Pure Botulinum Neurotoxin Is Absorbed from the Stomach and Small Intestine and Produces Peripheral Neuromuscular Blockade

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Botulinum neurotoxin (BoNT) is synthesized and released by the anaerobic, gram-positive bacterium Clostridium botulinum as seven related but serologically distinct proteins designated serotypes A, B, C, D, E, F, and G. BoNT (~150 kDa) is composed of a heavy chain and a light chain linked by a disulfide bond, and this dichain molecule is ordinarily a component of a complex formed by noncovalent association with other proteins, including a family of hemagglutinins (HA) and a single nontoxin, nonhemagglutinin subunit (11, 19).

BoNT serotype A (BoNT/A) is an unusually potent neurotoxin that causes the disease botulism, a form of flaccid paralysis that if left untreated can be fatal. Of the several possible routes that neurotoxin can use to enter the body, the oral route is the most common (9, 18, 25, 27). Intoxication usually occurs due to ingestion of preformed neurotoxin contaminating a meal or to ingestion of bacteria that may colonize the gut and produce neurotoxin. In either case, BoNT escapes the gastrointestinal (GI) system to reach the general circulation (lymph and blood). Neurotoxin in blood is then delivered to peripheral cholinergic nerve endings, which are the target for neurotoxin action.

Although absorption of neurotoxin from the gut is essential to the onset of disease, surprisingly little is known about specific mechanisms that contribute to absorption (1). A series of early studies indicated that the upper small intestine is the primary site of absorption (4, 5, 10, 16). This early work also established that neurotoxin which was administered orally or directly into the intestine appeared in lymph and blood. Absorbed neurotoxin was shown to be biologically active, as judged by assays for in vivo toxicity.

One of the obstacles that has hindered work on characterizing the mechanism of absorption is the finding that naturally occurring botulism is not due to pure neurotoxin but instead is due to neurotoxin that is part of a complex with auxiliary proteins. The serotype A complex is composed of several proteins, including neurotoxin, a family of hemagglutinins, and a nontoxin, nonhemagglutinin component. (There may also be a negligible amount of nonprotein, e.g., RNA.) Virtually all of the work that has been done to date indicates that auxiliary proteins probably play a role in protecting the neurotoxin from the harsh conditions of pH and proteolytic enzymes found in the gut (1, 3, 8, 20–22, 29, 30). Unfortunately, there is almost no work describing the role of these auxiliary proteins in the process of neurotoxin absorption (but see reference 7).

In the recent past, we have conducted two types of studies that may help to clarify both the mechanism by which neurotoxin is absorbed and the role of auxiliary proteins in absorption. In the first, molecular biological techniques were used to express a recombinant holotoxin that lacks the ability to poison nerve endings. This holotoxin was found to be an effective oral vaccine that elicited the production of systemic antibodies (13). In the second study, binding and transcytosis of neurotoxin was studied in T-84 cells, which are derived from the gut. This work demonstrated that native holotoxin bound to specific receptors on the mucosal side of cells and was actively transported to the serosal side of these cells (15). Taken together, these studies suggest that pure neurotoxin, in the absence of auxiliary proteins, can be absorbed from the gut.

To test the hypothesis that pure neurotoxin can be absorbed and to gauge the role of auxiliary proteins in this process, a
series of experiments were done in mice. Three neurotoxin preparations were examined as follows: (i) pure neurotoxin, (ii) neurotoxin in a complex that contained hemagglutinins, and (iii) neurotoxin in a complex that did not contain hemagglutinins. These preparations were injected directly into the stomachs or intestines of animals with or without ligation of the pylorus. The results of these studies help clarify the efficacy of neurotoxin absorption, both in the presence and in the absence of auxiliary proteins.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.), and additional laboratory supplies were obtained from Fisher Scientific (Malvern, Pa.). Two preparations, BoNT/A complex with hemagglutinins and pure neurotoxin from strain 62A, were purified by procedures described in the literature (6, 24, 28). A third BoNT/A complex without hemagglutinins from strain JMH-1-001 (honey isolate) was purified as follows.

