Analysis of the Mechanisms That Underlie Absorption of Botulinum Toxin by the Inhalation Route

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Botulinum toxin is a highly potent oral and inhalation poison, which means that the toxin must have an efficient mechanism for penetration of epithelial barriers. To date, three models for toxin passage across epithelial barriers have been proposed: (i) the toxin itself undergoes binding and transcytosis; (ii) an auxiliary protein, HA35, transports toxin from the apical to the basal side of epithelial cells; and (iii) an auxiliary protein, HA35, acts on the basal side of epithelial cells to disrupt tight junctions, and this permits paracellular flux of toxin. These models were evaluated by studying toxin absorption following inhalation exposure in mice. Three types of experiments were conducted. In the first, the potency of pure neurotoxin was compared with that of progenitor toxin complex, which contains HA35. The results showed that the rate and extent of toxin absorption, as well as the potency of absorbed toxin, did not depend upon, nor were they enhanced by, the presence of HA35. In the second type of experiment, the potencies of pure neurotoxin and progenitor toxin complex were compared in the absence or presence of antibodies on the apical side of epithelial cells. Antibodies directed against the neurotoxin protected against challenge, but antibodies against HA35 did not. In the final type of experiment, the potency of pure neurotoxin and toxin complex was compared in animals pretreated to deliver antibodies to the basal side of epithelial cells. Once again, antibodies directed against the neurotoxin provided resistance to challenge, but antibodies directed against HA35 did not. Taken collectively, the data indicate that the toxin by itself is capable of crossing epithelial barriers. The data do not support any hypothesis in which HA35 is essential for toxin penetration of epithelial barriers.

There are two mechanisms by which botulinum toxin (BoNT) can reach the general circulation, which is the compartment from which the toxin is distributed to vulnerable sites throughout the body (31, 32). In the first, the toxin (or the organism that makes the toxin) crosses ruptured barriers and is introduced directly into the body. Examples that illustrate this form of poisoning are (i) contamination of surgical wounds (5, 11, 14) and (ii) contamination of injection sites of illicit drug use (10, 17, 19, 24, 34). The second mechanism by which the toxin can reach the general circulation is penetration of epithelial barriers in the gut and airway, i.e., absorption (31). There is a consensus among investigators that absorption is a key step in the etiology of oral and inhalation botulism. However, there is no consensus on either the nature of the molecule or the nature of the mechanism that accounts for absorption.

Much of the confusion surrounding the absorptive process relates to the state of the neurotoxin molecule as it is found in nature. BoNT is typically encountered as part of a complex containing one or more auxiliary proteins. The best-characterized of these are the hemagglutinins (HA) with molecular masses of 15 to 17 kDa, 33 to 35 kDa, and 71 to 76 kDa and a nontoxin, nonhemagglutinin (NTNH), with a molecular mass of 130 kDa. There is agreement among investigators that auxiliary proteins can associate with certain serotypes of BoNT to form a complex that is highly resistant to proteolysis (4), which explains the ability of the toxin to survive endoproteases in the stomach (20). On the other hand, there is little agreement about the role of auxiliary proteins in the ability of the toxin to cross gut or airway epithelial barriers and reach the general circulation.

An examination of the literature suggests that the single biggest obstacle to establishing the true mechanism for absorption may be

the test systems that have been employed. Earlier work has been done mainly on *in vitro* preparations, and in many cases these preparations are not well suited for study of the absorptive process. In an attempt to overcome the deficiencies in these earlier reports, the present study examined the absorptive process *in vivo*, thus obviating questions about the appropriateness of *in vitro* models. *In vivo* studies on toxin absorption were done using the inhalation route, which provided two advantages. First, the airway does not have the same harsh conditions of low pH and proteolytic enzymes as the stomach. This afforded the opportunity to study not only the toxin complex, which is relatively resistant to metabolism, but also the pure neurotoxin. Second, the absence of harsh conditions in the airway permitted the use of antibodies as research tools. This would not have been possible with studies on oral absorption, due to the fact that antibodies are subject to gastric metabolism. There was one additional consideration that weighed in favor of analyzing the inhalation route, which is the fact that botulinum toxin is acknowledged to be more potent when administered via the airway than via the gut.

The use of *in vivo* inhalation poisoning as a model for analyzing

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the mechanisms that underlie absorption has, for the first time, allowed a critical assessment of the various models for toxin entry into the body. The data that have emerged from this work strongly support the premises that the neurotoxin can cross epithelial barriers without assistance from auxiliary proteins and that the underlying mechanism for absorption is transcytosis through cells rather than paracellular movement between cells.

MATERIALS AND METHODS

Animals. Female New Zealand White rabbits weighing 2 to 3 kg (Covance Research Products, Denver, PA) were used to generate antibodies. Female Swiss-Webster mice weighing 20 to 25 g (Ace Animals, Boyertown, PA) were used in toxicity experiments. All animals were housed in the animal facility at Thomas Jefferson University 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.

Toxin. Botulinum toxin type A (BoNT/A) and type E (BoNT/E) were purchased from Metabio (Madison, WI). BoNT/A was selected for study because it exists in two forms: pure neurotoxin and complete progenitor toxin complex (i.e., containing all three forms of HA). BoNT/E does not exist as a complete progenitor toxin complex (i.e., it does not possess any form of HA). Therefore, it was studied as the pure neurotoxin and as part of an incomplete complex that does not contain HA.

BoNT/A was obtained in a nicked and biologically active form. BoNT/E was isolated in the single-chain form and subsequently nicked, as previously described (29). The nicked toxin was collected, aliquoted, and stored at -20°C until use. A sample of the material was examined by electrophoresis to verify the degree of nicking and the integrity of the dischain toxin.

