Identification of the Factors That Govern the Ability of Therapeutic Antibodies to Provide Postchallenge Protection Against Botulinum Toxin: A Model for Assessing Postchallenge Efficacy of Medical Countermeasures against Agents of Bioterrorism and Biological Warfare

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ABSTRACT

Therapeutic antibodies are one of the major classes of medical countermeasures that can provide protection against potential bioweapons such as botulinum toxin. Although a broad array of antibodies are being evaluated for their ability to neutralize the toxin, there is little information that defines the circumstances under which these antibodies can be used. In the present study, an effort was made to quantify the temporal factors that govern therapeutic antibody use in a postchallenge scenario. Experiments were done involving inhalation administration of toxin to mice, intravenous administration to mice, and direct application to murine phrenic nerve-hemidiaphragm preparations. As part of this study, several pharmacokinetic characteristics of botulinum toxin and neutralizing antibodies were measured. The core obser-

vation that emerged from the work was that the window of opportunity within which postchallenge administration of antibodies exerted a beneficial effect increased as the challenge dose of toxin decreased. The critical factor in establishing the window of opportunity was the amount of time needed for fractional redistribution of a neuroparalytic quantum of toxin from the extraneuronal space to the intraneuronal space. This redistribution event was a dose-dependent phenomenon. It is likely that the approach used to identify the factors that govern postchallenge efficacy of antibodies against botulinum toxin can be used to assess the factors that govern postchallenge efficacy of medical countermeasures against any agent of bioterrorism or biological warfare.

Introduction

The potential use of botulinum toxin as a weapon in acts of bioterrorism or biological warfare has prompted vigorous efforts to develop medical countermeasures (Arnon et al., 2001; Lane et al., 2001; NIAID Blue Ribbon Panel on Bioterrorism and Its Implications on Biomedical Research, 2002 [http://www.nti.org/e_research/source_docs/us/congress/house_representatives/129.pdf]). The three categories of agents that are receiving the

greatest attention are vaccines, therapeutic antibodies, and pharmacologic antagonists (Casadevall, 2002; Dickerson and Janda, 2006; Simpson, 2009). Of these three, the only ones for which there is a reasonably clear understanding of the temporal relationship between efficacious administration of the countermeasure and challenge with toxin are vaccines. Vaccines must be administered to patients before challenge with toxin, and the temporal relationship that governs efficacy is the amount of time needed for any particular antigen and vaccination protocol to evoke a protective immune response.

The issues surrounding the use of therapeutic antibodies and pharmacologic antagonists are more complex. For example, each of these classes of agents can be used in either preincident or postincident scenarios. In a preincident scenario, the goal would be to protect individuals against subsequent exposure to pathologic levels of botulinum toxin. In a

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ABBREVIATIONS: PBS, phosphate-buffered saline; PBST, PBS with 0.5% Tween 20; NFDM, nonfat dry milk; ELISA, enzyme-linked immunosorbent assay.

postincident scenario, the goal would be to block, or perhaps more realistically diminish, the full impact of prior exposure to toxin. The temporal factors that govern efficacy of pre-incident and postincident administration of therapeutic antibodies and pharmacologic antagonists are not well understood.

To date, no pharmacologic antagonist of botulinum toxin has been described that is 1) notably effective in blocking the onset of toxin action *in vivo*, and 2) approved for human use or close to entry into human clinical trials. The situation with therapeutic antibodies is more promising. A polyclonal preparation of antibotulinum toxin antibodies has already been approved by the Food and Drug Administration for human use (Arnon et al., 2006, 2007). In addition, prospects are good that an oligoclonal preparation of therapeutic antibodies will soon enter clinical trials (Amersdorfer et al., 1997; Chen et al., 1997; Nowakowski et al., 2002). This suggests that it would be worthwhile to undertake experiments that can accomplish two things. First, it would be helpful to know the interval of time before or after exposure to any given dose of toxin that administration of therapeutic antibodies can provide protection. Second, it would be useful to identify the factors that govern these temporal relationships.

In this article, a series of experiments are presented that focus on the use of therapeutic antibodies in a postincident scenario. The factors that determine an efficacious outcome are described, and the underlying mechanisms that govern these factors are identified. In addition, a conceptual framework is presented that could ultimately be applied to pharmacologic antagonists if and when agents are discovered that are likely to have clinical utility in a postincident scenario.

Materials and Methods

Toxin. Botulinum toxin type A (complex and pure) was purchased from Metabio (Madison, WI). All of the experiments, with the exception of those shown in Fig. 4, were done with the toxin complex. The data in Fig. 4 were obtained using pure neurotoxin. Regardless of whether toxin complex or pure neurotoxin were given, all doses (amount of protein) are expressed in terms of neurotoxin content. Individual batches of toxin were assayed for neurotoxin content (see below) and bioassayed for potency (mouse lethality assay). For the various batches of material used, one mouse LD₅₀ was consistently 5 to 7 μ g of neurotoxin.

Animals. New Zealand white rabbits (female; 2–3 kg) were purchased from Covance (Denver, PA). Swiss-Webster mice (female; 20–25 g) were purchased from Ace Animals (Boyertown, PA). Rabbits and mice were housed separately in the animal care facility at Thomas Jefferson University, and all procedures involving animals were reviewed and approved by the university's Institutional Animal Care and Use Committee.

Methods of Administration. Both botulinum toxin and antiserum directed against the toxin were administered by the intravenous route and the inhalation route. Intravenous injections were given as a single bolus (50 μ l) via the tail vein. Inhalation administration was given as a single bolus (15 μ l) that was applied to the nares of mice. The use of a small volume maximized the likelihood that an administered dose of toxin or antiserum remained in the airway rather than entering the gastrointestinal system. During intravenous and inhalation administration, mice were lightly anesthetized (3% isoflurane; 30–90 s). This procedure diminished stress to the animals, and during inhalation administration it also reduced the possibility of sneezing and expulsion of administered material.

Assay for Toxicity. Botulinum toxin activity was bioassayed both *in vivo* (mouse lethality assay) and *in vitro* (mouse phrenic nerve-hemidiaphragm assay).

For the mouse lethality assay, the most characteristic outcome of botulinum toxin poisoning 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. 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 and Accreditation of Laboratory Animal Care guidelines (e.g., CO₂).

One mouse LD₅₀ is defined as the dose of botulinum toxin that causes death of 50% of a challenged populations within 4 days (5760 min). For the purposes of this study, survival (see Figs. 3–6) was measured as the absence of weakness or paralysis for 6000 min postchallenge.

Murine phrenic nerve-hemidiaphragm preparations were used as an *in vitro* bioassay for botulinum toxin activity (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- to 0.3-ms duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a 90% reduction in muscle twitch response to neurogenic stimulation.

