An Initial Assessment of the Systemic Pharmacokinetics of Botulinum Toxin

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ABSTRACT

Botulinum toxin is an extraordinarily potent molecule that has an unusually long duration of action. Despite this, there is little information available on natural mechanisms for metabolism or elimination and virtually no information on pharmacologically induced mechanisms for metabolism and elimination. Therefore, a number of experiments were performed on laboratory animals that addressed two major issues: 1) the effect of blood on the structure, function, and biologic half-life of the toxin, and 2) the effect of neutralizing antibodies on half-life and elimination of circulating toxin. In the first series of studies, the metabolic transformation of toxin was assessed by incubating it in blood for varying lengths of time. At each time point, aliquots were examined to determine the amount of toxin, the structure of toxin, the catalytic activity of toxin, and the neuromuscular blocking activity of toxin. This work demonstrated that blood did not alter any characteristic of the toxin molecule. Experiments were also done in which toxin was administered to mice and rats at doses that produced clinical poisoning. The results demonstrated that blood half-life for native (nonmetabolized) toxin in blood and serum was 230 to 260 min. During the second series of studies, the rate of elimination of circulating toxin was studied in the presence of antibodies directed against the carboxyl-terminal half of the toxin molecule. This work demonstrated that neutralizing antibodies enhanced clearance of toxin from the circulation and enhanced tissue accumulation of toxin, particularly in liver and spleen.

Botulinum toxin is one of the most remarkable molecules known to medicine and science. There are many reasons why this microbial toxin is viewed as special; however, two are particularly important. First, botulinum toxin is encountered in an unusual breadth of clinical settings. The molecule is now recognized as 1) the agent responsible for a naturally occurring disease (i.e., botulism) (Hatheway, 1995; Johnson and Goodnough, 1998); 2) an agent that can be used in acts of bioterrorism and biological warfare (Arnon et al., 2001); 3) an approved medication for the treatment of dystonia (Jankovic and Brin, 1991; Schantz and Johnson, 1997); 4) a widely administered medication to achieve various “esthetic” outcomes (Cather et al., 2002); 5) a transepithelial carrier for the creation of oral and inhalation drugs (Simpson et al., 1999); and 6) a neuronal carrier for delivery of drugs to cholinergic nerve endings (Zdanovskaia et al., 2000). There are few molecules that can rival botulinum toxin in terms of the breadth of clinical settings in which it is encountered.

A second and truly important characteristic of the molecule is its potency. Indeed, botulinum toxin is generally viewed as the most potent of all biological poisons (Lamanna, 1959; Gill, 1982). It is this very characteristic that underlies much of its prominence in medicine and science. For example, the fact that the toxin is lethal in minute quantities is the major reason for its being a potential agent of bioterrorism and biological warfare (Arnon et al., 2001).

The special status of botulinum toxin in the medical and the biological sciences has led to intense efforts to understand its structure and function. In terms of structure, the primary sequences of all seven serotypes (A to G) are known, and the crystal structures for certain serotypes have been reported (Lacy and Stevens, 1999; Swaminathan and Eswar-amoorthy, 2000). Botulinum toxin is a 150-kDa protein composed of a light chain (50 kDa) linked by a single disulfide bond to a heavy chain (100 kDa). The crystal structure of the molecule is distinguished by three lobes, which represent the light chain, the amino-terminal portion of the heavy chain, and the carboxyl-terminal portion of the heavy chain. In terms of function, the botulinum toxin molecule

ABBREVIATIONS: BoNT, botulinum neurotoxin; BoNT/A, BoNT type A; PAGE, polyacrylamide gel electrophoresis.
progresses through two sequences of events to produce its poisoning effect (Simpson, 2004). During the first, the toxin is absorbed across gut or airway epithelial cells to reach the circulation. In brief, the toxin binds to the apical surface of cells, undergoes endocytosis and transcytosis, and is released on the basal surface of cells. The toxin is then distributed throughout the periphery (Maksymowych and Simpson, 1998; Ahsan et al., 2005).

During the second sequence of events, the toxin blocks exocytosis at cholinergic nerve endings, such as those at the neuromuscular junction (Herreros et al., 1999; Humeau et al., 2000; Schiavo et al., 2000; Simpson, 2004). The carboxy-terminal portion of the heavy chain binds to the membrane of nerve terminals, the amino-terminal portion of the heavy chain promotes internalization to the cytosol, and the light chain acts as a metalloendoprotease to cleave polypeptides that are essential for transmitter release. The resulting blockade of transmission causes the muscle weakness or paralysis that is characteristic of the disease botulism.