Strain JMH-1-001, isolated from honey suspected in a case of infant botulism (15a), was grown in 2.0% NZ-Case TT (Quest)–1.0% yeast extract (Dilco)–0.5% glucose (Mallicknord) medium adjusted to pH 7.2. A 2-liter neurotoxin production culture was grown for 5 days at 37°C, and the neurotoxin was precipitated by addition of 3 N sulfuric acid to pH 3.2. The precipitated neurotoxin was collected by centrifugation and extracted in 0.2 M sodium phosphate buffer (pH 6.0) for 2 h. After centrifugation, the extracted neurotoxin was precipitated by making the solution 60% saturated with ammonium sulfate. The precipitated neurotoxin was collected by centrifugation, dissolved in 0.05 M sodium citrate buffer (pH 5.5), and then clarified by centrifugation and loaded on to a DEAE-Sephadex A-50 column equilibrated with 0.05 M sodium citrate buffer (pH 5.5). The neurotoxin-containing fractions were pooled and precipitated by making a solution 60% saturated with ammonium sulfate. A 2-mg sample of the precipitated neurotoxin was collected by centrifugation and dissolved in 1 ml of 30 mM sodium phosphate–0.2 M sodium chloride (pH 6.8). Neurotoxin samples were gel filtered on a Superose 6 (Pharmacia) column (1.6 by 90 cm), and the complex eluted in fractions representing a molecular size of 270 to 300 kDa.

Animals. Swiss Webster mice (female, 12 to 25 g), which were purchased from Ace Animals (Boyertown, Pa.), were housed in an accredited animal colony (American Association for Accreditation of Laboratory Animal Care) 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.

Surgical procedures. The preoperative protocol involved fasting animals for 18 h prior to surgery while allowing them free access to water. Preoperative preparation also included shaving the abdominal area and administering a prophylactic subcutaneous dose of gentamicin сулфат (6 mg/kg; Fujisawa USA, Inc., Deerfield, Ill.). On the day of surgery, the animals were transferred to a veterinary procedure room, and all subsequent steps were performed in an aseptic surgical environment.

Animals were anesthetized by administration of boflurane (Isno-thesia; Abbott Laboratories North, Chicago, Ill.)-oxygen, and the same inhalation anesthetic was administered throughout surgery. An abdominal laparotomy (~1.5 to 2.5 cm, depending on the size of the mouse) was performed, and either the stomach or the small intestine immediately proximal to the stomach was partially exteriorized. If required by protocol, a ligature with 3-0 Prolene (polypropylene suture; Ethicon, Inc., Somerville, N.J.) was placed immediately above (proximal to the stomach) the pyloric sphincter. Care was taken that this ligature was sufficient to prevent the flow of stomach juices into the intestine (or reverse flow of intestinal contents into the stomach) but not sufficient to cause mechanical damage to the tissues involved. Neurotoxin was administered through a 1-ml tuberculin syringe with a 0.5-in., 27-gauge needle. Injection volumes were kept constant at 0.1 ml per animal regardless of the site of administration (stomach or intestine). For all injections, the vehicle consisted of sterile Dulbecco's phosphate-buffered saline (pH 7.4) with 1 mg of bovine serum albumin per ml. Neurotoxin was administered into the lumen of the stomach by injection through the stomach wall at the greater curvature, with care being taken to avoid the gastro-epiploic vessels. Neurotoxin was administered into the lumen of the small intestine by oblique insertion of the needle parallel to the segment and always in a direction away from the stomach. The time of injection was recorded.