Luminescent Immunoassay for Botulinum Toxin and Antibodies. The concentrations of botulinum toxin in biological specimens were quantified with a luminescent sandwich immunoassay by using a human monoclonal antibody (4LCA) as a capture device (Adekar et al., 2008) and rabbit polyclonal antibodies as part of a reporter device (Stanker et al., 2008). Monoclonal antibodies were diluted in phosphate-buffered saline (PBS) to a concentration of 3 μ g/ml and coated on black Nunc Maxisorp plates (Nalge Nunc International, Rochester, NY). Plates were covered and stored overnight at 4°C, then antibody solutions were aspirated and discarded. Plates were blocked with 300 μ l per well of 2% nonfat dry milk (NFDM) in PBS with 0.05% Tween 20 (PBST) for 1 h at 37°C. Blocking solution was aspirated, and plates were washed three times with PBST.

Standards and experimental plasma samples were diluted 1:1 in PBS, then 50 μ l per well of diluted samples were added in triplicate to plates. Plates were covered and shaken slowly at room temperature for 1 h, then washed three times with PBST. Affinity-purified, biotinylated polyclonal anti-heavy chain antibodies were diluted in NFDM/PBST to a concentration of 3 μ g/ml and added to plates (50 μ l per well). Plates were incubated for 1 h at 37°C, then washed three times with PBST. A streptavidin poly-horseradish peroxidase conjugate was diluted to a concentration of 300 ng/ml in NFDM/PBST and added to plates (50 μ l per well). Plates were incubated for 30 min at 37°C, followed by nine washes with PBST. A luminol substrate (ThermoScie SuperSignal ELISA Femo Substrate; Thermo Fisher Scientific, Waltham, MA) was added to plates, and relative luminescence values were measured with a Biotek Synergy 2 Luminometer (BioTek Instruments, Winooski, VT). The limit of detection for the assay was 1 to 2 pg/ml.

A slight variation on the botulinum toxin assay was used to quantify antibodies directed against the toxin. Rather than adding monoclonal antibody 4LCA to plates as a capture device, the botulinum toxin heavy chain was used as a capture device. Affinity-purified, biotinylated polyclonal anti-heavy chain antibodies were administered to animals, and plasma samples from these animals were subsequently added to plates. The processing of these plates, and the

use of a streptavidin poly-horseradish peroxidase conjugate as a reporting device, was identical to that in the botulinum toxin assay, as described above.

Pharmacokinetics. The distribution half-life and elimination half-life of botulinum toxin were determined by methods similar to those described previously (Ravichandran et al., 2006; Al-Saleem et al., 2008). The toxin was administered intravenously to mice (50 μ l; tail vein), and at various times thereafter animals were sacrificed and blood was collected. Plasma was generated by adding heparin to blood, and the mixture was centrifuged in a clinical centrifuge (800 rpm; 20 min). Plasma was aspirated and stored at -20°C .

Experiments were also done to determine the levels of free toxin in blood before and after administration of a neutralizing dose of antibodies. Toxin was administered to mice as described above, and at various times before (1–20 min) and after (1–32 min) antibody administration, animals were sacrificed, blood was collected, and plasma was generated. Samples were stored at -20°C until analyzed.

The levels of free toxin in plasma were determined by a luminescent immunoassay (see above). The values were expressed as picogram of toxin per milliliter of sample.

Generation of Antibodies and ELISAs. Rabbits were actively immunized against the heavy chain of botulinum toxin. The reason for selecting rabbits was to generate high-titer antiserum preparations (ELISA dilution titers $>1,000,000$) that could subsequently be used as donor material in passive immunization experiments. Rabbits were vaccinated subcutaneously at a primary dose of 50 μ g (with 0.2% alum as adjuvant), followed by two booster doses on days 14 and 28 (50 μ g each; no adjuvant). Pooled rabbit antiserum was stored at -20°C until its use in clearance experiments.

Rabbit antiserum was titrated for antibody using a standard protocol (Takahashi et al., 2009). Flat-bottom 96-well microplates were coated with recombinant antigen (200 ng; 100 μ l/well) overnight at 4°C , followed by three washes with PBS containing 0.05% Tween 20, pH 7.4. Plates were blocked with 1% bovine serum albumin for 1 h at 37°C , after which 2-fold serially diluted serum samples were added, and plates were incubated for another 1 h at 37°C . Antigen-immunoglobulin complexes were treated with horseradish peroxidase-conjugated secondary antibodies (1: 1000 dilution in PBS) for 30 min at 37°C . Color was developed by adding substrate [2,2'-azinobis (3-ethylbenthiazoline-6-sulfonic acid)] in sodium citrate buffer, pH 5.0, containing 2 μ l of 30% H_2O_2 , (final concentration 0.3%) and incubating the plates for 30 min at 37°C . The reactions were stopped by adding 50 μ l of 2 N H_2SO_4 , and endpoint titers were derived as reciprocals of the last dilutions yielding an optical absorption equivalent to background (405 nm).

Results

Experimental Paradigms. There are several routes by which botulinum toxin and neutralizing doses of antiserum can be presented to peripheral cholinergic nerve endings, which are the target sites of toxin action (Fig. 1). The toxin can be delivered by the inhalation or oral routes, which are the ones that are most consistent with a potential bioweapons attack. For both of these routes of exposure, absorption of toxin depends on binding and transcytosis across epithelial cells (transport cells). For the present study, the inhalation route was selected as a model. This was deemed preferable to the oral route, for which low pH and gastric endoproteases serve as metabolic barriers to therapeutic antibody preparations.

The toxin can also be delivered directly into the general circulation. This is a less likely route of exposure for a bioweapons event, but nevertheless it possesses two noteworthy characteristics. First, it is the most straightforward exposure

Fig. 1. Postchallenge scenarios for analyzing therapeutic antibody efficacy. There are a variety of ways to expose cholinergic nerve endings to botulinum toxin, and each has its own experimental value. A, one approach is to administer the toxin by the inhalation or oral routes. This approach requires active absorption of toxin (i.e., binding and transport across epithelial cells), but it possesses the experimental value of mimicking a potential bioweapons attack. The inhalation route was selected as the experimental model for this study. B, a second approach is to administer toxin directly into the general circulation. The value of this approach is that it simplifies the process of analyzing the factors that influence postchallenge efficacy of neutralizing antibodies. C, a final approach is to apply the toxin directly to target cell nerve endings. This was accomplished by adding the toxin to isolated phrenic nerve-hemidiaphragm preparations.

paradigm to examine, and thus it can serve as a model for developing concepts to analyze other routes of exposure. Second, this is the route that would most likely be involved in clinical administration of therapeutic antibodies.

A third potential route of exposure would be direct application to peripheral cholinergic nerve endings, which would bypass absorption and bypass the general circulation. This route is the one that is least relevant to a bioweapons incident, but, interestingly, it is one that can be especially helpful in analyzing the sites and mechanisms of action of medical countermeasures.

