The Role of Systemic Handling in the Pathophysiologic Actions of Botulinum Toxin

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ABSTRACT

The ability of botulinum toxin to poison cholinergic nerve transmission is a dynamic phenomenon that involves not only the actions of the toxin on the body but also the actions of the body on the toxin. The former has been the subject of intense research, whereas the latter has received almost no attention. Therefore, a series of studies were performed to characterize systemic handling of botulinum toxin. The results indicated that the toxin reaches the general circulation (transcytosis across epithelial cells) without obvious changes in structure or biological activity. The general circulation acts as a holding compartment until there is adequate fractional distribution to neuromuscular junctions to produce blockade of transmission. During its transit through this compartment, the toxin 1) undergoes little biotransformation, 2) does not accumulate significantly in circulating cells, and 3) remains largely in the free state. In naive animals, the $t_{1/2}$ for toxin in the general circulation is approximately 10 h, and at any given point in time, there is little uptake in nontarget organs (liver, kidney, heart, and lung). In immunized animals, toxin clearance from the general circulation is rapid, and there is substantial accumulation of antibody-antigen complexes in liver. Thus, enhanced clearance from the circulation is a major mechanism by which active immunization can protect against poisoning.

Botulinum toxin (BoNT) occupies a unique position in the medical and biological sciences. There are a variety of factors that contribute to this special status, some of which relate to mechanism of action, and others of which pertain to clinical outcomes. In the category of mechanism of action, the toxin occupies a unique status by virtue of being the most potent of all biological toxins (Arnon et al., 2001). It is also somewhat unique due to the number and complexity of the steps through which it must progress to poison an organism (Simpson, 2004). In terms of clinical outcomes, BoNT is one of only a small number of substances that is both an agent known to cause disease (e.g., botulism) (Shapiro et al., 1998; Caya et al., 2004; Sobel, 2005) and an agent approved for the treatment of disease (e.g., dystonia) (Jankovic and Brin, 1991; Schantz and Johnson, 1997; Comella et al., 2003; Chaddock and Marks, 2006). As a corollary to this, it is the only substance on the Select Agent List that is both a potential agent of bioterrorism and biological warfare and a potential agent for the relief of an ever-widening array of clinical disorders, ranging from achalasia to migraine headaches.

The fact that the toxin is remarkably potent, and because it is encountered in a large number of clinical contexts, there have been intense efforts to determine how it acts in the body (Humeau et al., 2000; Schiavo et al., 2000; Simpson, 2004). The two principal areas of focus have been gut and airway epithelial cells, which carry the toxin into the body (i.e., transport cells), and peripheral cholinergic neurons, which are the site of toxin action (i.e., target cells). This work has culminated in a reasonably clear picture of toxin action at the cellular and subcellular levels, although there is much that remains to be done to resolve toxin action at the molecular level.

In contrast to the large and growing literature that describes toxin action on the body, there is only a small and incomplete literature describing the action of the body on the toxin. For example, efforts to describe the systemic pharmacokinetics of BoNT, including phenomena such as absorption, distribution, tissue accumulation, metabolism, and elimination, have only just begun (Ravichandran et al., 2006). As a result, many aspects of systemic and tissue handling of the toxin remain almost wholly unknown.

There are several reasons why it would be helpful to pos-

ABBREVIATIONS: BoNT, botulinum toxin; FRET, fluorescent resonance energy transfer; HC₅₀, carboxyterminal half of the heavy chain.
cess a better understanding of how the body handles and ultimately disposes of the toxin. To begin with, this information would contribute to a better understanding of the disease botulism and, most obviously, to issues such as rate of onset and rate of recovery. Likewise, this information could shed light on potential sites and mechanisms of action of medical countermeasures, such as vaccines and therapeutic antibodies. In a somewhat different realm, an understanding of how the body handles the toxin could lead to novel approaches to using the toxin as a medicinal agent.

In the report that follows, a series of studies are described that provide new information about the ability of the body to absorb, and then later eliminate, the toxin. Serotype B (BoNT/B) has been selected for study because it is one of the three serotypes most often implicated in human disease (namely, serotypes A, B, and E) (Arnon et al., 2001). Rodents have been selected for study because they are the animals most often used for research on mechanism of toxin action, on medical countermeasures against the toxin, and on therapeutic applications of the toxin. Furthermore, certain of the studies were conducted specifically in rats. These animals are especially appropriate for in vivo experiments on serotype B because they are relatively resistant to the neuropathic effects of this serotype (Burgen et al., 1949; Patarnello et al., 1993). Resistance to poisoning facilitates the ability to analyze disposition and metabolism of the toxin in animals that have not succumbed to illness.