In spite of the considerable progress that has been made in elucidating toxin structure and function, there are still considerable gaps in our knowledge. One area in which these gaps are especially large is the knowledge of the mechanisms for absorption, distribution, metabolism, and elimination. This in turn has hampered efforts to understand, and in some cases respond to, the various clinical settings in which the toxin is encountered. As an illustration, botulinum toxin causes a serious disease that can be either naturally occurring or the product of malice. In both cases, therapeutic intervention is hampered by a lack of information on the “window of opportunity” within which neutralizing antibodies can be administered to produce a beneficial effect. The data that are needed to establish window of opportunity, such as rate of metabolism and rate of elimination, are not available.

In this study, an effort has been made to initiate research that addresses the systemic fate of botulinum toxin. This has been done by focusing on three principal areas of concern: 1) developing techniques and establishing conditions for baseline systemic studies, 2) monitoring the natural fate of toxin after it has been delivered into the general circulation, and 3) monitoring the altered fate of toxin that has been exposed to neutralizing antibodies.

**Materials and Methods**

**Materials.** Sephadex G-25 gel filtration columns and reagents were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Other chemical reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and additional laboratory supplies were obtained from Fisher Scientific (Malvern, PA).

**Toxin.** Native BoNT type A (BoNT/A) was isolated from bacterial cultures as described previously (Sakaguchi, 1982; DasGupta and Sathyamoorthy, 1984; Simpson et al., 1988). The isolated neurotoxin had a homogeneity $\geq$98%, as determined by polyacrylamide gel electrophoresis (Laemmli, 1970). One inactive variant of the toxin was also used. To produce inactivation, native toxin was exposed to diethyl pyrocarbonate, which produced covalent modification of histidine residues, including the histidine residues that are essential for light chain catalytic activity. The toxin was modified as described previously (Rossetto et al., 1992).

The experiments in this study focused on the isolated neurotoxin (active or inactive), rather than progenitor toxin for three reasons. Firstly, there are no articles in the modern literature on botulinum toxin that demonstrate, either for laboratory animals or for human patients, that progenitor toxin reaches the general circulation. Secondly, even if the progenitor did reach the circulation, it could not persist. The main component of progenitor toxin is hemagglutinin, which would rapidly be absorbed by the gigantic excess of red blood cells. Finally, every investigator who has studied the phenomenon has reported that specific toxicity of neurotoxin is greater than that of progenitor toxin. The clear implication is that the toxin, not the progenitor, is distributed throughout the body to produce neuromuscular blockade.

**Antitoxin (Antiserum).** The carboxyl-terminal portion of the heavy chain of BoNT/A was cloned, expressed, and purified as reported previously (Maksymowych and Simpson, 2004). This material, which was used to evoke neutralizing antibodies, also had a homogeneity of $\geq$98%.

Antiserum was obtained by immunizing rabbits and rats. An initial injection of Alum-adsorbed antigen was given subcutaneously to rabbits (50 $\mu$g) and intranasally to rats (20 $\mu$g); three subsequent injections of nonadsorbed material (50 $\mu$g) 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 (Park and Simpson, 2003). For the experiments reported in this study, the circulating titer of IgG, as determined by dilution assay, was always 10$^4$ or higher.

**Iodination.** BoNT/A was iodinated using [125I]Bolton-Hunter reagent, essentially according to manufacturer's instructions. Pure neurotoxin (300 $\mu$g) in borate buffer (pH 7.5; 200 $\mu$l) was added to dried iodinated ester and reacted on ice for 15 min. The reaction was terminated by the addition of 50 $\mu$l of 1 M glycine in borate buffer for 15 min. The total reaction mixture (250 $\mu$l) and rinse (250 $\mu$l) were loaded onto a Sephadex G-25 column that was pre-equilibrated with filtration buffer (150 mM Na$_2$HPO$_4$, 150 mM NaCl, and 0.11% gelatin, pH 7.4). The labeled toxin was eluted with filtration buffer, and 0.5-ml fractions were collected. An aliquot of each fraction (5 $\mu$l) was assayed for radioactivity. The labeled toxin peak, which eluted at void volume, was pooled and stored at 3°C. Toxin concentration in the pooled fraction was determined spectrophotometrically at 278 nm using the following relationship: 1.63 A$_{278}$ = 1 mg/ml (DasGupta and Sathyamoorthy, 1984).