After administration of neurotoxin, the organs were gently reposeated and the incision in the abdominal muscle was sutured using 3-0 Prolene. The skin was closed with several small wound clips, after which the animals received a 2-mg/kg subcutaneous analgesic injection of Buprenorphine hydrochloride (Buprenex injectable; Reckitt & Colman Pharmaceuticals, Inc., Richmond, Va.) and another dose of gentamicin.

The surgical procedure lasted approximately 15 min per animal, and suspension of anesthesia resulted in full recovery within 10 to 15 min. The animals were then transferred to the laboratory, where they were monitored for assay endpoint. The time of death was recorded, and total elapsed time (in minutes) from time of injection to time of death was calculated.

**FIG. 1.** Comparison of BoNT components. Neurotoxin samples were prepared in reducing buffer and run on 7.5% polyacrylamide gels. Three samples of serotype A neurotoxin were used in this study. Lane 1, neurotoxin from strain 62A, HA· complex, showing nontoxin, nonhemagglutinin (NTNH), neurotoxin heavy-chain (HC) and light-chain (LC), and hemagglutinin bands. Lane 2, neurotoxin from strain 62A, pure neurotoxin, showing neurotoxin heavy and light chains. Lane 3, neurotoxin from strain JMH-1-001 neurotoxin, HA· complex, showing the nontoxin nontoxin nontoxin neurotoxin heavy and light chains.
Neurotoxin was administered in 100 μl of buffered saline solution (pH 7.4) that also contained 1 mg of protein (bovine serum albumin) per ml. The purpose of the ligation was to ensure that neurotoxin absorption could occur only at the site of injection (i.e., stomach or intestine). The purpose of the bolus of buffered solution with protein was to reduce the rate and extent of pH-dependent neurotoxin degradation.

In the second approach, neurotoxin was administered without ligation. This approach allowed the neurotoxin to move from the stomach to the intestine (as would occur in oral poisoning) or from the intestine to the stomach (as might occur following colonization of the bowel by Clostridium botulinum). As before, neurotoxin was administered in a buffered solution with protein to diminish pH-dependent neurotoxin degradation.

Absorption of neurotoxin complexes in ligated preparations. Experiments were done to assess the absorption of HA1 and HA2 complexes from the stomach and the intestine. The purpose of the experiments was to determine whether the rates of absorption in the stomach and intestine differed as a function of the presence or absence of hemagglutinin.

Figure 2 illustrates that the apparent toxicity of HA1 complex containing the equivalent of 1 mg of neurotoxin was the same whether administered into the stomach or into the intestine. The difference in survival time for the two groups was not statistically significant (P < 0.30). The figure illustrates that a qualitatively similar result was obtained when HA2 complex was injected into stomach or intestine. Once again, the difference in survival times was not statistically significant (P < 0.40). Thus, under conditions that maximize the opportunity for absorption while reducing the potential for degradation, the absorption of each complex was approximately equivalent at both sites of administration.

Absorption of pure neurotoxin isolated in ligated preparations. An identical series of experiments was performed with pure neurotoxin (1 mg/animal). The results, which are illustrated in Fig. 3, demonstrated two things. First, pure neurotoxin was absorbed from both sites, meaning that neither hemagglutinin nor the nontoxic, nonhemagglutinin is absolutely essential for uptake. Second, the survival times of animals receiving pure neurotoxin in the intestine were significantly shorter than those of animals receiving pure neurotoxin in the stomach (P < 0.01). This difference could be due to greater metabolism of pure neurotoxin in the stomach, lesser absorption in the stomach, or a combination of the two.

Absorption of pure neurotoxin and HA+ complex in nonligated preparations. Pure neurotoxin and HA+ complex (equivalent to 1 mg of neurotoxin/animal) were administered in a bolus of buffered solution into the stomach of animals in which the pyloric sphincter was not ligated. As shown in Figure 4, the toxicity of the HA+ complex appeared greater than that of pure neurotoxin. However, the difference in survival times did not attain statistical significance (P < 0.20).