To facilitate comparisons of potency, the text and the figures present dose-response data in terms of amount of administered neurotoxin. Thus, for any given dose that was administered, the amounts of protein for pure neurotoxin and for progenitor toxin complex were different, but the amounts of neurotoxin (i.e., moles of neurotoxin) were identical.

Assay for neurotoxin. A two-step procedure was used to quantify the neurotoxin content of the pure toxin preparation and the progenitor complex preparation: a capture immunoassay followed by a mouse challenge assay. For the initial procedure, the toxin was quantified with a luminescent immunoassay by using a sandwich of capture and biotinylated reporter antibodies. The immunoassay for quantification of pure and complex BoNT/A utilized a human monoclonal anti-light-chain antibody as a capture device and a biotinylated affinity-purified rabbit polyclonal anti-heavy-chain antibody as a reporter device. The immunoassay for quantification of pure or complex BoNT/E utilized an isolated heavy-chain rabbit IgG as a capture device and a biotinylated anti-heavy-chain rabbit IgG as a reporter device. For both assays, capture antibodies diluted in phosphate-buffered saline were coated onto black Nunc Maxisorp 96-well plates and stored overnight at 4°C . Plates were blocked with 2% nonfat dry milk in phosphate-buffered saline with 0.05% Tween 20 (NFDMPBST) for 30 min at 37°C and then washed with PBST. Botulinum toxin standards and samples diluted in NFDMPBST were added to plates and shaken slowly for 1 h at room temperature. Plates were washed with PBST, and biotinylated reporter antibodies diluted in NFDMPBST were added to washed plates and incubated for 30 min at 37°C . A streptavidin poly-horseradish peroxidase (poly-HRP) conjugate diluted in NFDMPBST was added to washed plates and incubated for 30 min at 37°C , followed by additional PBST washes. Plates were injected with luminol (Thermo Scientific SuperSignal ELISA Femto Substrate), and relative luminescence values were measured with a Biotek Synergy 2 Luminometer.

Preparations of pure toxin and complex toxin that appeared to be identical in their neurotoxin content when assayed by the luminescent procedure were subsequently bioassayed in a mouse challenge experiment (see below), in which the intraperitoneal 50% lethal dose (LD_{50}) was 5 to 7 pg. Comparisons of the inhalation potency of pure toxin preparations

and complex toxin preparations were performed only when the two preparations had the same neurotoxin content in both the luminescent immunoassay and the mouse challenge bioassay.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (18). Either pure neurotoxin or progenitor complex was run on 4 to 15% separating gels and then visualized by staining with Coomassie blue. The pure-neurotoxin forms of BoNT/A and BoNT/E were also run after partial reduction. The toxins were reduced by incubation in 20 mM HEPES buffer, pH 7.4, with 5.0 mM dithiothreitol, 0.25 mM ZnCl_2 , and 0.05% Tween 20 for 30 min at 37°C .

Pulldown assays. SDS-PAGE was used to determine whether the various components of the progenitor toxin complex were present. A pulldown assay was used to determine whether the components formed an intact complex.

Sepharose beads (0.5 mg, 4B, 90 μm ; GE Healthcare, Piscataway, NJ) were activated by suspension in 50 ml of 1 mM HCl buffer and then centrifuged at 1,500 rpm for 15 min at 4°C . The supernatant was discarded, and the procedure was repeated three times.

The coupling procedure was done by adding 2 mg of purified antibodies (see below) to activated beads in 5 ml of coupling buffer (0.1 M NaHCO_3 , pH 8.3, containing 0.5 M NaCl). The mixture was incubated overnight and then centrifuged and washed three times to remove free antibody. Any residual binding sites on the beads were blocked by incubation with 20 ml of 0.1 M Tris-HCl buffer (pH 8.0) for 2 h at 4°C . The mixture was centrifuged at 1,500 rpm for 10 min at 4°C , after which the beads were washed two times with two buffers: (i) 20 ml of 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0, and (ii) 20 ml of 0.1 M Tris-HCl buffer containing 0.5 M NaCl, pH 8.

For each experiment, 5 ml of phosphate-buffered saline (pH 6 or 7.4) was added to beads, and the mixture was transferred to 0.2- μm Centrux filters as described earlier. An initial wash with phosphate-buffered saline was performed to further ensure removal of any free antibody. Either free neurotoxin or toxin complex (final volume, 500 μl) was added to the column and incubated for 1 h at room temperature with slow shaking. Columns were centrifuged at 1,500 rpm for 2 min at 4°C , and the flow-through was collected. This material was used immediately, as described in Results.

Antibody (antiserum). The carboxy-terminal portion of the heavy chain of BoNT/A (HC50) was cloned, expressed, and purified as reported previously (22). This material, which was used to evoke neutralizing antibodies, had a homogeneity of $>98\%$ as determined by SDS-PAGE. The entire HA with a molecular mass of 35 kDa (HA35) was also cloned, expressed, and purified (16). This material too was used to evoke neutralizing antibodies.

Antiserum was obtained by immunizing rabbits. An initial injection of alum-adsorbed antigen was given subcutaneously to rabbits (50 μg); three subsequent injections of adsorbed material (50 μg each) were given at 2, 4, and 8 weeks. Fourteen days after the final booster, aliquots of blood were drawn and the circulating titer of IgG was determined by enzyme-linked immunosorbent assay (ELISA). For the experiments reported in this study, the circulating titer of IgG, as determined by dilution assay, was always 10^5 or higher.

Antibody purification. IgG was isolated from polyclonal antiserum through protein A-based affinity purification using a Montage Antibody Purification kit supplied by Millipore (Billerica, MA). Purified IgG in phosphate-buffered saline, pH 7.4, was stored with 5% glycerol at -20°C .