Iodinated samples were assayed in a gamma counter to ascertain disintegrations per minute. Sample concentration and associated counts were used to calculate specific activity.

**Iodinated Toxin Purity.** The homogeneity of 125I-BoNT/A was determined by SDS-PAGE. The BoNT/A samples were separated on a 10% polyacrylamide gel by the method of Laemmli (1970). Gels were run under nonreducing conditions and stained with Coomassie Blue stain followed by drying under vacuum (Gel Slab Dryer model 224; Bio-Rad, Hercules, CA) for 2 h. Gels were exposed to Hyperfilm ECL (GE Healthcare) for 24 to 48 h and developed using the Kodak X-OMAT film processor (Eastman Kodak Co., Rochester, NY). Developed films were analyzed to determine the presence of a 125I-BoNT/A band (\sim 150 KDa).

**Animals.** Swiss-Webster mice (female, 20–25g) and Sprague-Dawley rats (female; 200–250 g) were purchased from Ace Animals (Boytown, PA), and New Zealand White rabbits (female, 2–3 kg) were purchased from Covance (Denver, PA). All animals were housed in the animal care 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.

**Systemic Pharmacokinetics.** The biologic half-life of iodinated toxin in blood was determined in mice and in rats. The toxin was administered intravenously via the tail vein. Specimens were subsequently obtained by retro-orbital bleeding or by exsanguination. The amount of toxin in blood (or serum and plasma) was determined under various conditions and at various time points.

In one series of experiments, rats received both 125I-BoNT/A and
antiserum against the toxin. Three groups of animals were involved, as follows. 1) Toxin and antiserum were preincubated at room temperature for 30 min before administration; 2) toxin was administered to animals, and antiserum was given 10 min postchallenge; and 3) toxin was administered to animals, and antiserum was given 20 min postchallenge. As before, all injections were via the tail vein, and all specimens were obtained by retro-orbital bleeding.

In certain experiments, tissues were excised to determine the extent of local toxin accumulation. Rats were anesthetized by administration of Nembutal sodium (50 mg/kg; Abbott Laboratories, Chicago, IL), after which the thorax was opened to gain access to the heart. A butterfly needle (21-gauge needle) was inserted into the left ventricle, an incision was made in the right atrium, and the body was perfused with approximately 500 ml of phosphate-buffered saline with heparin (1 ml/500 ml of saline; American Pharmaceutical Partners, Schaumberg, IL). Several intact organs were removed from the body (liver, spleen, kidney, heart, and brain), and the accumulation of 125I-BoNT/A was determined.

**Serum Albumin Binding.** Ligand binding to rat serum albumin was quantified by affinity chromatography. Albumin was cross-linked to cyogen bromide activated-Sepharose 4B (agarose beads) according to the manufacturer's instructions (GE Healthcare). In each experiment, beaded albumin (2 x 10^9 M) was added to a 100-µl reaction volume that contained radiolabeled ligand, and the mixture was briefly stirred (rotary mixer, 10 rpm, 5 min). Albumin and radiolabeled ligand were then incubated at 37°C for 30 min followed by centrifugation at 12,000 rpm for 10 min. The supernatant was aspirated and discarded, after which the beads were washed three times (10 ml of phosphate-buffered saline, pH 7.4). The final pellet of cross linked albumin was used to determine amount of ligand binding.

**Challenge with Toxin.** 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. During pharmacokinetic experiments, as well as the associated experiments with antisera, animals received doses of toxin sufficient to produce poisoning. To minimize pain and suffering, animals were observed throughout the various protocols. When signs of neuromuscular weakness became apparent, animals were sacrificed in accordance with the Association for Assessment of Laboratory Animal Care guidelines (e.g., CO2).

**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 injected animals were also subjected to gel chromatography. In addition, a combination of studies that evaluated immunoprecipitation, enzymatic assays, and monitoring of neuromuscular transmission were done to ensure the structural and functional integrity of the toxin being measured (see below).