Materials and Methods

Materials. Radioisotopes were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Sephadex G-25 gel filtration columns, iodination reagents, and serum albumin binding reagents were purchased from GE Healthcare (Chalfont St. Giles, UK).

Toxin. Homogeneous BoNT/B was purchased from Metabolics (Madison, WI; specific toxicity, 1.3 x 10^9 MLD50/mg). The partially nicked, single-chain form of the toxin was converted to the fully nicked, dichain form by proteolytic activation with trypsin. The immobilized form of trypsin (4% beaded agarose; Pierce Chemical, Rockford, IL) was used to facilitate separation of activated toxin from trypsin. Nicked and immobilized enzyme (10:1 ratio) were incubated at 21°C for 1 h. After incubation, nicked toxin was separated from beaded trypsin by filtration through a 0.2-μm centrifugal filter (Cen-Beaded trypsin by filtration through a 0.2-

Iodination. BoNT/B was iodinated using the [125I] Bolton-Hunter reagent essentially according to the manufacturer’s instructions and as described previously (Maksymowych and Simpson, 1998). Assays to confirm retention of toxicity were also performed as described previously (Maksymowych and Simpson, 1998).

Before each experiment, the labeled toxin was refractionated to ensure separation of the iodinated holotoxin from free label and small polypeptides. Homogeneity of the toxin was confirmed by submitting samples to SDS-polyacrylamide gel electrophoresis to verify nicking and homogeneity of the final product.

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Immunoprecipitation and Autoradiography. To ensure homogeneity of the injected material, iodinated toxin was subjected to gel chromatography (G-25) before administration, thus eliminating free iodine or small degradation products. Biological samples obtained from animals that received injections were also subjected to gel chromatography. In addition, a combination of studies that entailed immunoprecipitation, enzymatic assays, and monitoring of

neuromuscular transmission were done to ensure the structural and functional integrity of the toxin being measured. The techniques that were used to accomplish these goals were identical to those previously reported (Ravichandran et al., 2006).

Enzyme Assay. The endoprotease activity of the holotoxin was measured using a fluorescent resonance energy transfer (FRET) assay that employed a synthetic substrate that has been described previously (60-SELDRADLAQAGASQFETSAAKLKKRYWWK-NL95; cleavage site, QT6-F77) (Schmidt and Stafford, 2000). BoNT/B was added to heparinized rat blood to achieve a final concentration of 10^-9 M. The mixture was incubated at 37°C for varying lengths of time. At the end of incubation, sample aliquots were centrifuged (2100 g) for 10 min. Supernatant (10 μl) was added to reaction buffer (final volume, 100 μl; 20 mM HEPES, pH 7.5, 0.25 mM ZnCl2, 1.25 mM dithiothreitol, 0.05% Tween 20) that contained the fluorescent substrate (10 μM). The mixture was incubated at 37°C for 2 h, after which the reaction was terminated by the addition of trifluoroacetic acid to a final concentration of 0.5%. The resulting fluorescence was measured at room temperature in a Luminescence Spectrometer LS 50B (PerkinElmer Life and Analytical Sciences) using FL Winlab software. Measurements were made at an excitation of 390 nm and emission of 480 nm, with an integration time of 3 s. The results were expressed as arbitrary fluorescence units.

Neuromuscular Transmission. Murine phrenic nerve hemidiaphragm preparations were used to bioassay the residual toxicity of BoNT in biological specimens (Maksymowych and Simpson, 2004; Simpson et al., 2004). Tissues were excised and suspended in physiological buffer that was aerated with 95% O2 and 5% CO2 and maintained at 35°C. The physiological solution contained 137 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1.0 mM MgSO4, 24 mM NaHCO3, 1.0 mM NaH2PO4, 11 mM D-glucose, and 0.01% gelatin. Phrenic nerves were stimulated continuously (0.2 Hz; 0.1–0.3-ms duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a 90% reduction in muscle twitch response to neuromuscular stimulation.

Cell Culture and Transcytosis Assay. T-84 human epithelial cells were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (1 g/l D-glucose) and Ham’s F-12 nutrient medium supplemented with 5% newborn calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 15 mM HEPES. Cultures were maintained at 37°C in 6% CO2, 95% air. T-84 cells were fed every 3 days and passaged (1:2) when 95% confluent, approximately every 6 days. Passages 65 through 90 were used for experiments described in this article.