ELISA. Antibody titers were determined by standard procedures. Briefly, flat-bottom, 96-well Corning plates (Corning Incorporated, Corning, NY) were coated with the HC50 or HA35 polypeptides (300 ng/well) and incubated at 4°C overnight, followed by washing with phosphate-buffered saline plus Tween 20 (0.1%), pH 7.4. The plates were blocked with 1% bovine serum albumin (BSA). Twofold serial dilutions of serum samples were added to the plates and incubated at 37°C for 60 min. IgG titers were determined using peroxidase-conjugated goat anti-rabbit

IgG (Sigma-Aldrich, Inc., St. Louis, MO). Secondary antibodies were diluted 1:1,000 in phosphate-buffered saline. The primary and secondary antibodies were incubated for 60 min at 37°C, after which an *ortho*-phenylenediamine substrate solution in sodium citrate buffer (pH 5.0) containing 2 μ l of 30% H₂O₂ was added as a substrate, and the plates were incubated for an additional 10 to 15 min at 37°C. Endpoint titers were determined as the reciprocal of the last dilution yielding an absorbance at 450 nm that was above the control value (preimmune serum).

Assay for hemagglutination. Chicken red blood cells were used to conduct an agglutination assay, essentially as previously described (28, 36). Freshly drawn blood was mixed with Alsever's (anticoagulant) solution and then centrifuged at 800 $\times$ g for 10 min. The pellet was washed three times with 0.8% NaCl. Immediately prior to the assay, red blood cells were diluted with 0.8% NaCl to give a 1% suspension.

Recombinant HA35 and bovine serum albumin (negative control) were each diluted in 75 mM sodium phosphate buffer, pH 7.2, containing 75 mM NaCl. A 50- μ l aliquot of each protein was added to the wells of a U-bottom microtiter plate (Costar, Corning, Inc., NY), and this was followed with a 50- μ l aliquot of the red blood cell suspension. The plate was allowed to stand at 4°C for 45 to 60 min, after which the presence or absence of agglutination was scored.

For inhibition assays, anti-HA35 antibodies were incubated with HA35 for 1 h at room temperature. An aliquot of this mixture was added to the U-bottom plates, and the agglutination was conducted as just described.

Assays for toxicity. BoNT was administered by the intranasal (i.n.), intravenous (i.v.), and intraperitoneal (i.p.) routes. For i.n. administration, mice were lightly anesthetized with isoflurane (3 to 3.5%). A single bolus (10 to 20 μ l) was applied slowly to the nares. For i.v. administration, mice were similarly anesthetized with isoflurane. Toxin was given as a single bolus (50 μ l) via the tail vein. For i.p. administration, toxin (100 μ l) was administered to nonanesthetized animals.

BoNT was administered in three experimental paradigms. In the first, pure neurotoxin or toxin complex was administered by the i.v. and i.n. routes. The goal of the experiments was to determine the relative potencies of the two forms of the toxin when given by a route that does not require penetration of epithelial membranes (i.v.) or when given by a route that does require penetration of epithelial barriers (i.n.). In the second experimental paradigm, pure neurotoxin and toxin complex were preincubated with antibodies directed against HC50 or HA35. These mixtures were then administered to mice by the i.n. route. The goal of the experiments was to determine whether HC50 or HA35 played an essential role on the apical (luminal) side of airway epithelial cells.

In the third experimental paradigm, antibodies directed against HC50 and HA35 were administered i.v. to mice 18 h before intranasal challenge with toxin. This lengthy interval ensured that there was adequate time for antibodies to distribute to the basal (systemic) side of airway epithelial cells. This approach is feasible, because the half-life for anti-botulinum toxin antibodies in mice is longer than 2 weeks (2). Pure neurotoxin and toxin complex were then administered by the i.v. and i.n. routes. The goal of the experiments was to determine whether HC50 or HA35, when present on the basal side of epithelial cells, played an essential role in the absorption of toxin.

Measuring toxicity outcome. The most characteristic outcome of botulinum toxin action is neuromuscular blockade. This outcome is easily discernible as weakness and eventual paralysis of the muscles of locomotion and the muscles of respiration. When signs of serious neuromuscular weakness became apparent, animals were sacrificed in accordance with the American Veterinary Medicine Association recommendations (e.g., CO₂). The duration of survival was noted in minutes or hours depending on the type of experiment.

RESULTS

Characterization of the toxin. Experiments were done with BoNT/A as pure neurotoxin and as complete progenitor toxin

complex, and with BoNT/E as pure neurotoxin and as incomplete progenitor toxin complex (i.e., without HA). The molecular composition of the pure neurotoxins was confirmed by SDS-PAGE. As shown in Fig. 1A, the neurotoxins migrated as single bands under nonreducing conditions (molecular weight [MW], ~150,000), but the emergence of two new bands under mildly reducing conditions was seen (heavy chain, ~100,000; light chain, ~50,000). In neither case was there evidence for the presence of NTNH or any of the HAs.

The BoNT/A progenitor toxin complex is known to contain the neurotoxin, three hemagglutinins, and a single NTNH. There does not exist a comparable BoNT/E complex. Figure 1B illustrates a gel for BoNT/A complex that was processed under nonreducing conditions. The gel confirms that the progenitor complex possessed all of its respective components.

Dose-response experiments. Mice were challenged with BoNT/A and BoNT/E in a Latin-square paradigm (pure versus complex; intravenous route versus inhalation route). A comparison of the dose-response characteristics of pure neurotoxin given by the i.v. and i.n. routes is shown in Fig. 2A and B; a comparison of the dose-response characteristics of complete and incomplete progenitor toxin complex given by the same two routes is also shown in Fig. 2C and D.