The structural integrity of administered botulinum toxin was assessed by combining immunoprecipitation and autoradiography. Biological specimens were incubated overnight at 4°C with an excess of anti-carboxyl-terminal fragment antibody raised in rabbits (see above). This mixture was subsequently incubated with Sepharose Protein A (Pierce, Rockford, IL) at 4°C for 1 h to immobilize the antigen + antibody complex. The reaction mixture was then centrifuged (3,000g; 10 min), washed once with phosphate-buffered saline (200 µl, pH 7.4), and recentrifuged (3000g, 10 min). The resulting complex was submitted to SDS-PAGE (10% gels, nonreducing conditions). The gels were dried under vacuum (2 h) and then exposed to Hyperfilm ECL for 24 to 48 h and developed using a Kodak O-XMAT film processor.

**Enzyme Assay.** The endopeptase activity of the holotoxin was measured using a fluorescent resonance energy transfer assay that employed a synthetic substrate that has been described previously (Schmidt and Stafford, 2003). BoNT/A 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 (2100g) 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; a gift from Dr. James Schmidt, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD). 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, Boston, MA) using FL Winlab software. Measurements were made at excitation of 390 and emission of 480, with an integration time of 3 s. The results were expressed as arbitrary fluorescence units.

**Neuromuscular Transmission.** Murine phrenic nerve-hemidaphragm preparations were used to bioassay the residual toxicity of botulinum toxin 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, 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 50% reduction in muscle-twitch response to neurogenic stimulation.

**Results**

**Iodination of Toxin.** The botulinum toxin complex was isolated from cultures of *Clostridium botulinum* (strain 62A), and the complex was further fractionated to obtain homogeneous neurotoxin. The Bolton-Hunter reagent was subsequently used to generate iodinated toxin, and exclusion chromatography was used to separate iodinated polypeptide from free iodine.

Botulinum toxin type A was iodinated three times during the course of the study. The average specific activity of the preparations was approximately 2.2 x 10^4 Ci/mmol. Each preparation was used for approximately 60 days. Aliquots of material for individual experiments were submitted to additional chromatography (G-25) before use to ensure removal of free isotope and of small polypeptide fragments.

A variety of experiments were performed to determine the structural integrity and biological activity of iodinated toxin immediately after collection of biological specimens (see below). However, it is important to note that, when bioassayed for activity before pharmacokinetic experiments, the in vivo toxicity of iodinated material was the same as that of native material.

**Structural Integrity of the Botulinum Toxin Molecule.** Botulinum toxin is absorbed either from the gut or from the airway to enter the general circulation. It is then distributed to peripheral cholinergic nerve endings, where it blocks the airway to enter the general circulation. It is then distributed out of blood are essential to tissue and/or function of the toxin molecule. To gauge the likelihood that blood can alter the toxin, a series of ex vivo experiments were performed.
Iodinated toxin was added to rat blood that was maintained at 37°C for 120 or 240 min (final concentration, 1 \times 10^{-7} \text{ M}). Samples were then immunoprecipitated with toxin-specific antisera. Aliquots were submitted to nonreducing polyacrylamide gel electrophoresis, and the gels were subsequently prepared for autoradiography. Control specimens were processed identically, with the exception that \(^{125}\text{I}-\text{BoNT/A} was added to blood immediately before immunoprecipitation.

The results, which are shown in Fig. 1, indicate that blood was not capable of significant endoprotease or exoprotease action on the toxin. Both the amount of material, as well as the apparent molecular weight of the material, was maintained over the course of the experiment. Thus, blood did not produce major structural changes in toxin.

**Biological Activity of the Toxin.** Two types of experiments (cell-free enzyme assay; neuromuscular junction bioassay) were done to assess whether toxin recovered in blood was biologically active and capable of producing blockade of exocytosis. In the first, botulinum toxin was added to rat blood, and this mixture was incubated for varying lengths of time. At each time point, aliquots of blood were withdrawn and assayed for metalloendoprotease activity.

The results of these experiments, which are shown in Fig. 2A, support two conclusions. Firstly, the enzymatic activity of the toxin did not diminish over the course of the experiment. Secondly, there was nothing in blood that interfered with the enzyme assay, and there was nothing in blood that sequestered toxin or otherwise made it unavailable for detection of catalytic activity.