All three routes of exposure were used in this study. Direct administration into the general circulation was used to develop an analytical approach for evaluating antibody action. Inhalation exposure was used to approximate an authentic bioweapons incident, and the resulting data were evaluated using the analytic approach developed during administration into the general circulation. Direct application to nerve endings was used to confirm the factors that govern the ability of antibodies to afford protection against exposure to the toxin.

Dose-Response Data. To ensure that the data had broad applicability, several doses of toxin spanning more than two orders of magnitude were tested (unless otherwise indicated, all experiments were done with toxin complex). For intravenous studies, the doses of neurotoxin were: 10 pg (approximately 2 MLD₅₀), 50 pg (approximately 10 MLD₅₀), 500 pg (approximately 100 MLD₅₀), and 5 ng (approximately 1000 MLD₅₀). For inhalation studies, the doses were 10, 50, and 500 ng and 5 µg. The differences between intravenous and inhalation doses reflect the differences in toxin potency administered by these two routes (Park and Simpson, 2003; F. H. Al-Saleem, D. M. Ancharski, S. G. Joshi, A. K. Singh, and L. L. Simpson, submitted for publication).

A rabbit antiserum preparation with a high circulating titer of antibotulinum toxin antibodies (dilution titer >10⁶) was used throughout these studies. Dose-response experiments were conducted to determine an amount of antiserum that would completely neutralize the intravenous doses of toxin being tested. Toxin was incubated with antiserum for 60 min at room temperature and then administered by the intravenous route to mice (*n* = 10). As shown in Fig. 2, 10 µl of antiserum completely neutralized 5 ng of toxin (i.e., all challenged mice survived). Both 3.3 and 10 µl of antiserum completely neutralized 500 pg of toxin. At lower doses of toxin (50 and 10 pg), all doses of antiserum (0.3–10) produced complete protection.

Fig. 2. Neutralization of botulinum toxin. Various amounts of rabbit polyclonal antiserum were incubated with botulinum toxin complex (neurotoxin content: 5 ng, ■; 500 pg, ●; 60 min at room temperature), then administered by the intravenous route to mice (*n* = 10). The antiserum produced dose-dependent neutralization of toxin.

Postchallenge Paradigm: Intravenous Toxin Followed by Intravenous Antibody. An initial dose-response experiment was conducted in which mice (*n* = 6 or more) were challenged with several doses of toxin (5 ng and 500, 50, and 10 pg). The results of this dose-response experiment are illustrated in Fig. 3. The survival times of animals ranged from approximately 105 min at the highest dose to approximately 1650 min at the lowest dose.

A family of curves was generated by administering each of the four toxin doses to mice (intravenously) and at various times thereafter (10–640 min) administering a neutralizing dose of antiserum (10 µl; intravenously). At the highest dose of toxin tested (5 ng), none of the animals that received antiserum survived (Fig. 3A). Even when the interval between toxin administration and antibody administration was only 10 min the survival rate was zero. When the dose of antiserum was increased from 10 to 100 µl, there were still no survivors.

Although the antibodies were not able to provide complete protection, they did produce interval-dependent prolongation of time to death. This afforded the opportunity to generate a quantitative measure of reduction in toxin potency (e.g., 90%), which will be referred to as potency (0.1). The data in Fig. 3A can be used to illustrate how this measure of potency was derived.

Control animals that received only toxin at a dose of 5 ng lived approximately 105 min. Control animals that received only toxin at a dose one order of magnitude lower (500 pg) lived approximately 250 min. When the interval between challenge with 5 ng of toxin and administration of antibody was 10 min, the time to death was approximately 400 min. This represents a reduction in toxin potency of more than 90%. By locating the point on the descending slope of the postchallenge interval curve that was equal to 250 min (i.e., the survival time associated with a 90% reduction in toxin dose; 500 pg), and by extrapolating to the x-axis, one obtained a postchallenge interval of approximately 16 min. Therefore, when the challenge dose of toxin was 5 ng, the postchallenge interval for antibody administration that diminished toxin potency by one order of magnitude was 16 min.

This same approach could be used to quantify the potency (0.1) value for administration of any dose of toxin, followed by postchallenge administration of neutralizing antibodies. Thus, the window of opportunity can be expressed either as the postchallenge interval of time within which neutralizing antibodies provide complete protection (survival) or the postchallenge interval of time within which neutralizing antibodies produce a quantifiable reduction in apparent toxin potency [90%; potency (0.1)].

A similar analysis was performed for the three remaining doses of toxin. When the challenge dose was 500 pg (Fig. 3B), there were intervals of time within which administration of antibody produced complete protection against poisoning. The window of opportunity associated with survival was 20 min. The window of opportunity associated with an apparent reduction in potency of one order of magnitude was 50 min.

When the challenge dose of toxin was 50 pg (Fig. 3C), the efficacy of antiserum administration was considerably greater. The window of opportunity associated with survival was 80 min. At this dose of toxin it was not possible to determine a potency (0.1) value, because a one order of magnitude reduction in potency would apply to a dose of 5 pg.

This is the LD₅₀ for botulinum toxin type A, meaning that on average half of the animals would die and half would survive. The fact that half of the population would live obviates any attempt to determine a potency (0.1) value. However, a close approximation can be achieved by using the data for 10 pg. This would represent an 80% rather than 90% reduction in potency. Using this as a measure, the potency (0.2) value was 98 min.

At the lowest dose of toxin tested (10 pg; Fig. 3D), the efficacy of antibody was dramatically greater. The window of opportunity associated with survival was 320 min. It was not possible to deduce a meaningful reduction in potency value [i.e., potency (0.1)] because of the very low dose of toxin being tested.

Examination of the window of opportunity data for all four doses of toxin reveals that as the challenge dose of toxin decreased the window of opportunity for obtaining any advantage from antibody administration increased. The lowest dose of toxin tested was 10 pg, or 2×1 LD₅₀. This is a close approximation to one lethal dose, and therefore the window of opportunity associated with survival (320 min) is probably a close approximation of the widest window of opportunity for survival that is attainable, at least in a mouse model.

Postchallenge Paradigm: Intravenous Toxin (Pure Neurotoxin) Followed by Intravenous Antibody. To ensure that complex toxin and pure neurotoxin are equivalent, when normalized for neurotoxin content, the intravenous postchallenge paradigm illustrated in Fig. 3 was reproduced, except that pure neurotoxin was administered as the challenge agent. As before, a family of curves was generated by administering four toxin doses to mice (5 ng and 500, 50, and 10 pg) by the intravenous route, followed at various times thereafter by a neutralizing dose of antiserum (10 μ l: intravenously). Examination of the data (Fig. 4) revealed that the results with pure neurotoxin were strikingly similar to those for the complex toxin (Fig. 3). The windows of opportunity for survival were identical for the four challenge doses of toxin, and the windows of opportunity for apparent reduction in potency (0.1 and 0.2) were closely comparable. The data indicate that the potency for complex toxin and pure neurotoxin given by the intravenous route, when doses were normalized for neurotoxin content, are similar.