An analysis of the dose-response data reveals two important points. First, the potency of BoNT given as an i.v. bolus was greater than that given as an i.n. bolus. This was true both for pure neurotoxin (Fig. 2A and B) and for toxin complex (Fig. 2C and D). In the case of pure neurotoxin, the magnitude of the disparity between i.v. and i.n. potency was in the range of 500- to 1,000-fold. The magnitude of the disparity for toxin complex was virtually the same. Interestingly, this was true for both the complete toxin complex (BoNT/A) and the incomplete toxin complex (BoNT/E).

The second major point to emerge from the dose-response experiments was that pure neurotoxin and progenitor toxin complex, when normalized for amount of toxin, were approximately equivalent in potency when assayed by the i.n. route (i.e., a route that requires penetration of epithelial barriers). A comparison of the dose-response curves in Fig. 2A and C and those in Fig. 2B and D indicated little difference between the two forms of the toxin.

Absorption of toxin. The hypothesis that HA35 can act as a transport protein to carry BoNT into the general circulation (7–9) and the hypothesis that HA35 can disrupt epithelial barriers to permit paracellular flux of toxin (15, 23) arise from studies done on BoNT/A. BoNT/E does not contain HA35, and thus it cannot be used to implicate HA35 in toxin absorption (see Discussion).

As a sequel to the studies showing that equivalent amounts of pure neurotoxin and progenitor toxin complex produce equivalent levels of toxicity, experiments were done to compare equivalent amounts of pure neurotoxin and progenitor toxin complex in terms of toxin absorption.

Pure BoNT/A and progenitor complex BoNT/A (each containing 500 ng of the neurotoxin) were administered by the inhalation route to mice. At various times thereafter (20 to 180 min), animals were sacrificed, blood was collected, plasma was generated, and the circulating titers of toxin were determined. As shown in Fig. 3, the rate and extent of neurotoxin absorption for the pure BoNT/A and for the progenitor complex BoNT/A were very similar. There was no evidence to suggest that the presence of HA35 in the progenitor complex had a significant effect on toxin absorption.

An inspection of the data in Fig. 3 indicates that a pseudo-

FIG 1 The components of pure toxin and progenitor toxin complex were identified. (A) BoNT/A and BoNT/E were submitted to SDS-PAGE (4 μ g of each toxin per lane) under nonreducing (N) and partially reducing (R) conditions. The locations of the holotoxin, heavy chain (HC), and light chain (LC) polypeptides are indicated. Note that each neurotoxin was essentially homogenous and that there was no presence of auxiliary proteins. Also note that reducing conditions led to diminished amounts of the holotoxin and to the appearance of its constituent chains. (B) BoNT/A in the progenitor complex form was submitted to SDS-PAGE under nonreducing conditions. Note that the complex possessed all of its constituent polypeptides. Also note that the high-molecular-mass HA from the toxin complex (HA 70-72) spontaneously dissociated into two components of ca. 52 kDa and 19 to 20 kDa, as previously reported (7). The molecular masses (in kDa) for each of the polypeptide components of the BoNT/A complex are given.

steady state for absorption was obtained between 60 and 120 min. Therefore, a second test was performed to assess the equivalence of pure toxin and progenitor toxin complex in terms of neurotoxin absorption. In this case, the administered dose of pure toxin and progenitor toxin complex contained 5 μ g of neurotoxin (i.e., 1 order of magnitude higher than that used in the previous experiment). The circulating titers of neurotoxin were measured at 60 min and 100 min (Fig. 4). Once again, the data indicated a close comparability between pure toxin and progenitor toxin complex in terms of neurotoxin absorption into the general circulation.

Generation of antibodies. Rabbits were immunized with recombinant versions of the two polypeptides that are reportedly essential for absorption of toxin: BoNT/A HC50 (i.e., the carboxy-terminal half of the toxin heavy chain); and HA35 (i.e., the entire polypeptide). The sera from immunized animals were assayed by ELISA. The dilution titers for anti-HC50 and anti-HA35 were both in the range of 5×10^5 .

Molecular structure of toxin complex. The purported value of antibodies as research tools hinges on the interrelationship of the various components in the toxin complex. As shown in Fig. 1B, all of the polypeptides that are constituents of the BoNT/A complex were present. However, these polypeptides must remain in association with one another for the antibody experiments to be meaningful.

Tests for integrity of the complex were performed by covalently linking anti-HC50 antibodies or anti-HA35 antibodies to beads and then incubating toxin complex with these beads. After incubation, the mixtures were centrifuged to remove beads with antibody-antigen complex from the solution. The supernatant

was administered i.p. to mice to determine the amount of soluble (unbound) toxin.

When beads with anti-HC50 antibodies were incubated with increasing doses of BoNT/A complex (neurotoxin content, 50 pg, 100 pg, 200 pg, or 400 pg) at pH 6.0, all of the neurotoxin was removed from the solution. Thus, administration of supernatant failed to cause death or even illness in any of the mice (Fig. 5A). When beads with anti-HA35 antibodies were incubated with toxin and then processed identically to the beads with anti-HC50 antibodies, the same result was obtained. All challenged animals survived without signs of illness. In contrast, when beads linked to gelatin rather than antibody were used, 100% of the challenged animals succumbed to the injection of supernatant. In this case, the added toxin was not removed from the solution.

A second and procedurally similar set of experiments were done with beaded antibodies at pH 7.4 (Fig. 5B). When anti-HC50 antibodies were involved, all of the toxin at all of the tested doses was removed from the solution, and as a result all of the challenged animals survived. When anti-HA35 antibodies were involved, little if any of the toxin was removed from the solution. Even at the low dose of 50 pg, all of the challenged animals succumbed to poisoning. As before, beads that were linked to gelatin had no ability to extract the toxin from the solution.