In the second type of experiment, toxin was added to blood as before, and aliquots were drawn at the same time points. This material was then tested for its ability to block neuromuscular transmission in phrenic nerve-hemidiaphragm preparations. The results of these experiments were supportive of the earlier experiments on catalytic activity. Thus, toxin that was incubated in blood retained its ability to block neuromuscular transmission (Fig. 2B). By deduction, one can conclude that the binding domain, the translocation domain, and the catalytic domain of the molecule were structurally and functionally intact.

![Fig. 1. \(^{125}\text{I}-\text{BoNT/A} was added to rat blood and incubated at 37°C for 120 or 240 min. At the end of incubation, the specimen was submitted to immunoprecipitation with antibody directed against the carboxyl-terminal half of the heavy chain. An aliquot of this material was run in nonreducing polyacrylamide gel electrophoresis, after which the gel was analyzed by autoradiography. Lane 1 has a control \(^{125}\text{I}-\text{BoNT/A} sample that was added to blood for 0 min. Lane 2 has the toxin sample that was incubated with blood for 120 min. Lane 3 has a toxin sample that was incubated in blood for 240 min. The last lane indicates the position of molecular weight markers (gel electrophoresis without autoradiography).](image)

![Fig. 2. Unlabeled botulinum toxin (1 \times 10^{-7} \text{ M}) was incubated at 37°C in rat blood for varying lengths of time (0–256 min) and then assayed for catalytic activity and neuromuscular blocking activity. A, the catalytic activity was assayed using a synthetic substrate and a fluorescent resonance energy transfer technique (Schmidt and Stafford, 2003). The results are presented as arbitrary fluorescence units. For each time point, the data are illustrated for substrate added to rat blood (open bars) and for substrate plus toxin added to rat blood (filled bars). B, neuromuscular blocking activity was assayed on the murine phrenic nerve-hemidiaphragm preparation. The results are expressed as twitch response to neurogenic stimulation. Toxin was bioassayed for residual activity after varying lengths of incubation (0, 32, 64, 128, and 256 min). However, there was no loss of activity at any time point. The figure illustrates the data for the 0-min time point (●●●; n = 16) and for the 256-min time point (●●●; n = 4).](image)
fore, work was done to assess the percentage of the total toxin in blood that was free and thus available for redistribution.

For these experiments, labeled toxin (5 × 10⁻⁹ M) was added to a 100-µl reaction volume that contained ca. 1 mg of rat serum albumin bound to agarose beads (i.e., 1–2 × 10⁻⁴ M). After incubation, the beads were washed to separate bound toxin from free toxin. Similar experiments were done with [³⁵S]digoxin, which was used as a comparator that displays low-affinity binding to albumin (Plotz et al., 1974), and with [³⁵S]warfarin, which displays high-affinity binding to albumin (Olsen et al., 2004).

The serum albumin binding assay produced results that are in keeping with past reports. Approximately 5 to 6% digoxin was bound to albumin, meaning that more than 90% was free. Approximately 85% warfarin was bound to albumin, meaning that little was free. When the binding albumin assay was done with ¹²⁵I-BoNT/A, the data revealed that approximately 27% was bound; thus, more than 70% was free.

Biologic Half-Life. In an initial series of experiments, ¹²⁵I-BoNT/A (36 ng/animal) was administered to mice (group n = 7). After various intervals of time, aliquots of blood were obtained to quantify the amount of toxin still in the circulation. The results of this initial series of experiments revealed three things. Firstly, animals became seriously ill after ca. 90 min, at which point they were sacrificed. Secondly, the blood and serum levels of ¹²⁵I-BoNT/A were similar over this period of time. Thirdly, an experiment lasting ca. 90 to 100 min was not sufficiently long to reveal an actual, as opposed to an extrapolated half-life (>200 min; see below).

In the next series of experiments, mice (group n = 5) received ¹²⁵I-BoNT/A (36 ng/animal) that had been previously inactivated by covalent modification of histidine residues that are essential for toxin catalytic activity (i.e., diethyl pyrocarbonate-induced inactivation). As before, aliquots of blood were obtained at various time points to quantify the amount of toxin, and these aliquots were fractionated as described above to ensure that the toxin was intact. The results, which are shown in Fig. 3, indicate that the half-life in blood was ~239 min. In keeping with the experiments described earlier, the fractional recovery in serum was ~95% of that in blood, and this fractional recovery remained stable throughout the time course of the experiment. The half-life of toxin in serum was ~231 min.