Postchallenge Paradigm: Intranasal Toxin Followed by Intravenous Antibody. Administration of botulinum toxin by the inhalation route mimics one of the exposure scenarios thought to be relevant to potential acts of bioterrorism and biological warfare. Therefore, this is a particularly good scenario for examining the factors that govern the efficacy of therapeutic antibodies in a postincident paradigm.

The inhalation exposure experiments followed the same progression as the intravenous exposure experiments, beginning with a dose-response curve. In keeping with previous findings (Park and Simpson, 2003; F. H. Al-Saleem, D. M. Ancharski, S. G. Joshi, A. K. Singh, and L. L. Simpson, submitted for publication), the potency of toxin given as a bolus by the intranasal route in mice was approximately 500- to 1000-fold less than toxin given as a bolus by the intravenous route. The doses of toxin chosen for study (5 μ g to 10 ng) and the corresponding survival times are shown in Fig. 5.

A family of curves was generated by administering four inhalation doses of toxin and at various times thereafter a

standard dose of antiserum (10 μ l; intravenously). For each of the four doses, the windows of opportunity data were as follows: 5 μ g of toxin [survival = none; potency (0.1) = 36 min]; 500 ng toxin [survival = 20 min; potency (0.1) = 38 min]; 50 ng of toxin [survival = 80 min; potency (0.1) = 115 min]; and 10 ng of toxin [survival = 160 min; potency (0.1) = not determined].

A comparison of the data in Figs. 3 and 4 with those in Fig. 5 must take two points into account. First, the estimate that intravenous potency of botulinum toxin is greater than inhalation potency is based on a bioassay (e.g., *in vivo* survival data). Second, the measure of window of opportunity is a secondary bioassay based on the primary potency bioassay. When one takes these two points into consideration, it seems reasonable to conclude that the windows of opportunity for intravenous antibody to provide protection against equipotent doses of intravenous toxin and inhalation toxin are comparable. Furthermore, for both routes of exposure it is clear that the window of opportunity for obtaining any advantage from antibody administration increases as the challenge dose of toxin decreases.

Postchallenge Paradigm: Intranasal Toxin Followed by Intranasal Antibody. This sequence of experiments followed the same progression as the last three, but with one dose-related difference. The ability of antibody to alter postchallenge survival of mice given 5 μ g of toxin was not examined, because of the inability of the standard dose of antibody to provide complete protection at this toxin dose (Fig. 5A).

A family of curves was generated by administering 500, 50, and 10 ng of toxin and at various times thereafter a standard dose of antiserum. The results of these experiments (Fig. 6) were strikingly different from those in which toxin was given by the inhalation route and antibody was given by the intravenous route (e.g., Fig. 5). At toxin doses of 500 and 50 ng, there was no window of opportunity associated with survival. At a toxin dose of 10 ng there were also no survivals, although the time to death was greatly prolonged. At a toxin dose of 500 ng there was no window of opportunity associated with an apparent reduction of potency of one order of magnitude; at a toxin dose of 50 ng the potency (0.1) value was 14 min; and at a toxin dose of 10 ng the potency (0.1) value could not be determined.

Pharmacokinetics of Botulinum Toxin. Several doses of botulinum toxin were administered by the intravenous route to mice, and at various times thereafter animals were sacrificed, blood was collected, plasma samples were generated, and the circulating levels of toxin were determined. The doses of toxin that were examined were 10 and 1 ng and 100 pg. At the highest dose tested (10 ng), the anticipated survival time of mice was approximately 80 min. Therefore, the duration of the pharmacokinetic experiments was set at 64 min, which minimized any impact the onset of paralysis might have on disposition of toxin.

The baseline pharmacokinetics for intravenously administered botulinum toxin are shown in Fig. 7. The data indicate that for each dose of administered toxin there were two first-order kinetic processes: an initial distribution phase, and a subsequent elimination phase. The distribution phase probably represents the time for the toxin to be distributed throughout the vasculature, then the peripheral extravascular, extracellular space (e.g., the compartment that presents toxin to vulnerable nerve endings). The elimination phase

Fig. 3. Postchallenge paradigm of intravenous toxin followed by intravenous antibody. Mice were challenged with four doses of botulinum toxin complex (neurotoxin amounts: 5 ng, ■; 500 pg, ●; 50 pg, ▲; and 10 pg, ◆; C = control, toxin but not antibody). The survival times for control animals receiving these doses of toxin are shown. Experimental animals received the same doses of toxin, and at various times after toxin administration a neutralizing amount of polyclonal antiserum was administered (open symbols). The efficacy of antibody (i.e., window of opportunity within which

represents the time for the toxin to be eliminated by natural processes from the central compartment.

The rate constants for these two processes for each administered dose of toxin are given in Table 1. The most obvious point to emerge from these data is that the two rate constants were notably similar across doses. This means that doses of toxin that differed by two orders of magnitude did not differ by even 2-fold in their respective distribution rate constants or elimination rate constants.

Extrapolation of the elimination curves for each of the toxin doses to the y-axis (i.e., time 0) allows one to deduce the apparent volumes of distribution. The values for these volumes of distribution, which are given in Table 1, were comparable for all three toxin doses. Given that the elimination rate constants were independent of dose, and the apparent volumes of distribution were independent of dose, one can deduce that the rates of total body clearance were independent of dose.

As indicated above, the duration of the pharmacokinetic experiments was governed by the expected survival time of mice receiving the highest toxin dose (10 ng; 80 min). As a result, the $t_{1/2}$ for elimination had to be determined by extrapolation of the curves in Fig. 7. An examination of the data for the distribution rate constants for the three doses of toxin that were tested in Fig. 7 gave an average value of 16.6 min (Table 1). This means that the amount of time necessary for the distribution process to approximate completion (i.e., approximately 90%) was approximately 55 min (i.e., 3.3×16.6). This represents a large proportion of the total length of the pharmacokinetic experiment shown in Fig. 7, and therefore the estimated half-life for elimination may have been influenced by this.

To obtain a truer estimate of the half-life for elimination, one experiment was performed in which biological samples were collected for a substantially longer period of time. Mice were injected intravenously with a dose of 500 pg, and samples were obtained for 200 min (note: the expected survival time for these mice was approximately 250 min; see Fig. 3B). The $t_{1/2}$ for elimination in this experiment was approximately 408 min (see Fig. 8). Thus, when the duration of the experiment was sufficiently long to minimize the impact of the distribution process (relatively rapid) on the elimination process (relatively slow), the half-life for elimination was slightly increased.

In Vivo Clearance of Botulinum Toxin. The major mechanism by which circulating antibodies neutralize botulinum toxin is induced clearance, in which antigen-antibody complexes are removed from the general circulation and accumulate in liver and spleen (Ravichandran et al., 2006; Al-Saleem et al., 2008). This antibody-driven clearance is a prelude to metabolism and elimination.