For the transcytosis assays (Maksymowych and Simpson, 1998, 2004), cells were grown in Transwell porous bottom dishes on poly-carbonate membranes with a 0.4-μm pore size. The growth area within each insert was 1.12 cm2. The Transwells were coated with 10 μg/cm2 rat tail collagen type 1. The coated wells were allowed to dry at room temperature overnight (18 h). After drying, the wells were sterilized under UV light for 1 h, followed by preincubation with cell culture medium (30 min). The preincubation medium was removed immediately before addition of cells and fresh medium.

T-84 cells were plated at confluent density (approximately 1.5 x 10^5 cells) into the Transwells with 0.5 ml of medium in the upper chamber and 1.0 ml in the lower chamber. Medium in the upper chamber bathed the apical (or mucosal) surface of cells, and medium in the lower chamber bathed the basolateral (or serosal) surface of cells. Culture medium was changed every 2 days. The cultures were allowed to differentiate for a minimum of 10 days before assay of transcytosis. The formation of tight junctions was experimentally confirmed by measuring transepithelial electrical resistance. Experiments were performed on cultures that were between 10 and 15 days old.

The transcytosis assay was initiated by adding BoNT to the upper chamber. Transport was monitored for 18 to 20 h by collecting all of the medium from the bottom chamber. An aliquot (0.5 ml) from each sample was filtered through a Sephadex G-25 column, and 0.5-ml fractions were collected. The amount of polypeptide that crossed
epithelial monolayers was quantified in various ways, as described under Results.

**Animals.** Swiss-Webster female mice (18–20 g; pharmacokinetic experiments requiring sensitive animals) and Sprague-Dawley female rats (150–170 g; pharmacokinetic experiments requiring resistant animals) were purchased from Ace Animals (Boyertown, PA). BALB/c female mice (16–20 g; immunologic experiments) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). New Zealand white rabbits (female, 2–3 kg; generation of immune serum) were purchased from Covance Research Products (Princeton, NJ). All procedures involving animals were reviewed and approved by the Institute of Laboratory Animal Resources (1996).

**Active and Passive Immunization.** The carboxyl-terminal portion of the heavy chain of BoNT/B (HC_{50}) was cloned, expressed, and purified to a homogeneity of >98%, according to previously reported techniques (Maksymowycz and Simpson, 2004). This portion of the molecule, which possesses the domain that binds to receptors on the surface of epithelial cells and on peripheral cholinergic nerve endings (Simpson, 2004), was used as an antigen to evoke neutralizing antibodies in rabbits. These neutralizing antibodies served as the antiserum source for the passive immunization of rodents used in systemic clearance experiments.

The immunization paradigm consisted of one prime immunization and three booster immunizations. The prime immunization consisted of alum-adsorbed antigen (50 μg) given subcutaneously to rabbits. The booster immunizations (50 μg each; nonadsorbed) were given at 2, 4, and 8 weeks. Fourteen days after the final boost, aliquots of blood were drawn, and the circulating titer of IgG was determined by enzyme-linked immunosorbent assay (Park and Simpson, 2003; Ravichandran et al., 2007). The circulating titers for the serum used in all neutralization and clearance experiments exceeded a dilution factor of 10^{4}.

Active immunization was evoked in mice by intranasal administration of the HC_{50} domain of BoNT/B. The antigen (20 μg/administration) was administered by application of a 20-μl phosphate-buffered saline solution, pH 7.4, to the nares of animals. The prime dose was administered on day 0, and booster doses were given on days 14 and 28. Each dose of antigen was given in combination with a mucosal adjuvant [vitamin E d-α-tocopheryl polyethylene glycol 1000 succinate, 5% (w/v)]. The neutralization titers obtained in these animals also exceeded a dilution factor of 10^{4}.

**Enzyme-Linked Immunosorbent Assay.** Antibody titers in serum were determined by using procedures that have been described previously (Ravichandran et al., 2007).

**Challenge with Toxin.** The most characteristic outcome of botulinum toxin action is neuromuscular blockade. This outcome is easily discernable as weakness and eventual paralysis of the muscles of locomotion and the muscles of respiration. During pharmacokinetic experiments and the associated experiments with antiserum, animals received doses of toxin sufficient to produce poisoning within minutes. The use of death as an endpoint for laboratory research has become an increasingly unacceptable practice. Indeed, certain nations do not permit experiments that use death as an intended experimental outcome. Therefore, to minimize pain and suffering, animals were observed throughout the various protocols. When signs of serious neuromuscular weakness became apparent, animals were sacrificed in accordance with the Association for Assessment of Laboratory Animal Care guidelines (e.g., CO_{2}).