A similar series of experiments was performed in rats, and again the administered dose of iodinated toxin was 36 ng/animal (group n = 7). As before, specimens were obtained at various time points, the individual specimens were fractionated, and the amount of toxin in blood and serum was quantified. The results (see Fig. 3) revealed that the half-lives in blood and serum were closely similar (260 and 255 min, respectively).

Neutralization with Specific Antiserum. The data in the preceding sections demonstrate that the elimination half-life for toxin in blood is ca. 4 h. During this time, the toxin molecule remains intact; i.e., there is no evidence of significant metabolism. In addition, the majority of the biologically active toxin is free and available for distribution to vulnerable nerve-endings. These findings set the stage for studying the phenomenon of induced metabolism and elimination.

One obvious pharmacologic intervention that can alter the disposition and biological activity of toxin is antibody treat-
becoming ill, although the time to onset of illness was prolonged. An interval of 20 min also resulted in illness, and the time to onset of illness was less prolonged.

One might argue that failure of chase administration of antiserum to provide full protection was merely a dilution artifact. More precisely, the administration of antiserum into the total blood volume of experimental rats may have produced a concentration that was too low to bind toxin. Therefore, the same animals that were used for toxicity experiments were also used for systemic pharmacokinetic experiments. The results were very helpful in interpreting the meaning of the challenge data.

Figure 5A shows the levels of toxin in blood over time for control animals. The data for experimental animals (i.e., toxin preincubation with antitoxin) were strikingly different. Within minutes, the levels of toxin in blood fell ca. 90%.

Interestingly, chase administration of antiserum at 10 (Fig. 5B) and 20 min (Fig. 5C) also had a dramatic effect. In both cases, there was a prompt fall in blood levels of toxin. Clearly, the administered dose of antiserum was adequate to find and alter the disposition of toxin that was still in the circulation.

**Clearance of Toxin and Tissue Accumulation.** The data in Fig. 5, A to C, show that rabbit antiserum promotes clearance of toxin from the circulation, which in turn would mean that there is enhanced tissue accumulation. The two likely candidates for accumulation are the liver and the spleen. To test this hypothesis, either free toxin or antiserum-pretreated toxin (0.01 ml; room temperature; 30 min) was administered to rats. Thirty minutes after administration, animals were anesthetized and extensively perfused. The livers and spleens were then excised, and the fractional accumulation of 125I-BoNT was determined (Fig. 6). For control animals, the accumulation of toxin (i.e., percentage of total administered dose) in liver and spleen was approximately 7 and 1%, respectively. When toxin was incubated with antitoxin before administration, these values increased substantially. Accumulation in liver was between 30 and 40%; accumulation in spleen was between 5 and 7%.

**Heterologous Antiserum versus Homologous Antiserum.** The possibility that toxin-specific antibodies can greatly promote clearance of toxin from the circulation has important therapeutic implications (see Discussion). Therefore, experiments were done to gauge whether enhanced clearance could be attributed 1) solely to the toxin-antitoxin reaction or 2) to the toxin-antitoxin reaction but only if the antibodies are heterologous in origin.

Experiments were done identically to those reported above, with the exception that both rabbit antiserum and rat antiserum were tested (note: the strain of rat used to raise antibodies was the same as that used in challenge experi-
ments). The results of this experiment are shown in Fig. 6. The data show that 1) tissue accumulation of free toxin in all tissues of control animals ranged from low to undetectable, 2) antibody promoted tissue accumulation in liver and spleen but not in kidney, heart, or brain, and 3) the effects of heterologous antiserum and homologous antiserum on tissue accumulation were comparable.

**Target Organ Protection.** The antitoxin used in the experiments described in the last section were generated by immunizing animals with the carboxyl-terminal half of the heavy chain (see Materials and Methods). This portion of the molecule is known to possess the domain that binds to receptors on the surface of peripheral cholinergic nerve endings (Simpson, 2004). This raises the possibility that at least one of the clonal antibodies in the antiserum might associate with toxin at, or close to, the receptor binding domain.

To test the possibility that antiserum could occlude receptor binding, unlabeled toxin was incubated without (control) or with (experimental) rabbit antiserum (0.01 ml; 30 min; room temperature) and then added to murine phrenic nerve-hemidiaphragm preparations to produce a final concentration of $1 \times 10^{-11}$ M. The paralysis time for control tissues was $91 \pm 7$ min ($n = 6$). Experimental tissues ($n = 6$) that were incubated with immune serum showed no evidence of neuromuscular blockade over an equivalent amount of time (Fig. 7).