Clearance is presumably the major mechanism by which therapeutic antibodies act in a postchallenge paradigm. This

premise was tested by administering botulinum toxin by the intravenous route and at various times thereafter administering antibodies by the intravenous route. The first experiment involved the administration of 500 pg of toxin, a neutralizing amount of antiserum (10 μ l; see Fig. 2), and a window of opportunity associated with survival of all challenged animals (20 min; Fig. 3B). Biological samples were obtained at 1, 2, 4, 8, 16, and 20 min after toxin administration and 1, 2, 4, 8, 16, and 32 min after antibody administration.

The levels of free toxin measured in plasma before antibody administration were comparable with those observed in control mice examined in pharmacokinetic experiments (e.g., Figs. 7 and 8). The levels of free toxin in plasma after antibody administration fell rapidly and dramatically. Within minutes the levels had fallen more than one order of magnitude (Fig. 9A). By extension, one can deduce that the amounts of free toxin available for distribution to nerve endings had fallen by an order of magnitude.

In the next experiment the dose of administered toxin was increased to 5 ng. The dose of antiserum was maintained at 10 μ l, and the interval between administration of toxin and administration of antiserum was maintained at 20 min. The critical distinction between this experiment and the previous one is that, in this case, the antibody would not be expected to produce survival (Fig. 3A). It is noteworthy that the antiserum was fully active in terms of evoking clearance (Fig. 9B). Just as in the previous experiment, there was a rapid and dramatic reduction in the circulating levels of free toxin.

The final experiment in the series involved the administration of 500 pg of toxin and 10 μ l of antiserum. The interval between administration of toxin and antiserum was increased to 60 min, which is an interval that is associated with no survival (Fig. 3B). Similarly to the previous two cases, the antibody still evoked a dramatic reduction in the levels of free toxin (Fig. 9C). Thus, antibodies retained their characteristic ability to bind to antigen and evoke clearance, even when the dose of toxin was too large to permit survival (Fig. 9B), and when the interval between administration of toxin and administration of antiserum was too long to permit survival (Fig. 9C).

In Vitro Clearance of Botulinum Toxin. The in vivo scenario in which neutralizing antibodies were used to eliminate free toxin from the fluid compartment can be mimicked in vitro. In this case, the toxin can be applied directly to target nerve endings in a tissue bath. Elimination of free toxin can be achieved simply by replacing the fluid compartment, without the need for neutralizing antibodies.

Various doses of botulinum toxin (3×10^{-10} to 3×10^{-13} M) were added to isolated phrenic nerve-hemidiaphragm preparations, and at various times thereafter the baths were emptied, tissues were washed, and baths were replenished with medium without added toxin (Fig. 10). The results of these experiments were qualitatively similar to those ob-

antibody was effective) was measured in two ways: survival (no deaths by 6000 min), and a quantifiable reduction in apparent toxin potency (See *Postchallenge Paradigm: Intravenous Toxin Followed by Intravenous Antibody* for explanation). A, at a toxin dose of 5 ng, postchallenge administration of antibody did not produce survival, and there was only a short interval within which it could reduce apparent toxin potency by 90% [potency (0.1) = 16 min]. B and C, at toxin doses of 500 pg (B) and 50 pg (C), postchallenge administration of antibody did produce survival (20- and 80-min postchallenge interval, respectively) and quantifiable reductions in apparent potency (50- and 98-min postchallenge interval, respectively). D, at a toxin dose of 10 pg, there was a dramatic widening of the window of opportunity within which antibody produced survival (320 min). Because of the low toxin dose, it was not possible to obtain a potency (0.1) value (ND, not determined). A comparison of the data for all toxin doses revealed that the efficacy of antibody administration increased as the dose of toxin decreased. Each data point represents n of 6 or more, and the S.E.M. for each data point was equal to or less than 8% of the mean for that data point.

Fig. 4. Postchallenge paradigm of intravenous toxin followed by intravenous antibody. The methods and data analysis are identical to those in Fig. 3, except that pure neurotoxin was administered rather than toxin complex. The windows of opportunity for survival and for apparent reduction in potency are given. Each data point represents n of 6 or more, and the S.E.M. for each data point was equal to or less than 9% of the mean for that data point.

Fig. 5. Postchallenge paradigm of intranasal toxin followed by intravenous antibody. These experiments were conducted and analyzed identically to those in Fig. 3, except that toxin was administered by the inhalation route. A, when toxin was given at a dose of 5 µg, subsequent administration of antibody did not produce survival, although there was a window of opportunity associated with an apparent reduction in potency [potency (0.1) = 36 min]. B and C, at toxin doses of 500 ng (B) and 50 ng (C), postchallenge administration of neutralizing antibody did produce survival and decreases in the apparent potency of toxin. D, at the lowest dose of toxin (10 ng), there was the widest window of opportunity for postchallenge administration of antibody to produce survival. It was not possible to obtain a potency (0.1) value because of the low dose of toxin tested (ND, not determined). Each data point represents *n* of six or more, and the S.E.M. for each data point was equal to or less than 11% of the mean for that data point.

Fig. 6. Postchallenge paradigm of intranasal toxin followed by intranasal antibody. The data from these experiments were analyzed identically to those in Fig. 3. Botulinum toxin was administered at doses of 500 ng (●), 50 ng (▲), and 10 ng (◆). Postchallenge administration of antibody did not produce survival at any toxin dose. Postchallenge administration of antibody was not sufficiently efficacious to produce a 90% reduction in apparent toxin potency at a toxin dose of 500 ng, but it did produce a 90% reduction in apparent toxin potency at a dose of 50 ng (14 min). At a toxin dose of 10 ng, a potency (0.1) could not be determined (ND). Each data point represents n of three or more, and the S.E.M. for each data point was equal to or less than 9% of the mean for that data point.

tained in the various in vivo postchallenge paradigms (Figs. 3–6). Postexposure clearance of toxin from the bathing medium resulted in protection against poisoning that was time- and dose-dependent. As the dose of toxin decreased, the window of opportunity within which clearance of toxin afforded protection increased.

At the highest dose of toxin tested (3×10^{-10} M), which caused paralysis of tissues within 40 to 50 min, there was only minimal protection, even when washing occurred within 2.5 min. At a toxin dose of 3×10^{-11} M, there was a measurable window of opportunity. The potency (0.1) value was approximately 9 min.

An excised murine phrenic nerve-hemidiaphragm is usable for only approximately 7 h. When tissues were exposed to a toxin concentration of 3×10^{-12} M, washing provided the maximum protection that is measurable in an isolated tissue (e.g., approximately 7 h) for postexposure intervals up to 10 min, and the potency (0.1) value was 13 min. At a toxin concentration of 3×10^{-13} M, the maximum protection that is measurable (7 h) was obtained for postexposure intervals up to 20 min. A potency (0.1) value could not be obtained at this low dose of toxin.

