure of botulinum neurotoxin type A. The toxin molecule is composed of a heavy chain (dark blue; 100 kDa) and a light chain (light blue; 50 kDa). Both chains have numerous exposed lysine residues (yellow). The figure illustrates two views of the toxin molecule and the surface lysine residues (i.e., 180° rotation).
Figure 1
Figure 2

Fluorescence Arbitrary Units

Concentration (molar)

$10^{-6}$ $10^{-7}$ $10^{-8}$ $10^{-9}$
Figure 3

The graph shows the relationship between time (in minutes) and fluorescence (in arbitrary units). The data points are depicted with error bars, indicating the variability or uncertainty in the measurements. The trend line suggests a linear increase in fluorescence over time.
Figure 4
Figure 6

Top

0 min. | 5 min. | 10 min. | 20 min. | Phase

Bottom