**Systemic Pharmacokinetics.** The biologic half-life of iodinated toxin in blood was determined in rats, using techniques similar to those previously reported for mice (Ravichandran et al., 2006). Experiments were also done with serum and with plasma. Serum samples were generated by placing blood at 4°C for 2 to 3 h to allow for coagulation. Samples were centrifuged in a clinical centrifuge for 10 min, after which serum was aspirated and stored at –20°C. Plasma was generated by adding heparin to blood to prevent coagulation. The mixture was centrifuged in a clinical centrifuge for 10 min, after which plasma was collected and stored at –20°C.

**Serum Albumin Binding.** Ligand binding to rat serum albumin was quantified as previously reported (Ravichandran et al., 2006).

**Blood and Organ Levels of Toxin.** Iodinated BoNT/B was administered intravenously to mice via the tail vein, and 30 min later, specimens were obtained by retro-orbital bleeding. Experiments were done in control animals (toxin only) and in experimental animals (toxin preincubated with antiserum from vaccinated animals). The goal of the experiments was to determine the extent of toxin clearance in animals that had a circulating titer of antibody adequate to produce complete neutralization of the administered dose of toxin.

In addition to monitoring the levels of toxin in blood, one set of experiments determined the extent of local toxin accumulation in tissues. Mice were anesthetized by administration of pentobarbital (Nembutal) sodium (50 mg/kg dose), after which the thorax was opened, and the heart was exposed. A butterfly needle (23 gauge) was inserted into the left ventricle, an incision was made in the right ventricle, and the body of the animal was perfused with a heparinized solution of phosphate-buffered saline (45–60 ml). Four major organs were removed from the body (liver, kidney, lung, and heart), and the accumulation of iodinated toxin was determined.

**Statistical Analyses.** For all data points in all figures, the group n was 3 or more. The S.E.M. for each data point was equal to or less than 10% of the value for that data point.

**Results**

**Structural and Functional Stability of BoNT/B (Laboratory Animal Model).** Iodinated BoNT/B was added to rat blood that was incubated at 37°C for 240 min. The starting concentration for the toxin was 1 × 10^{-7} M. Aliquots of this material were immunoprecipitated with antisera raised against the heavy-chain component of the toxin. This material was submitted to polyacrylamide gel electrophoresis, after which it was analyzed by autoradiography. Control samples were processed identically, except that the iodinated toxin was added to blood immediately before immunoprecipitation.

The results of this experiment are shown in Fig. 1. The data indicate that both the amount of toxin and the apparent mol. wt. of the toxin are the same in the control preparation (0 min) and in the incubated preparations (120 and 240 min). There was no evidence that the toxin had undergone any major structural change.

Companion experiments were done to ensure that the toxin was functionally intact. BoNT/B (unlabeled) was added to rat blood at 37°C for 256 min, and at various time points throughout the experiment, aliquots of material were with-
drawn and examined in two bioassays: a FRET-based assay to quantify catalytic activity and an isolated tissue-assay to measure neuromuscular blocking activity. The results of the catalytic assay demonstrated that the metalloendoprotease activity of the toxin remained stable over the time course of the experiment (Fig. 2A). The results of the isolated tissue experiment were supportive of this outcome. Thus, the neuromuscular blocking activity of the toxin remained intact throughout the incubation period in blood (Fig. 2B).

**Structural and Functional Stability of BoNT/B (Human Model).** T-84 cells were grown in a Transwell apparatus, as described under Materials and Methods. Transepithelial electrical resistance was monitored to ensure patency of the monolayer. BoNT/B (1 × 10^-8 M) was added to the apical surface of cells, and the accumulation of toxin on the basal side of cells was monitored. In keeping with earlier reports (Maksymowych and Simpson, 1998; Maksymowych et al., 1999; Ahsan et al., 2005), the toxin was shown to bind and penetrate epithelial barriers (Fig. 3). It is particularly noteworthy that transcytosed material crossed monolayers without undergoing any detectable metabolism. Thus, the material collected on the basolateral side of cells appeared as a single band rather than multiple bands, and the mol. wt. of the transcytosed band was indistinguishable from that of native, nontranscytosed toxin (Fig. 3, lane 1).

Samples of toxin collected from the basal chamber of the Transwell apparatus were added to human blood at room temperature for 0, 128, and 256 min. These specimens were then treated with anti-BoNT/B antibodies to immunoprecipitate the holotoxin (plus any putative metabolites). The isolated polypeptides were subsequently analyzed by Western blots (Ravichandran et al., 2007).

As shown in Fig. 3, BoNT/B seemed to be stable in human blood over the time course of the experiment. The evidence to support this premise is as follows: 1) the intensities of the bands at 128 and at 256 min were indistinguishable from that at 0 min; 2) the mol. wt. values of the toxin bands at all three time points were indistinguishable from that of native toxin not previously exposed to blood; and 3) there were no
low mol. wt. bands (namely, metabolites) in any of the samples obtained from human blood.