Prechallenge Paradigm: Intravenous Antibody Followed by Intravenous Toxin. In one set of experiments the sequence of toxin/antibody administrations was reversed. A standard dose of antibody (10 μ l) was administered to mice, and at various times thereafter the animals were challenged with four doses of toxin.

The data from this experiment, which are shown in Table 2, illustrate a profound difference between the window of opportunity in a prechallenge paradigm and the window of opportunity in a postchallenge paradigm (e.g., Figs. 3 and 4). In a postchallenge paradigm, the window of opportunity for providing complete protection was measured in minutes. In

the prechallenge paradigm, the window of opportunity was measured in days.

Pharmacokinetics of Antibody. By definition, one mouse LD₅₀ is the amount of toxin that causes death of 50% of a population within 4 days. In an earlier set of experiments, the pharmacokinetics of several doses of botulinum toxin were examined (Fig. 7; Table 1). Those studies revealed that the $t_{1/2}$ for systemic elimination of toxin was measured in minutes. This is far less than the survival time associated with one LD₅₀. Therefore, companion experiments were done to determine the systemic fate of antitoxin antibodies over 4 days. Five micrograms of biotinylated antibody was administered intravenously to mice, and at various times thereafter animals were sacrificed, plasma samples were generated, and the circulating titers of antibodies were measured. The results of this experiment revealed that there was less than 20% reduction in the circulating titer of antibodies over 4 days (Fig. 11). Stated differently, the $t_{1/2}$ for elimination of antitoxin antibodies is orders of magnitude longer than the $t_{1/2}$ for elimination of botulinum toxin.

Discussion

In the wake of the terrorist attacks that occurred with commandeered aircraft on September 11, 2001, and the bioterrorist attacks with anthrax toxin that followed shortly thereafter, there have been intense efforts to develop protective measures that will safeguard both civilian and military populations. In the context of potential bioweapons attacks, one of the agents of major concern is botulinum toxin. Thus, there have been intense efforts to develop medical countermeasures such as vaccines, therapeutic antibodies, and phar-

Fig. 7. Pharmacokinetic characteristics of botulinum toxin. Three doses of botulinum toxin (10 ng, ●; 1 ng, ■; 100 pg, ▲) were administered intravenously to mice (tail vein; $n = 5$ or more per data point). At various times thereafter the animals were sacrificed, and plasma samples were analyzed for toxin by luminescent immunoassay. For each toxin dose there seemed to be two first-order rate constants: a distribution rate constant (dashed line) and an elimination rate constant (solid line). The half-life values for these two rate constants are given in Table 1. Inspection of the data here and the half-life values in Table 1 show that the pharmacokinetic characteristics of the toxin were similar over toxin doses that spanned two orders of magnitude.

TABLE 1

Pharmacokinetic constants for botulinum toxin administered by the intravenous route

Pharmacokinetic constants were extracted from the graphic data in Fig. 7. Note that the $t_{1/2}$ values for distribution are comparable, whereas the $t_{1/2}$ values for elimination are more variable. This likely reflects the fact that experiments were sufficiently long to encompass the entire distribution process, but not sufficiently long to encompass the entire elimination process (see *Pharmacokinetics of Botulinum Toxin* and Fig. 8). The apparent volume of distribution for each dose of toxin was quantified by extrapolating each curve describing an elimination process to the y-axis, then dividing the resulting value (in pg/ml) by the corresponding administered dose of toxin.

Pharmacokinetic Constant	Toxin Dose			X
	10 ng	1 ng	100 pg	
Distribution $t_{1/2}$ (min)	18.2	14.5	17.2	16.6
Elimination $t_{1/2}$ (min)	266	364	231	287
Apparent volume of distribution (ml)	4.8	5.0	4.6	4.8

macologic antagonists that will block poisoning caused by botulinum toxin.

Progress toward clinical evaluation and ultimate approval for human use of these three classes of countermeasures has been somewhat slow. Nevertheless, there has been measured progress in the area of antibody-mediated

countermeasures. For example, a recombinant vaccine (carboxyl-terminal half of toxin heavy chain) is currently in clinical trials (Smith, 2009), one therapeutic antibody preparation (human immunoglobulin) has progressed through the regulatory process and obtained approval for human use (Arnon et al., 2006, 2007), and another thera-

Fig. 8. Pharmacokinetic characteristics of botulinum toxin. The duration of the experiment in Fig. 7 was governed by the survival time of mice that received the highest dose of toxin (10 ng; approximately 80 min). For the data to the right, mice ($n = 10$ per data point) were injected intravenously with 500 pg of toxin (expected survival approximately 250 min), and biological samples were collected for 200 min. The $t_{1/2}$ for the distribution phase (dashed line; 14.3 min) was similar to those obtained in the prior and shorter experiments (Table 1). The $t_{1/2}$ for the elimination phase (solid line; 408 min) was slightly longer than those obtained in the shorter experiments.

Fig. 9. Antibody-induced elimination of free toxin from the general circulation. Three clearance paradigms were examined: 500 pg of toxin and postchallenge administration of antibody at 20 min (A), 5 ng of toxin, followed by antibody at 20 min (B), and 500 pg of toxin, followed by antibody at 60 min (C). In each paradigm biological samples were collected at various time points before and after administration of antibody ($n = 3$ or more per data point). The distribution phase (dashed line) and the elimination phase (solid line) from pharmacokinetic experiments are superimposed on each of the three parts of the figure. Note that in each paradigm the intravenous administration of neutralizing antibody is associated with a rapid and dramatic decline in the levels of free toxin (pg/ml; plasma). This occurred for the paradigm that is associated with survival of mice (A), but also for the two paradigms that are not associated with survival (B and C; compare with data in Fig. 3).

peutic antibody preparation (oligoclonal combination of humanized antibodies) is about to enter clinical trials (Amersdorfer et al., 1997; Chen et al., 1997). In contrast to antibody-based countermeasures, there is no pharmacologic antagonist that has been approved for human use or has entered human trials.

The temporal issues that govern vaccine efficacy are relatively straightforward. In a postchallenge incident there is no interval within which a vaccine can afford protection. In a prechallenge incident, the temporal relationship that governs efficacy is the amount of time needed for any particular antigen and vaccination protocol to evoke a protective immune response.

The temporal factors that govern therapeutic antibody efficacy are more complex. In a prechallenge incident, the temporal factor of paramount importance is the amount of time needed to administer antibody and achieve neutralizing titers in the general circulation. The duration of protection will then be governed by the pharmacokinetics of the antibody preparation, because the elimination half-life for antibodies (Fig. 11) is much longer than that of botulinum toxin (Fig. 7; Table 1).