Addition of antibodies to the apical side of epithelial cells. Antibodies were used to evaluate the possibility that different proteins can act on the apical side of epithelial cells to influence the absorption of toxin. BoNT/A plus antibodies directed against the HC50 and HA35 polypeptides were used in this analysis.

As before, animals were challenged as part of a Latin-square

FIG 2 Pure BoNT/A (A), pure BoNT/E (B), complete BoNT/A progenitor complex (C), and incomplete BoNT/E progenitor complex (D) were administered by the intravenous route (—●—) or by the inhalation route (—■—). Each data point represents the mean value obtained from a group of 10 or more. For each data point, the standard error of the mean was <15% of the mean for that data point. The magnitude of the shift in the intravenous versus inhalation dose-response curves for pure toxin (A and B) indicates that the intravenous route was ca. 500- to 1,000-fold more potent than the intranasal route. The magnitude of the shift between the intravenous route and the intranasal route for progenitor complex (C and D) was ca. 500-fold. To facilitate the comparison of data in the four parts of the figure, the *x* axes reflect the amount of neurotoxin rather than the total amount of protein (e.g., toxin + auxiliary proteins) that was administered.

FIG 3 Pure BoNT/A (dashed line) and progenitor complex BoNT/A (solid line) were administered by the inhalation route to mice. For both forms of the toxin, the neurotoxin content was 500 ng. Animals (*n* = 6 per group) were sacrificed at various times after toxin administration, and plasma samples were assayed for neurotoxin. The results indicated that the initial rates of absorption and the subsequent pseudo-steady states were closely comparable for the two forms of the toxin.

paradigm (pure neurotoxin versus progenitor toxin complex; pH 7.4 versus pH 6.0). This paradigm reflects the fact that the toxin complex remains intact at acid pH but dissociates at neutral or physiologic pH (Fig. 5). The Latin-square paradigm was performed twice, reflecting the use of two antibodies (anti-HC50 and anti-HA35).

In all experiments, mice received pure neurotoxin or toxin complex amounts that were equal in terms of neurotoxin content (50 ng). The preparations were incubated without or with antiserum (10 μ l) at room temperature for 60 min at the indicated pH. A total volume of 20 μ l was then administered by the i.n. route, and animals were monitored for 96 h. The data are presented in Tables 1 and 2.

Antibodies directed against the HC50 domain afforded complete protection both against pure neurotoxin and against complex (Table 1). This protection was evident regardless of whether incubation was at pH 7.4 or pH 6.0.

The results with anti-HA35 serum were strikingly different (Table 2). To begin with, antibodies in this preparation did not protect against challenge with pure neurotoxin at either pH. How-

FIG 4 Pure BoNT/A (open bars) and progenitor complex BoNT/A (filled bars) were administered to mice by the inhalation route (neurotoxin content for both forms of the toxin, 5 µg). Animals (*n* = 6 per group) were sacrificed at 60 min or 100 min, and plasma samples were assayed for neurotoxin. At both time points, the circulating titers of the neurotoxin were comparable for the two forms of administered toxin.

ever, pure neurotoxin does not possess the antigen, so failure to provide protection was the anticipated outcome. Anti-HA35 antibodies also failed to protect against toxin complex incubated at pH 7.4, and this may or may not be viewed as an expected outcome. At pH 7.4, the complex may dissociate, and this could mean that antigen-antibody binding would not influence absorption of the neurotoxin. The most provocative finding occurred when complex was incubated with anti-HA35 antibodies at pH 6.0, which is a condition under which the complex would be expected

TABLE 1 Effect of soluble antibodies (anti-HC50) on inhalation poisoning with BoNT/A^a

Form of toxin	Antibody	pH	No. of surviving mice/total no.
Pure	—	7.4	0/20
Pure	+	7.4	20/20
Pure	—	6.0	0/20
Pure	+	6.0	20/20
Complex	—	7.4	0/20
Complex	+	7.4	20/20
Complex	—	6.0	0/20
Complex	+	6.0	20/20

^a The dose of pure neurotoxin was 50 ng; the dose of toxin complex was adjusted to produce an identical 50-ng dose of neurotoxin. The amount of antiserum was 10 µl. Mice were observed for at least 96 h after challenge. The animals in the four antibody groups did not develop any signs of poisoning.

to remain intact (Fig. 5A). Even under these conditions, the anti-serum still failed to provide protection.

Addition of antibodies to the basal (systemic) side of epithelial cells. *In vivo* experiments were done to test the premise that anti-HC50 antibodies or anti-HA35 antibodies can act on the basal (systemic) side of epithelial cells to influence absorption of toxin. Mice (*n* = 5 or more per group) were challenged i.v. with BoNT/A complex (neurotoxin content, 500 pg). Animals were injected i.v. with various doses of anti-HC50 serum 18 h before administration of the challenge dose of toxin. The results of this experiment indicated a dose-response relationship between the amount of administered antibody and the resistance to i.v. challenge with toxin (Fig. 6A). The 50% effective dose (ED₅₀) for protection against challenge was approximately 2.0 µl of antiserum, and complete neutralization was observed at 3.3 µl and higher.

FIG 5 Beaded antibodies (anti-HC50 and anti-HA35) were incubated with various amounts of BoNT/A complex as described in the text. The beads were then removed by centrifugation, and the supernatant was administered to mice (*n* = 10). (A) At pH 6.0, beaded anti-HC50 and anti-HA35 both removed toxin from the reaction mixtures. As a result, all challenged animals survived. (B) At pH 7.4, beaded anti-HC50 removed toxin from the solution, and thus all challenged animals survived. At this pH, beaded anti-HA35 did not remove any dose of toxin from the solution, and all challenged animals succumbed to poisoning. Beaded gelatin was used as a negative control. It failed to remove toxin from solution at either pH.