In parallel experiments, transcytosed toxin that was incubated in human blood for 0 and 256 min was added to murine phrenic nerve hemidiaphragm preparations. The amount of time necessary for onset of neuromuscular blockade was monitored. Similar to the data shown in Fig. 2 for rat blood, data for incubation of toxin in human blood did not reveal loss of neuromuscular blocking activity (data not shown). When combined with the preceding data, these results demonstrate that the toxin was structurally and functionally intact.

Disposition of Biologically Active Toxin in Blood. Experiments were done to quantify the amount of active toxin in blood that was available for distribution to vulnerable cells. These experiments were done in two steps: 1) quantification of toxin in serum and plasma versus blood, to gauge whether formed blood elements were accumulating toxin, and 2) quantification of free versus bound toxin to determine whether serum albumin was sequestering toxin. For the first set of experiments, 125I-BoNT/B (230 ng) was administered to rats (n = 3), and 32 min later, the animals were anesthetized and exsanguinated. A portion of the whole blood was reserved, and the balance was used to generate serum and plasma. Analysis of the various specimens indicated that the fractional recoveries of toxin in plasma and in serum were approximately 90.1 and 76.9%, respectively.

For the second set of experiments, 125I-BoNT/B (2.4 × 10^−9 M) was added to a 100-μl reaction volume that contained approximately 1 mg of rat serum albumin bound to agarose beads (approximately 1.2 × 10^−4 M). After incubation, the beads were washed to separate bound toxin from free toxin. The results indicated that approximately 26% of the toxin was bound, meaning that approximately 74% was free.

Biological Half-Life. 125I-BoNT was administered i.v. to rats at a dose of 230 ng. At various times after injection, animals (n = 5 per time point) were sacrificed, blood was collected, and the concentration of toxin in blood was determined. As shown in Fig. 4, the loss of toxin from the circulation could be described by two rate constants. The early and rapid loss had a t_{1/2} of approximately 61 min, and the later and slower loss had a t_{1/2} of approximately 600 min.

Dose-Response Characteristics of Toxin in Blood. When administered to rats at a dose of 230 ng, the toxin concentration in the general circulation at pseudo-steady state fell within the range of approximately 5.0 (200 min) to approximately 1.9 (1000 min) ng/ml. This represents a concentration range of approximately 3.3 to 1.2 × 10^{−11} M. It seems unlikely that the toxin could be saturating a metabolic process or an elimination pathway at such low concentrations. To ensure that this assumption was correct, toxin was administered i.v. to rats at doses that span approximately 1 order of magnitude (0.3 × 230, 230, 3.0 × 230 ng), and the blood levels of toxin were determined (64 min). The resulting values were as follows: 1.91 ng of toxin per ml of blood at an administered dose of 77 ng; 6.0 ng of toxin per ml of blood at an administered dose of 230 ng; and 17.6 ng of toxin per ml of blood at an administered dose of 690 ng. These data make clear that saturation phenomena were not adversely affecting pharmacokinetic analyses (e.g., Fig. 4).

Induced Changes in Biologic Half-Life: Active Immunization. One possible mechanism to alter the half-life of toxin in the general circulation is to induce a circulating titer of neutralizing antibodies. To achieve this outcome, mice (n = 10) were vaccinated with the carboxyterminal half of the heavy chain of BoNT/B, as described previously (Ravichandran et al., 2007). Seven days after completion of the vaccination protocol, aliquots of blood were obtained and the circulating levels of IgG were determined. The vaccination protocol evoked a robust immunoglobulin response (50% serum dilution titer, approximately 3 × 10^4).

125I-BoNT/B (36 ng/animal) was administered i.v. to immunized mice (n = 10), and the concentration of toxin in blood was measured. The procedures were similar to those described above for control rats (e.g., Fig. 4). There was no reason to be concerned about survival of mice because vaccinated animals were resistant to poisoning. As shown in Fig. 5, prior vaccination of animals had a major effect on the disposition of administered toxin. There was an initial and rapid decline in blood levels of the agent. By implication,

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Fig. 4. 125I-BoNT/B was administered i.v. to rats (230 ng/animal), and at various times thereafter, animals (n = 5 per data point) were sacrificed, blood was collected, and the levels of toxin were quantified. The resulting data could be resolved into at least two rate constants, as follows: K_s t_{1/2} ~ 61 min; and K_p t_{1/2} ~ 600 min.