The temporal factors that govern efficacy of antibodies in a postchallenge paradigm are more difficult to analyze. However, it is possible to identify one core observation that is critical to understanding antibody efficacy. In all three of the *in vivo* paradigms that were studied, the window of opportu-

Fig. 10. In vitro clearance of botulinum toxin. Several doses of botulinum toxin, from 3×10^{-10} to 3×10^{-13} M, were added to murine phrenic nerve-hemidiaphragm preparations ($n = 4$ per time point). At various times after the addition of toxin, tissues were washed and immersed in medium without toxin. The paralysis times of tissues (90% reduction in twitch amplitude) were then measured. It is not practical to measure "survival" with an isolated tissue, but it is appropriate to measure apparent reductions in toxin potency [e.g., potency (0.1)]. Therefore, the paralysis times of tissues were monitored as a function of the interval of time between addition of toxin to the tissue bath and subsequent washing to remove free toxin. The potency (0.1) values for each toxin dose are listed. Note that the in vitro clearance experiments (washing) mimic the in vivo clearance experiments (antibody); thus, the lower the toxin dose, the wider the window of opportunity for clearance to afford protection. ND, not determined.

TABLE 2

Survival rates of animals that received antibody before challenge with toxin

Animals group ($n = 6$) were injected with neutralizing antibody (10 μ l), and at various times thereafter (1–32 days) they were injected with the indicated dose of toxin. Both injections were given via the tail vein. The percentage of animals that survived is presented.

Time (days)	Toxin Dose			
	50 ng	5 ng	500 pg	50 pg
	% survived			
1	100	100	100	100
2	100	100	100	100
4	100	100	100	100
8	100	100	100	100
16	0	100	100	100
20		0	50	100
24			0	50
28				50
32				0

nity within which antibodies could provide protection against poisoning increased as the challenge dose of toxin decreased. This was true both for survival and potency (0.1). Any proposed model to account for antibody activity must be consistent with this core observation.

There is yet another fact that must be taken into account when analyzing the window of opportunity for antibody administration. Botulinum toxin possesses a highly efficient mechanism for binding and internalization at nerve endings, whereas natural antibodies possess no such mechanisms. This means that the efficacy of an antibody molecule hinges on its ability to locate and associate with a toxin molecule before the latter is internalized by a nerve cell. Any model to account for antibody activity must be consistent with the differing neuronal fates of toxin and neutralizing antibody.

Governing Factors. An analysis of the pharmacokinetics of botulinum toxin is a potentially powerful way to identify the specific factors that govern antibody action. For example, toxin that reaches the general circulation undergoes a distribution phenomenon and an elimination phenomenon. The distribution phase probably involves the movement of toxin out of the vasculature and into the extravascular, extracellular space. This is the fluid compartment through which the toxin must pass to reach vulnerable nerve endings. This process is relatively rapid, and therefore one might assume that it could govern the window of opportunity for antibody administration. However, an examination of the data makes clear that this cannot be true. The $t_{1/2}$ for the distribution phase was virtually identical for all toxin doses that spanned two orders of magnitude. Given that the window of opportunity for antibody administration increases as toxin dose decreases, any pharmacokinetic process that is constant across toxin doses cannot be a governing factor.

A similar argument applies to the elimination phase. The $t_{1/2}$ for toxin elimination did not change significantly for toxin doses that covered two orders of magnitude. This result is not surprising. The concentrations of toxin that were measured in biological samples were in the picomolar to subpicomolar range. It is highly unlikely that concentrations in this range would saturate any metabolic process or elimination process. Thus, the elimination half-lives were very similar across doses, and this similarity of rates could not be the factor that governs dose-dependent windows of opportunity.

There is yet another pharmacokinetic process that can be discounted as a governing factor. The principal mechanism of antibody action in the general circulation is evoked clearance of toxin. This is a two-step process in which 1) antibody molecules bind to toxin molecules, and in the process cause a loss of free toxin, and 2) antibody-toxin complexes are cleared from the circulation by uptake in liver and spleen (Ravichandran et al., 2006; Al-Saleem et al., 2008). The neutralizing dose of antibody used in this study certainly did eliminate free toxin from the circulation. For example, when antibody was administered to mice under conditions that led to survival of challenged animals, the levels of free toxin fell dramatically. However, it was also true that antibody eliminated free toxin from the circulation when the dose of toxin was too high to permit survival, and when the postchallenge interval before administration of antibody was too long to permit survival. The latter observations mean that antibody retains its ability to evoke clearance of toxin, even after paralyzing doses of toxin have entered nerve endings.

Although the phenomenon of clearance is not the temporal factor that governs antibody efficacy, the clearance experiments themselves are very revealing. They demonstrate that free toxin can locate, enter, and poison vulnerable nerve endings long before significant amounts of toxin are elimi-

Fig. 11. Pharmacokinetic characteristics of antitoxin antibodies. Affinity-purified antitoxin antibodies (5 μ g) were administered intravenously to mice (tail vein; $n = 4$ per data point), and at various times thereafter animals were sacrificed and plasma samples were obtained. Over 4 days, the fractional loss of antibodies from the general circulation was low (<20%). This very long half-life probably accounts for the lengthy preincident window of opportunity within which antibodies were effective in blocking the onset of poisoning (see Table 2).

nated from the body. As an illustration, the $t_{1/2}$ for elimination of toxin is several hundred minutes. This should be contrasted with the finding that clearance of free toxin from the circulation 20 min after administration of 5 ng of toxin does not lead to survival. In other words, at a time when there has been only a small fractional elimination of toxin, there has already been sufficient fractional accumulation of toxin in nerve endings to produce a paralyzing outcome.

The observations on fractional accumulation are especially revealing and, by deduction, they explain the underlying temporal factor that governs postchallenge efficacy of therapeutic antibodies. The rate constant for distribution of toxin to nerve endings is constant across doses, whereas the amount of toxin that causes a fatal outcome is fixed (one mouse LD_{50} is approximately 5 μ g). This means that the fractional redistribution of toxin into nerve endings that is needed to produce a fatal outcome will increase as the administered dose of toxin decreases. Likewise, the amount of time that will be needed for a lethal dose of toxin to bind and enter nerve endings will increase as the challenge dose of toxin decreases. By extension, one can deduce that the postchallenge window of opportunity for administration of an efficacious dose of antibody will be governed by the amount of time needed for fractional redistribution of a neuromuscular quantum of toxin into nerve endings.

This concept is consistent with the core observation that the window of opportunity within which antibodies can provide protection increases as the challenge dose of toxin decreases. This is a direct outcome of the fact that the lower the challenge dose of toxin, the greater will be the amount of time necessary for a lethal dose to reach and enter nerve endings, and therefore the greater will be the amount of time within which antibodies can act.

If the concepts that were just outlined are correct, they should be applicable to any intervention that acts on the outside of nerve endings to remove toxin from the fluid compartment. Thus, if a nonantibody agent should be identified that promotes clearance, the principal factor that will govern the temporal aspects of its postchallenge efficacy will still be the amount of time needed for fractional redistribution of a neuromuscular amount of toxin from the extraneuronal to the intraneuronal space. And indeed, there does not even have to

be an agent for one to observe this phenomenon. As demonstrated with excised phrenic nerve-hemidiaphragm preparations, removal of free toxin from the bathing solution of tissues by washing conforms to the same principles as removal of free toxin from the general circulation of animals by antibody-mediated clearance. For both the in vivo and in vitro situations, the data demonstrated that the lower the ambient toxin concentration, the longer the interval of time within which removal of free toxin would afford some protection. This, in turn, is a reflection of the fact that the lower the toxin concentration, the longer will be the amount of time needed for fractional redistribution of a paralyzing dose of toxin into nerve endings.