Dose dependence of pure neurotoxin and HA+ complex uptake in nonligated preparations. The data presented above indicate that (i) pure neurotoxin can be absorbed from the stomach and (ii) auxiliary proteins may protect neurotoxin, thus giving a greater apparent toxicity for an equivalent amount of neurotoxin. If these points are correct, they suggest that the ap-
parent difference in potency between pure neurotoxin and neurotoxin in complex would be greatest at low neurotoxin concentrations (i.e., the rate of metabolism of pure neurotoxin may exceed the rate of absorption). Therefore, experiments similar to those in the preceding section were done but with a lower neurotoxin concentration (25 ng/animal). In addition, injections were administered in the stomach and in the intestine.

As shown in Table 1, injections of pure neurotoxin and HA<sup>+</sup> complex into the stomach produced strikingly different results. There were no deaths among animals receiving pure neurotoxin whereas there was only one survival among 10 animals receiving HA<sup>+</sup> complex. By contrast, when pure neurotoxin and HA<sup>+</sup> complex were injected into the intestine, the end result was the same (one survival after 4 days).

**DISCUSSION**

BoNT is a remarkably potent substance that acts on the peripheral nervous system to cause a disease characterized by flaccid paralysis. In the overwhelming majority of cases, the disease is due to ingestion of neurotoxin or to ingestion of bacteria that produce the neurotoxin (9, 18, 25, 27). In either case, the neurotoxin escapes the GI system to reach the general circulation and eventually the peripheral nervous system. It acts on cholinergic nerve endings to block the release of acetylcholine, and this in turn produces the characteristic outcome of flaccid paralysis (12, 17, 19, 23, 26).

The fact that the oral route is the primary mode of neurotoxin entry into the body makes it obvious that there must be an effective mechanism for it to cross gut membranes. Unfortunately, there is little information available on the mechanism that accounts for penetration of these membranes. One of the major impediments to studying transcellular movement of the neurotoxin is that this substance by itself rarely if ever causes disease. In nature, botulinum neurotoxin is ordinarily found in a complex with one or more auxiliary proteins (11, 19). These proteins almost certainly help protect the neurotoxin from degradation by low pH and proteolytic enzymes in the gut (1, 3, 8, 20–22, 29, 30). The presence of these proteins in a complex with the neurotoxin, coupled with their ability to protect it from degradation, complicates efforts to assess how and where the neurotoxin is absorbed.

In the present study, an effort was made to gain insight into the mechanisms that govern neurotoxin absorption. For this purpose, three different preparations were used: pure neurotoxin, HA<sup>+</sup> complex, and HA<sup>-</sup> complex. In most experiments, these preparations were administered to animals in which the pylorus was ligated, thus ensuring that neurotoxin absorption could occur only in the vicinity of its administration. In addition, a buffered solution with protein was used as a vehicle to reduce pH-dependent degradation and thus maximize the opportunity for absorption.

The most important observation to emerge from the study was that pure neurotoxin is capable of being absorbed from both the stomach and the intestine. Neither the hemagglutinin proteins nor the nontoxin nonhemagglutinin protein is absolutely essential for absorption. This observation appears to be at odds with the work of Fujinaga et al. (7), who reported that hemagglutinin plays a critical role in intestinal absorption of BoNT/C. However, the observation is consistent with a larger body of findings. To begin with, BoNT from strain JMH1-001 is capable of producing oral poisoning in humans. Indeed, this strain was isolated from honey implicated in an outbreak of infant botulism (15a). Interestingly, the genome of strain JMH1-001 does not contain the gene for hemagglutinin, and therefore it is impossible that hemagglutinin can participate in—let alone be essential for—poisoning due to neurotoxin from this strain.

In a separate line of investigation, Kiyatkin et al. (13) used techniques of molecular biology to generate a botulinum holotoxin that was modified in a site that governs neurotoxicity. Oral administration of this modified holotoxin did not produce any of the signs of botulism but did elicit the production of systemic antibodies that neutralized native neurotoxin. This result strongly implies that the expression product, which was entirely free of hemagglutinin, was capable of being absorbed from the gut.