Fig. 5. 125I-BoNT/B (36 ng/animal) was administered to control mice (□) and to immunized mice (■), and the levels of toxin in blood were determined at various times thereafter (n = 5 per time point). The results demonstrate that there was an initial and rapid clearance of toxin from blood in animals that had a high titer of circulating antibodies. The curve describing clearance in immunized animals is extrapolated back to the origin, based on the premise that control animals and immunized animals received the same initial dose of toxin.
there was also a major reduction in the levels of free toxin available for distribution to cholinergic nerve endings.

Previous studies with BoNT/A have demonstrated that the principal reason for clearance of toxin from blood is accumulation of antigen-antibody complexes in tissues that participate in processing of immune complexes, such as liver and spleen (Ravichandran et al., 2006). This phenomenon was confirmed for BoNT/B, using the liver as a test organ. Mice were challenged with BoNT/B as described above, and 25 min later, they were anesthetized and perfused for subsequent removal of organs. The results of the experiment demonstrated that the circulating titer of antibody in immunized animals caused a marked increase in liver accumulation of toxin (Fig. 6). There was no such enhanced accumulation in tissues that play little role in processing of immune complexes (e.g., heart, kidney, and lung).

Dose-Response Characteristics of Toxin Accumulation in Liver. Antibody-mediated clearance from the general circulation is one of the key mechanisms that contributes to neutralization produced by active immunization and passive immunization. The importance of this mechanism highlights the need to determine whether hepatic clearance mechanisms can be saturated. This issue was addressed by performing dose-response experiments similar to those described above. Rats rather than mice were selected for study because of their resistance to BoNT/B. This allowed the experiments to be performed in the presence of relatively large amounts of toxin.

Rats received injections i.v. with $^{125}$I-BoNT/B at doses of 77, 230, and 690 ng. Each dose was administered simultaneously with 100 µL of rabbit antiserum. Animals ($n = 3$ or more per group) were sacrificed at 64 min for surgical removal of the entire liver, as described under Materials and Methods.

The results of these experiments, which are shown in Fig. 7, indicate that antibodies produced a dose-dependent accumulation of toxin in the liver. The nature of the dose-response data indicate that, within the range of toxin doses tested, the ability of the liver to accumulate antigen-antibody complexes was not saturated.

**Discussion**

An examination of the literature on the structure and biological activity of the botulinum toxin molecule reveals two interesting and inter-related trends. First, research in the past has focused almost exclusively on peripheral cholinergic nerve endings, which are the site of toxin action (Huemeau et al., 2000; Schiavo et al., 2000). However, more recent work has sought to describe all events associated with pathophysiology, from absorption to metabolism and elimination (Simpson, 2004). Second, earlier work dealt mainly with toxin action on the body, whereas more recent studies have broadened the focus to incorporate the action of the body on the toxin (Ravichandran et al., 2006).

Maintaining a perspective that incorporates both directions of action (toxin on the body; body on the toxin) is essential to a true understanding of botulinum toxin and botulism. There is a well known example that is a compelling argument in support of this premise. The toxin molecule causes paralysis of transmission of cholinergic nerve endings by cleaving cytosolic polypeptides that are essential for exocytosis. However, this action of the toxin on the body could not occur without an antecedent event. The catalytic domain of the toxin must be released from the rest of the holotoxin before it can exert its enzymatic effects, and release requires reduction of a disulfide bond (Schiavo et al., 2000; Simpson, 2004). Reduction of that bond is an example of the body acting on the toxin.

There are a number of studies that discuss the role of individual nerve cells in activating (e.g., disulfide bond re-
duction) and inactivating (e.g., metabolism) the toxin molecule. In contrast, there is almost a complete absence of studies that address systemic handling of the toxin molecule. This is unfortunate because studies on systemic handling of the molecule could contribute importantly both to an understanding of pathophysiology and to the search for medical countermeasures.

In a previous report, an effort was made to initiate pharmacokinetic studies on botulinum toxin, using serotype A (BoNT/A) as an example. Work in the present study continues that effort, with a focus on BoNT/B. A comparison of these two serotypes seems justified because BoNT/A and BoNT/B: 1) differ in their primary sequences by more than 50%, 2) evoke antibodies that do not cross-react (i.e., are serologically distinct), 3) bind to different polypeptide receptors on the neuronal membrane, and 4) cleave different substrates in the neuronal cytosol. However, the present study does more than merely compare serotypes. To the contrary, there are three new concepts that have been introduced. To begin with, this study uses a human model for studying absorption of toxin into blood. This model entails the use of gut epithelial cells, in which the binding and transcytosis of BoNT have already been characterized kinetically (Maksymowych and Simpson, 1998, 2004) and visually (Ahsan et al., 2005). Next, the study introduces a relatively resistant animal model (rat) to the analysis of BoNT pharmacokinetics. The rat is resistant to BoNT/B because of a point mutation at the cleavage site of the intraneuronal substrate (Patarnello et al., 1993). This mutation is not likely to alter the extracellular fate of BoNT/B; thus, the rat is an excellent model in which to examine systemic pharmacokinetics. Finally, this study takes advantage of the pharmacokinetic findings to help clarify the underlying bases for antibody-mediated clearance of toxin from blood.