**Discussion**

The general circulation plays a key role in the poisoning due to botulinum toxin. It is the link between the normal site of toxin absorption (e.g., gut or airway epithelial cells) and the major site of toxin action (e.g., neuromuscular junction). In spite of this, research on the systemic pharmacokinetics of botulinum toxin remains a largely unexplored field. To the extent that work has been done, it is mainly a byproduct of research on the spread of toxin after injection in the vicinity of nerve endings (i.e., therapeutic administration; Tang-Liu et al., 2003).

In the present study, an attempt has been made to initiate certain types of pharmacokinetic experiments. This work was governed by four major considerations. First, botulinum toxin type A was selected as a prototype. This is one of the three serotypes (A, B, and E) that has been identified as being of greatest concern in the context of bioterrorism and biological warfare (Arnon et al., 2001). Second, the doses of serotype A chosen for study were within the range that produces illness in laboratory animals. This confirmed that the data would be relevant to clinical poisoning. Third, all doses of toxin were administered directly into the circulation. This eliminated issues of concern, such as gastrointestinal metabolism and fractional absorption, and instead placed emphasis on distribution and elimination. Finally, attention was limited to the intact and biologically active holotoxin. The fate of metabolites, if any, was not addressed (see Simpson et al., 2005).

**Baseline Pharmacokinetic Studies.** The data support two conclusions about the interaction between blood and the circulating titer of botulinum toxin. 1) Blood, with its constituent cells and proteases, does little to attack or metabolize the toxin, and 2) no component of blood tightly binds or in any other way sequesters the toxin.

In relation to structure and biological activity, blood was shown to have little effect on the toxin molecule, at least during the time frame that is relevant to poisoning. Immunoprecipitation combined with SDS-PAGE autoradiography demonstrated that the amount and molecular weight of toxin that had been incubated in blood were indistinguishable from these same measures for control preparations. Bioassays for enzymatic activity and for neuromuscular blocking activity similarly demonstrated that blood had little impact. The studies on phrenic nerve-hemidiaphragm preparations were particularly revealing. Botulinum toxin must progress through many steps to block transmission, and these various steps depend on different functional domains within the molecule (Simpson, 2004). The retention of neuromuscular blocking activity is a strong indicator that toxin in blood is structurally and functionally intact.

The data also revealed that blood does not tightly bind or otherwise sequester toxin. Approximately 85 to 95% toxin in blood could be recovered in plasma or serum, and this fractional recovery remained constant over time. In addition, there was nothing in plasma or serum to which the toxin became tightly associated.

When taken in the aggregate, the data show that blood has little ability to alter the toxin (viz., metabolize) or to alter its bioavailability (viz., high-affinity binding) within the time frame that is relevant to the onset of poisoning. Instead, blood is mainly a conduit through which toxin is delivered to other sites in the body.

**Systemic Pharmacokinetics.** When administered intravenously to mice, a lethal dose of botulinum toxin had a blood and serum half-life of 230 to 240 min. The fact that the values were the same for both implies that formed blood elements were not accumulating toxin over time. The absence of evidence for accumulation reinforces the earlier observation that toxin in blood is relatively free for delivery to other sites. When studied in rats, BoNT/A had a half-life in blood similar to that of mice, and the half-life in blood was mirrored by that in serum. For the reasons given earlier, this equivalence of half-lives 1) rules out selective accumulation over time in formed blood elements and 2) reinforces the concept that toxin in blood is relatively available for delivery to target organs.

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Fig. 7. Botulinum toxin activity was bioassayed on the mouse phrenic nerve-hemidiaphragm preparation. The figure illustrates the results for control tissues (final toxin concentration, $1 \times 10^{-11}$ M; $n = 6$) and for experimental tissues (toxin preincubated with antitoxin for 30 min at room temperature; final toxin concentration, $1 \times 10^{-11}$ M; $n = 6$). Note that preincubcation with antitoxin caused loss of toxin activity within the time frame of the experiment.
The Effects of Neutralizing Antibody on Toxin Distribution and Activity. The baseline pharmacokinetic data indicate that there is little or no metabolism of toxin in blood during a time interval equivalent to the initial half-life for elimination. Furthermore, this unmodified toxin remains substantially free and thus available for distribution to cholinergic nerve endings. These findings naturally raise questions about pharmacologic interventions that can be used to promote metabolism and/or elimination. Neutralizing antibodies are the most obvious choice to achieve this outcome.