TABLE 2 Effect of soluble antibodies (anti-HA35) on inhalation poisoning with BoNT/A^a

Form of toxin	Antibody	pH	No. of surviving mice/total no.
Pure	—	7.4	0/20
Pure	+	7.4	0/20
Pure	—	6.0	0/20
Pure	+	6.0	0/20
Complex	—	7.4	0/20
Complex	+	7.4	0/20
Complex	—	6.0	0/20
Complex	+	6.0	0/20

^a The dose of pure neurotoxin was 50 ng; the dose of toxin complex was adjusted to produce an identical 50-ng dose of neurotoxin. The amount of antiserum was 10 μ l. Mice were observed for at least 96 h after challenge. All of the animals in all of the groups succumbed to poisoning.

As described earlier, equiactive doses of toxin administered by the i.v. route and the i.n. route differ 500- to 1,000-fold. Therefore, mice ($n = 5$ or more per group) were challenged i.n. with BoNT/A complex (neurotoxin content, 500 ng). As before, animals were injected i.v. with various doses of anti-HC50 antiserum 18 h prior to the challenge dose of toxin. The results from this experiment, which are shown in Fig. 6B, indicated that the dose-response curve for antibody neutralization of i.n. challenge was comparable to that for neutralization of an equivalent i.v. challenge.

In the next set of experiments, the efficacy of anti-HA35 antibodies was assessed. Various doses of anti-HA35 antiserum were administered i.v. to mice ($n = 5$ or more per group) 18 h before i.v.

challenge with BoNT/A complex (neurotoxin content, 500 pg). The resulting data indicated that pretreatment of animals with as much as 10 μ l of antiserum provided no measurable level of protection (Fig. 6A).

In a variation of this experiment, mice ($n = 5$ or more per group) received i.v. doses of anti-HA35 antiserum 18 h prior to i.n. challenge with BoNT/A complex (neurotoxin content, 500 ng). Once again, the results showed that no test dose of antiserum produced measurable protection against challenge with toxin (Fig. 6B).

Neutralizing activity of anti-HA35 antibodies. Anti-HC50 antibodies acted on both the apical and the basal (systemic) sides of epithelial cells to neutralize toxin activity. Anti-HA35 antibodies had no activity on either side of epithelial barriers. This can be interpreted as evidence that HA35 plays no essential role in absorption of the toxin, but an alternative and speculative explanation is that anti-HA35 antibodies do not possess neutralizing activity. To rule out the latter possibility, a neutralization assay was performed utilizing a known action of HA35, which is hemagglutination.

Recombinant HA35 was prepared at a concentration of 100 μ g/ml, and serial 2-fold dilutions were assayed for their ability to agglutinate chicken red blood cells. The minimum concentration that produced full agglutination was 25 μ g/ml (ca. 7×10^{-7} M) (Table 3). The same experiment was then done in the presence of serial dilutions of anti-HA35 antiserum. At a 1:3 dilution, the anti-HA35 antiserum completely neutralized the agglutinating activity of HA35, even at a concentration of HA35 that was 4-fold higher than the minimum needed for the molecule to agglutinate

FIG 6 Mice were passively immunized with anti-HC50 serum or anti-HA35 serum 18 h prior to challenge with BoNT/A complex. Antiserum was administered by the intravenous route (filled symbols, anti-HC50 serum; open symbols, anti-HA35 serum), and toxin complex was administered by the intravenous route (neurotoxin content, 500 pg) (A) or by the inhalation route (neurotoxin content, 500 ng) (B). Note that anti-HC50 produced dose-dependent protection against toxin by either route of administration, but anti-HA35 serum provided no protection against either route of administration. Each data point represents a population of 5 or more. The survival times of animals were monitored for 1 week. All mice that lived at least 96 h ultimately survived.

TABLE 3 Neutralizing activity of anti-HA35 antibodies on hemagglutination^a

Concn ($\mu\text{g/ml}$)	BSA	HA35	HA35 + antibodies ^b at dilution of:			
			1:3	1:6	1:12	1:24
100	—	+	—	+	+	+
50	—	+	—	—	+	+
25	—	+	—	—	—	+
12.5	—	—	—	—	—	—
6.2	—	—	—	—	—	—

^a Recombinant HA35 and BSA (negative control) were prepared at an initial concentration of 100 $\mu\text{g/ml}$, and 2-fold serial dilutions were tested. HA35 agglutinated red blood cells at concentrations of 100, 50, and 25 $\mu\text{g/ml}$. No concentration of BSA caused agglutination. +, agglutination occurred; —, no agglutination.

^b Antiserum was incubated with HA35 at the indicated dilutions prior to addition to red blood cells. At a 1:3 dilution, antibodies blocked the ability of HA35 to cause hemagglutination. When the antibodies were progressively diluted, their ability to block hemagglutination was progressively diminished.

red blood cells (100 $\mu\text{g/ml}$) (Table 3). Serial dilutions of antibody had a progressively diminished effect. At a serum dilution of 1:24, neutralizing activity of the antibodies was lost.

DISCUSSION

The ability of botulinum toxin to act as an oral or inhalation poison depends on the ability of the toxin molecule to cross barriers in the gut and airway. There is agreement among investigators that epithelial cells are integral to the process of absorption, but there is no consensus on the mechanisms that account for penetration of epithelial barriers. Thus, three models for toxin absorption have been proposed, and these models differ in terms of the molecule that purportedly mediates the process, as well as the mechanism that underlies the process. One model envisions that the toxin molecule crosses epithelial barriers by the process of binding and transcytosis (1, 21, 22). This model identifies the toxin itself as the molecule that recognizes epithelial receptors. Furthermore, the model envisions that the toxin can cross epithelial barriers in the complete absence of auxiliary proteins (e.g., HA and NTN1).