**Stability of the Toxin.** There are a variety of routes by which the toxin can naturally enter the body, with the most important being the oral and inhalation routes. In both cases, the toxin penetrates epithelial barriers to reach the general circulation (Maksymowych and Simpson, 1998; Maksymowych et al., 1999; Park and Simpson, 2003). The toxin is too large to be eliminated by filtration in the kidney, and it is only poorly transcytosed across renal epithelial cells (Maksymowych and Simpson, 1998). In addition, it is unlikely that this large protein is eliminated mainly in expired air or mainly in perspiration. On the other hand, hepatic uptake and biotransformation are possible preludes to elimination. This means that, until such time as there is hepatic disposition of the molecule, the general circulation is essentially a “holding compartment” in which the toxin is retained while sufficient amounts are distributed to vulnerable nerve endings to cause poisoning.

It is interesting that this holding compartment does little to alter the structure or function of the toxin molecule. When BoNT/B was incubated in rat blood for an amount of time adequate to allow distribution to nerve endings, there were no obvious changes in the structure of the molecule. Both the amount of protein and the mol. wt. of this protein were unchanged. Likewise, there were no obvious reductions in biological activity. Whether assayed in a cell-free system (FRET assay for catalytic activity) or in an intact cell system (twitch response in phrenic nerve hemidiaphragm preparation), the toxin retained its distinctive biological properties. This result is in keeping with that previously reported for BoNT/A (Ravichandran et al., 2006).

The clinical relevance of the work was enhanced by performing comparable experiments on human tissues, in the process adding one important element. The toxin was added to monolayers of human gut epithelial cells, and material recovered on the basolateral side of cells was immediately examined to ensure there had been no intracellular processing. A sample of the collected material was also added to human blood to assess whether there would be significant biotransformation. The combined use of human epithelial cells and human blood to study absorption and processing of toxin represents a novel contribution to the literature. The results from this combined model demonstrate that neither the cellular transport compartment nor the fluid holding compartment, or the two in sequence, has a significant impact on the structure or function of the toxin molecule. To the contrary, they are tissue compartments with which the toxin interacts exploitatively to reach its site of action at cholinergic nerve endings.

**Biologic Half-Life.** Toxin that reached the general circulation of rodents displayed two rate constants. The initial and rapid rate ($K_p, t_{1/2}$, approximately 61 min) may represent a distribution phenomenon, during which toxin within the vasculature is distributed to the extravascular, extracellular space. The subsequent and slower rate ($K_p, t_{1/2}$, approximately 600 min) is likely linked to metabolism and elimination. A similar set of phenomena (initial and rapid rate; subsequent and slower rate) has been reported after i.v. administration of tetanus toxin, which is structurally and functionally related to botulinum toxin (Habermann, 1970). An initial rapid and subsequent slow rate of elimination has also been reported after i.v. administration of IgG to rats (Bazin-Redureau et al., 1997). It may be noteworthy that botulinum toxin and IgG are proteins with similar mol. wt. values.

The data obtained after administration of BoNT/B to rats, which are relatively resistant to this serotype, may prompt a reassessment of earlier work with BoNT/A that was administered to sensitive animals (Ravichandran et al., 2006). The apparent $t_{1/2}$ of approximately 4 h for metabolism and elimination of BoNT/A may be an underestimate due to the following: 1) the influence of the distribution half-life, which would tend to shorten the apparent elimination half-life; and 2) the absence of time points long after toxin administration because animals were succumbing to the effects of poisoning, which would also tend to shorten the apparent half-life. Experiments to reassess this matter are underway.

The fractional recovery of toxin in plasma and serum was approximately 90 and 77%, respectively. This is a strong indication that the toxin was not being accumulated in any of the various cells that circulate in blood. Furthermore, the slight loss in toxin during preparation of serum or plasma was almost surely due, at least in part, to packing of cells during the separation process. This means that the amount of toxin in the fluid phase was probably >90%.