When neutralizing antibody was mixed with toxin before administration, it abolished neurotoxicity. When antibody was administered after toxin, the ability to produce neutralization decreased as the lag time to administration increased. Thus, a lag time of 10 min abolished the ability of the antibody to prevent death, although there was prolonged survival. A lag time of 20 min almost abolished protective activity.

There was an interesting disparity between the survival data and the pharmacokinetic data. Increasing the lag time before administration of antibody diminished antibody-induced protection but had little effect on antibody-induced changes in systemic pharmacokinetics. The explanation for this difference in outcomes is not hard to deduce. Fractional redistribution during the lag time may have been relatively small; nevertheless, it was sufficient for lethal concentrations of a high dose of toxin to reach vulnerable sites. Thus, subsequent administration of antitoxin could influence the disposition of toxin still in the circulation yet not reach or neutralize toxin that had associated with cholinergic nerve endings.

The combined data on toxicity and pharmacokinetics demonstrate that, after a substantial challenge dose, the window of opportunity to protect against poisoning is rather small. However, both the fractional redistribution that produces a terminal outcome and the duration of the window of opportunity are likely to vary as a function of dose. For example, lower doses would require a proportionately larger fractional redistribution to produce a terminal outcome and therefore would allow a proportionately longer window of opportunity to administer protective antibodies. Experiments to address this specific point are currently underway.

Mechanism of Inactivation. One final aspect of the study stands in some contrast to most of the previous literature on in vivo neutralization of botulinum toxin. Many previous reports have documented that antibodies can neutralize toxin, but no underlying mechanisms were described. By contrast, the present study documents that neutralizing antibodies raised against the carboxyl-terminal half of the heavy chain provided two layers of protection. The first layer of protection is related to pharmacokinetics. The association of antibody with toxin led to enhanced clearance from the circulation, which in turn was due to enhanced accumulation in certain tissues, particularly in the liver and spleen. Antibody-decorated toxin that was sequestered in liver and spleen was no longer available for distribution to vulnerable nerve endings.

The second layer of protection occurred at cholinergic nerve endings. Pretreatment of toxin with antiserum before addition to phrenic nerve-hemidiaphragm preparations greatly prolonged the paralysis times of these tissues. Neither clearance from blood nor accumulation in non-neural tissues can account for this finding. Instead, the antibody-toxin reaction must have blocked or delayed one or more steps in toxin-induced paralysis. Blockade of binding to membrane receptors is the most obvious possibility, but blockade of productive internalization is also a possibility. Given that the antibodies were raised against a portion of the heavy chain, blockade of light chain endoprotease activity could not be a possibility.

There is a subtle but perhaps important underlying distinction between these two layers of antibody-induced neutralization. The botulinum toxin molecule is a large protein that has numerous linear and conformational epitopes (Chen et al., 1997; Oshima et al., 1997; Atassi and Oshima, 1999). Many, and perhaps all, of these epitopes are potential antibody binding sites that can mark the toxin molecule for enhanced clearance from the circulation. This is very different from the state of affairs that applies to the nervous system. The binding domain of the toxin molecule is quite small compared with the total size of the toxin molecule. This is a strong indicator that the number of epitopes in or immediately juxtaposed to the binding site would also be small. This carries a substantial clinical implication. The prospect of achieving neutralization of toxin either by active means (e.g., administration of vaccine) or by passive means (e.g., administration of therapeutic antibodies) will be greater if one aims to achieve enhanced systemic clearance as well as blockade of receptor binding.

In the context of trying to identify mechanisms of antibody-induced neutralization of toxin, it is important to note that both heterologous antiserum (equine) and homologous immune globulin are available for human administration (Arnon et al., 2006). The underlying mechanism by which these preparations produce neutralization of circulating toxin has not been reported. Thus, it would be interesting to quantify the respective roles of enhanced clearance from the circulation and blockade of neuronal binding in the action of equine and human antibody preparations. Work that addresses these clinically relevant issues is currently underway.

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References


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