A second model, developed mainly from oral studies, agrees with the first in terms of mechanisms (binding and transcytosis across epithelial barriers), but it differs in its structure-function characteristics (7–9). According to this model, it is an HA component that binds to epithelial cells, and particularly the HA of intermediate molecular mass (33 to 35 kDa). This HA component of the complex acts to transport the noncovalently attached toxin from the lumen of the gut into the general circulation.

The third model, which also arises from oral studies, differs from the first two in terms of mechanism of penetration of epithelial barriers but may overlap them in terms of structural determinants (15, 23). This model suggests that there is an initial penetration step across epithelial barriers, after which some fraction of the absorbed dose of toxin complex reaches the basal side of epithelial cells. The complex dissociates to give free HA35, which acts to disrupt tight junctions. This allows residual toxin on the apical side of cells to diffuse between cells, meaning that absorption is primarily a paracellular rather than transcellular process.

In an attempt to determine which of the three models is the closest to the authentic absorption process, the present study has focused on an *in vivo* model. BoNT, either in pure form or in complex, was administered by the i.n. route to mice. The inhala-

tion route was selected because (i) it represents one of the natural routes of poisoning (13, 25) and (ii) it is associated with toxin absorption across epithelial barriers. An added advantage is that the airway does not have the same harsh conditions of low pH and proteolytic enzymes as the stomach, and this permitted the use of antibodies as research tools in structure-function analyses.

Dose-response data and model 1. The pure and complex forms of BoNT/A and BoNT/E were compared for their respective potencies when given by the i.v. and i.n. routes. In each case, the toxin was administered as a bolus rather than continuously (i.e., i.v. infusion or i.n. aerosol), and this greatly facilitated the analysis of data.

There were two revealing findings that emerged from the dose-response data. First, the pure BoNT/A neurotoxin and the pure BoNT/E neurotoxin were both active by the inhalation route (Fig. 2). This means that each of these neurotoxins possesses the inherent ability to cross epithelial barriers. In addition, preliminary studies on the BoNT/B neurotoxin have shown that it too is an inhalation poison (F. H. Al-Saleem, D. M. Ancharski, and L. L. Simpson, unpublished data). Thus, all three of the botulinum toxins that are most often associated with human illness can penetrate epithelial barriers and enter the general circulation in the complete absence of HA.

These experimental findings are strongly reinforced by clinical observation. The BoNT/E toxin complex is composed of neurotoxin and NTN1, but there are no HA components (26, 27). Nevertheless, the BoNT/E complex produces oral poisoning in humans (3, 6, 30, 33, 35). This means that both for inhalation poisoning (this report) and for oral poisoning (clinical observation), BoNT/E does not require any HA component to traverse gut or airway epithelial barriers.

A second finding to emerge from the dose-response experiments was that pure neurotoxin and progenitor toxin complex, when administered by the same route and in equimolar amounts, possessed comparable potencies. For example, when pure BoNT/A and complex BoNT/A were administered by the i.n. route in equimolar amounts, they displayed closely similar potencies (Fig. 2).

One possible concern associated with this work is that assays for toxicity represent an indirect method to deduce the rate and extent of toxin absorption. This indirect approach has been used extensively in the field of botulinum toxin research, but with the advent of sensitive techniques to quantify the toxin molecule this is no longer necessary. Therefore, the present study has measured the actual rates and extents of toxin absorption after i.n. administration. This is the first time such studies have been reported.

Two types of experiments were performed. In the first, BoNT/A in the pure form and in the complex form, both at a neurotoxin dose of 500 ng, were administered by the inhalation route. At various times ranging from 5 min to 180 min, plasma samples were obtained and assayed for the toxin molecule. An examination of the data at early time points (5 min to 20 min) indicated that the rates of absorption were similar for the two forms of the toxin. An examination of the data at later time points indicated that the extents of absorption were also comparable.

These data appear to show that absorption is not significantly influenced by the absence or presence of HA components. However, there is a possibility that this outcome could be a by-product of saturation. If absorption were occurring at a maximal rate at a dose of 500 ng, any putative ability of HA to enhance the process

might be difficult to detect. To rule out this possibility, a second experiment was done. The pure and complex forms of BoNT/A were administered at an equivalent neurotoxin dose of 5 μg (i.e., 1 order of magnitude higher than that used in the previous experiment). Plasma samples were obtained and assayed at the 60-min and 100-min time points. The data revealed that absorption was the same for the two forms of the toxin, and the circulating titers were much higher than those obtained at the lower dose. This result makes clear that the earlier findings on the rate and extent of toxin absorption were not adversely affected by saturation.

The assays for toxicity and the assays for the toxin molecule, when taken together, form the basis for strong conclusions. Thus, the fact that pure BoNT/A and BoNT/E can cause inhalation poisoning means that the neurotoxin by itself can penetrate epithelial barriers *in vivo* (model 1). Conversely, these observations rule out the possibility that HA35 is required as a device for transport (model 2) or as a device to disrupt tight junctions (model 3). The fact that the inhalation toxicity curves and inhalation absorption curves for equimolar amounts of pure toxin and complex are nearly identical lends further support to these conclusions. They demonstrate that the neurotoxin by itself can cross *in vivo* epithelial barriers and that the auxiliary proteins, and HA35 in particular, are not required.

Antibody experiments on the apical surface of epithelial cells. A conceptually different approach was used to gain further insight into the relative merits of the three models for toxin absorption. In this approach, two antibodies were used to help identify the molecules and the mechanisms that account for the ability of botulinum toxin to cross epithelial barriers. One antibody was directed against a recombinant polypeptide that represents the binding domain within the neurotoxin molecule (HC50); the other was directed against a recombinant auxiliary protein that has been implicated in BoNT absorption (HA35). Both antigens evoked high systemic levels of IgG when administered to rabbits, and the resulting antisera were used to analyze toxin action.