In addition to cell association, there can also be protein association. Experiments on serum albumin binding revealed that only a small proportion of toxin in blood was bound to albumin (24% bound, 76% free). When taken together, these two phenomena (cell binding, protein binding) would lead one to believe that binding is not a trap that captures and retains

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It is interesting that this holding compartment does little to alter the structure or function of the toxin molecule. When BoNT/B was incubated in rat blood for an amount of time adequate to allow distribution to nerve endings, there were no obvious changes in the structure of the molecule. Both the amount of protein and the mol. wt. of this protein were unchanged. Likewise, there were no obvious reductions in biological activity. Whether assayed in a cell-free system (FRET assay for catalytic activity) or in an intact cell system (twitch response in phrenic nerve hemidiaphragm preparation), the toxin retained its distinctive biological properties. This result is in keeping with that previously reported for BoNT/A (Ravichandran et al., 2006).

The clinical relevance of the work was enhanced by performing comparable experiments on human tissues, in the process adding one important element. The toxin was added to monolayers of human gut epithelial cells, and material recovered on the basolateral side of cells was immediately examined to ensure there had been no intracellular processing. A sample of the collected material was also added to human blood to assess whether there would be significant biotransformation. The combined use of human epithelial cells and human blood to study absorption and processing of toxin represents a novel contribution to the literature. The results from this combined model demonstrate that neither the cellular transport compartment nor the fluid holding compartment, or the two in sequence, has a significant impact on the structure or function of the toxin molecule. To the contrary, they are tissue compartments with which the toxin interacts exploitatively to reach its site of action at cholinergic nerve endings.

**Biologic Half-Life.** Toxin that reached the general circulation of rodents displayed two rate constants. The initial and rapid rate ($K_p, t_{1/2}$, approximately 61 min) may represent a distribution phenomenon, during which toxin within the vasculature is distributed to the extravascular, extracellular space. The subsequent and slower rate ($K_p, t_{1/2}$, approximately 600 min) is likely linked to metabolism and elimination. A similar set of phenomena (initial and rapid rate; subsequent and slower rate) has been reported after i.v. administration of tetanus toxin, which is structurally and functionally related to botulinum toxin (Habermann, 1970). An initial rapid and subsequent slow rate of elimination has also been reported after i.v. administration of IgG to rats (Bazin-Redureau et al., 1997). It may be noteworthy that botulinum toxin and IgG are proteins with similar mol. wt. values.

The data obtained after administration of BoNT/B to rats, which are relatively resistant to this serotype, may prompt a reassessment of earlier work with BoNT/A that was administered to sensitive animals (Ravichandran et al., 2006). The apparent $t_{1/2}$ of approximately 4 h for metabolism and elimination of BoNT/A may be an underestimate due to the following: 1) the influence of the distribution half-life, which would tend to shorten the apparent elimination half-life; and 2) the absence of time points long after toxin administration because animals were succumbing to the effects of poisoning, which would also tend to shorten the apparent half-life. Experiments to reassess this matter are underway.

The fractional recovery of toxin in plasma and serum was approximately 90 and 77%, respectively. This is a strong indication that the toxin was not being accumulated in any of the various cells that circulate in blood. Furthermore, the slight loss in toxin during preparation of serum or plasma was almost surely due, at least in part, to packing of cells during the separation process. This means that the amount of toxin in the fluid phase was probably >90%.

In addition to cell association, there can also be protein association. Experiments on serum albumin binding revealed that only a small proportion of toxin in blood was bound to albumin (24% bound, 76% free). When taken together, these two phenomena (cell binding, protein binding) would lead one to believe that binding is not a trap that captures and retains
toxin in the general circulation. Instead, it is more likely a transient state that does little to alter the basic concept that toxin in blood is largely free and available for distribution to nerve endings.

Medical Countermeasures and Windows of Opportunity. As stated in the Introduction, one of the values of establishing the basic pharmacokinetics of the toxin molecule is to help understand the mechanisms of action of medical countermeasures. This is particularly true when the countermeasures are ones that can alter the disposition of toxin. Thus, antibody action during active immunization or passive immunization should be linked to toxin pharmacokinetics.

One well known outcome of active immunization is to evoke a circulating titer of antibodies that form complexes with antigen. These antibody-antigen complexes are cleared from the circulation by organs involved in the processing of immune complexes. This is precisely the result that was obtained in immunized mice. Administration of toxin to immune complexes. This is precisely the result that was obtained in immunized mice. Administration of toxin to immune complexes caused clearance of toxin from blood and accumulation of toxin in liver. This induced change in the systemic pharmacokinetics of the toxin is an important contributing factor in the phenomenon of antibody-induced resistance.

References


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