Finally, Maksymowych and Simpson (15) have reported that homogeneous BoNT binds specifically to T-84 cells and is actively transported from the apical (luminal) to the basal (serosal) side of cells. The product that is released on the basal side is native neurotoxin capable of producing in vivo and in vitro blockade of neuromuscular transmission. Once again, the data indicate that no auxiliary protein is absolutely essential for the neurotoxin to cross gut membranes.

The fact that auxiliary proteins are not required for absorption does not necessarily mean that they do not participate in

**TABLE 1. Toxicity of** C. botulinum **serotype A pure neurotoxin and neurotoxin complex injected into the stomach or intestine**

<table>
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<tr>
<th>Time (h)</th>
<th>Survival (no. alive/total no.) after injection of:</th>
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<tbody>
<tr>
<td></td>
<td>Pure neurotoxin</td>
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<tr>
<td></td>
<td>Stomach</td>
</tr>
<tr>
<td>24</td>
<td>0/10</td>
</tr>
<tr>
<td>48</td>
<td>0/10</td>
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<td>72</td>
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<tr>
<td>96</td>
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<sup>a</sup> Mice were injected with pure neurotoxin or HA<sup>+</sup> complex. Samples were introduced directly into the stomach or directly into the intestine. No ligature was used to restrict neurotoxin movement. Animals were monitored, and the number of deaths was recorded at the end of each time period. Pure neurotoxin (25 ng/animal) and HA<sup>+</sup> complex (equivalent to 25 ng of pure neurotoxin/animal) were used.
absorption. The data support the conclusion that pure neurotoxin can cross gut membranes, but they do not rule out the possibility that the neurotoxin-auxiliary protein complex can also cross membranes. It is conceivable that both free neurotoxin and neurotoxin in complex have an exposed domain that can associate with receptors that mediate transcytosis. It would be desirable to determine the comparative rates for transcytosis of free neurotoxin and of neurotoxin-auxiliary protein complex. Experiments on rates of association with cell surface receptors and rates of transcytosis would more appropriately be done on isolated cell systems (e.g., T-84 cells) than on live animals.

It should be noted that nothing in this study challenges the premise that the intestine is a major site, and perhaps the principal site, of neurotoxin absorption. What the work does demonstrate is that under the proper circumstances, the stomach as well as the intestine can be a site of absorption. Furthermore, nothing in the study challenges the widely held belief that auxiliary proteins protect the neurotoxin from degradation, especially in the stomach. However, the work shows that the importance of this protection is dose dependent.

The fact that the protective role of auxiliary proteins depends on the administered dose is not surprising. When pure neurotoxin enters the stomach, it is subject to two processes: (i) metabolism, which reduces the likelihood of poisoning, and (ii) absorption, which increases the likelihood of poisoning. Each of these processes is governed by its respective affinity constants and rate constants. If affinity constants favor metabolism over absorption, then low doses of pure neurotoxin are more likely to be degraded than to produce neuromuscular blockade. Moreover, as the concentration of pure neurotoxin in the stomach approaches or exceeds the affinity constants for metabolism, the potential protective effect of auxiliary proteins would not be as obvious. These expectations were reflected in the experimental findings. When administered at a dose of 25 ng/animal, the protective effect of auxiliary proteins was obvious in the survival data (Table 1). Conversely, when the dose was incremented substantially (1 µg/animal), the protective effect of auxiliary protein was difficult to detect.

As indicated above, this work provides the first clear demonstration that homogeneous BoNT/A can be absorbed from the gut to produce peripheral neuromuscular blockade. The work also highlights the importance of using different experimental preparations and experimental conditions to separate the role of auxiliary proteins in the process of absorption from the role of these proteins in the process of metabolism.

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REFERENCES