In a series of experiments with the anti-HC50 antibody, the results were in accord with the neutralizing activity of the material (Table 1). Antibody that was free and soluble produced complete protection against challenge with toxin that was pure as well as with toxin in complex, and this activity was apparent at both pH 7.4 and pH 6.0. The results with soluble anti-HA35 were completely different. There was no condition (pure versus complex; pH 7.4 versus pH 6.0) in which the antibody provided protection against challenge (Table 2). The results for the pure toxin are in accord with the absence of antigen. The results for the complex are more difficult to explain. Anti-HA35 was inactive against the heteromolecular complex, even when the antigen was present.

One possible way to explain the data is that the complex was not intact. If auxiliary proteins were present but not in association with neurotoxin, then the anti-HA35 antibodies might not be able to antagonize transport of toxin. To test this possibility, a different type of experiment was performed. Rather than using free and soluble antibodies, these experiments utilized immobilized antibodies. And rather than administering the entire reaction mixture to animals, these experiments entailed administration of only the unbound fraction (Fig. 5).

The results with the immobilized anti-HA35 antibodies were consistent with the characteristics of the BoNT/A complex. Thus, beaded antibody did not capture and remove toxin from solution at pH 7.4. At physiologic pH, the complex is known to dissociate, so one would not expect anti-HA35 antibodies to capture any-

thing other than antigen. However, beaded antibodies did capture and remove toxin from solution at pH 6.0. A result like this could have occurred only if the complex were intact. This premise is reinforced by the purported structure of the complex (12). The neurotoxin and HA35 do not have direct contact with one another. Instead, the neurotoxin is thought to associate with NTN_H, which in turn associates with HA70, which then is in association with HA35. Therefore, the only way that an antigen-specific antibody directed against HA35 can remove neurotoxin from solution is if the elements of the complex are intact.

The findings with immobilized anti-HA35 can now be used to help explain the data with the soluble antibody. Anti-HA35 failed to act at pH 7.4, because the complex dissociated under these conditions. Soluble anti-HA35 also failed to act at pH 6.0, but this cannot be attributed to dissociation of the complex. The data with immobilized anti-HA35 make clear that the complex was intact, so some other explanation must be sought. There are two possibilities that are worth considering. First, HA35 may not play a role in transport of the toxin, so there is no reason to believe that anti-HA35 antibodies should prevent absorption of toxin. The second possibility is that the airway has substantial buffering capacity. Even if the i.n. bolus is administered in pH 6.0 solution, the airway might buffer this to a physiological pH, which would lead to dissociation of the complex. Once again, there would be no reason to expect anti-HA35 antibodies to block absorption of toxin.

The antibody-based experiments, like the dose-response experiments, form the basis for strong conclusions. In every situation in which anti-HC50 antibodies were used, inhalation poisoning was blocked; in every situation in which anti-HA35 antibodies were used, inhalation poisoning was not affected. These findings are consistent with the hypothesis that the neurotoxin is the agent responsible for its own transport across epithelial barriers (model 1). The data offer no support for any involvement of HA35, either as a transport device (model 2) or as a membrane-disrupting device (model 3).

Passive immunization on the basal side of epithelial cells. A third and final experimental approach was used, mainly to assess the plausibility of model 3. This approach did not make any assumptions about the mechanism by which HA35 might reach the basal surface of epithelial cells.

Anti-HA35 antibodies were administered by the intravenous route ("passive immunization") to create a binding trap for any HA35 that was absorbed. The antibody was administered well in advance of toxin challenge (18 h), which afforded ample time for distribution to the general circulation on the basal side of epithelial cells (2). When mice were passively immunized with anti-HA35 antibodies and then challenged i.n. with 500 ng of complex, none of the animals survived. For purposes of comparison, one should note that when mice were passively immunized with anti-HC50 antibodies and then similarly challenged with complex, all of the animals survived. Data such as these suggest that an antigen binding trap for HA35 does not influence the ability of BoNT to reach the circulation. By extension, this suggests that absorption of BoNT is not dependent on prior absorption of HA35 that facilitates toxin diffusion between epithelial cells.

One possible objection to this conclusion is that the anti-HA35 antibodies do not actually possess anti-HA35 properties. However, there are three separate and independent experimental observations that argue against this point. First, the ELISA titers, which were based on the use of HA35 as the coating antigen, indicated that the antiserum had a high titer of antibodies that recognize HA35. Second, the

pulldown experiments that utilized beaded anti-HA35 and toxin complex revealed that the antibodies did recognize antigen and did pull it out of solution. Third, the agglutination experiments demonstrated that HA35 possessed the ability to agglutinate red blood cells and anti-HA35 antibodies blocked HA35-induced agglutination. These three separate and independent experiments leave little doubt that the anti-HA35 antibodies can recognize and neutralize HA35. The logical extension of these findings is that the failure of anti-HA35 antibodies to block the purported systemic effects of HA35 is not due to absence of activity on the part of the antibodies. It is due to an absence of systemic effect of HA35 in promoting toxin absorption (e.g., model 3).

Summary. Three fundamentally different types of *in vivo* experiments were performed to assess the three models that have been proposed for BoNT absorption across epithelial barriers. These included (i) experiments in which toxin was administered alone, (ii) experiments in which toxin was administered in the presence of test antibodies on the luminal side of epithelial barriers, and (iii) experiments in which toxin was administered in the presence of test antibodies on the systemic side of epithelial barriers. It is noteworthy that the data for all types of experiments were compatible with model 1, in which it is envisioned that the neurotoxin by itself is competent to cross epithelial barriers. None of the data were compatible with model 2 or model 3. In fact, the data are not supportive of any model that envisions an essential role for auxiliary proteins in the absorption of BoNT from the airway.

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