Implications of the Data. This study represents the first effort to make a detailed analysis of the temporal factors that govern the efficacy of therapeutic antibodies against botulinum toxin. Indeed, it is the first study to describe and quantify the factors that govern postchallenge efficacy of therapeutic antibodies directed against any bioweapons agent.

The concepts and the data that emerged from the study have their own inherent value, but they also have important implications. Most obviously, the approach used in this study helps to establish a platform for analyzing the circumstances under which any antitoxin countermeasure would display a beneficial effect. This means that the same approach used here could ultimately be applied to pharmacologic antagonists of the toxin, if and when truly efficacious antagonists are identified.

There are additional implications that derive from the study, two of which are particularly deserving of attention.

Development of Therapeutic Antibodies. There is one therapeutic antibody preparation that has been approved for human use (Arnon et al., 2006, 2007). Baby BIG, which is a human immunoglobulin preparation, is intended for infants with type A or B botulism. There are many other therapeutic antibody preparations under development, and at least one of these is about to enter clinical trials (Amersdorfer et al., 1997; Chen et al., 1997; Nowakowski et al., 2002). However, one of the noteworthy implications of the data and concepts discussed above is that no therapeutic antibody preparation will prove superior to any other therapeutic antibody preparation in terms of postchallenge efficacy. Assuming that each

of the antibody preparations is administered intravenously at a dose that is authentically neutralizing, they will all behave the same when analyzed in terms of postchallenge window of opportunity. This inescapable conclusion is based on the fact that the temporal factor that governs efficacy is not related to antibody behavior, but instead is the product of toxin behavior (i.e., redistribution to the cytosol of target nerve cells).

Clinical Utility of Therapeutic Antibodies. It is a well established principle that therapeutic antibodies cannot reverse the signs and symptoms of botulism. Nevertheless, even patients who are already ill can benefit from therapeutic antibody administration. The beneficial outcome is not immediate reversal of muscle weakness or paralysis; instead, the benefit is reduction in the ultimate severity and duration of illness (Arnon et al., 2006, 2007). It is likely that the data in the present study can provide a mechanistic basis to explain this clinical outcome.

There are two observations that act together to provide the explanation. First, the time necessary for redistribution of a paralyzing dose of toxin into nerve endings is very short compared with the elimination half-time for the toxin. This means that there will still be a substantial body burden of toxin when signs of muscle weakness begin to emerge. Second, a neutralizing dose of antibody can evoke clearance of toxin, even at time points beyond those at which the antibody can block onset of toxin action. When combined, these two observations mean that 1) some portion of the large residual titer of toxin that is still in the body after emergence of signs and symptoms can continue to accumulate in nerve endings, and 2) administration of antibodies after emergence of signs and symptoms will lead to clearance of the residual titer, which, in turn, will block further accumulation of toxin in nerve endings. Thus, administration of antibodies will not act immediately to reverse muscle weakness, but it will act to prevent additional accumulation of toxin in nerve cells. This in turn will diminish the ultimate severity and duration of illness.

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Authorship Contributions

Participated in research design: Al-Saleem, Olson, and Simpson.

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Performed data analysis: Al-Saleem, Nasser, Olson, and Simpson.

Wrote or contributed to the writing of the manuscript: Simpson.

References

- Adekar SP, Jones RM, Elias MD, Al-Saleem FH, Root MJ, Simpson LL, and Dessain SK (2008) A human monoclonal antibody that binds serotype A botulinum neurotoxin. *Hybridoma* **27**:11–17.
- Al-Saleem FH, Ancharski DM, Ravichandran E, Joshi SG, Singh AK, Gong Y, and Simpson LL (2008) The role of systemic handling in the pathophysiologic actions of botulinum toxin. *J Pharmacol Exp Ther* **326**:856–863.
- Amersdorfer P, Wong C, Chen S, Smith T, Deshpande S, Sheridan R, Finnern R, and Marks JD (1997) Molecular characterization of murine humoral immune response to botulinum neurotoxin type A binding domain as assessed by using phage antibody libraries. *Infect Immun* **65**:3743–3752.
- Arnon SS (2007) Creation and development of the public service orphan drug human botulism immune globulin. *Pediatrics* **119**:785–789.
- Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, et al. (2001) Botulinum toxin as a biological weapon: medical and public health management. *JAMA* **285**:1059–1070.
- Arnon SS, Schechter R, Maslanka SE, Jewell NP, and Hatheway CL (2006) Human botulism immune globulin for the treatment of infant botulism. *N Engl J Med* **354**:462–471.
- Casadevall A (2002) Passive antibody administration (immediate immunity) as a specific defense against biological weapons. *Emerg Infect Dis* **8**:833–841.
- Chen F, Kuziemko GM, Amersdorfer P, Wong C, Marks JD, and Stevens RC (1997) Antibody mapping to domains of botulinum neurotoxin serotype A in the complexed and uncomplexed forms. *Infect Immun* **65**:1626–1630.
- Dickerson TJ and Janda KD (2006) The use of small molecules to investigate molecular mechanisms and therapeutic targets for treatment of botulinum neurotoxin A intoxication. *ACS Chem Biol* **1**:359–369.
- Lane HC, Montagne JL, and Fauci AS (2001) Bioterrorism: a clear and present danger. *Nat Med* **7**:1271–1273.
- Maksymowych AB and Simpson LL (2004) Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. *J Pharmacol Exp Ther* **310**:633–641.
- Nowakowski A, Wang C, Powers DB, Amersdorfer P, Smith TJ, Montgomery VA, Sheridan R, Blake R, Smith LA, and Marks JD (2002) Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. *Proc Natl Acad Sci USA* **99**:11346–11350.
- Park JB and Simpson LL (2003) Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. *Infect Immun* **71**:1147–1154.
- Ravichandran E, Gong Y, Al Saleem FH, Ancharski DM, Joshi SG, and Simpson LL (2006) An initial assessment of the systemic pharmacokinetics of botulinum toxin. *J Pharmacol Exp Ther* **318**:1343–1351.
- Simpson LL (2009) Botulinum toxin, in *Vaccines for Biodefense and Emerging and Neglected Diseases* (Barrett ADT and Stanberry L eds) pp 891–917, Elsevier Inc., San Diego.
- Simpson LL, Maksymowych AB, Park JB, and Bora RS (2004) The role of the interchain disulfide bond in governing the pharmacological actions of botulinum toxin. *J Pharmacol Exp Ther* **308**:857–864.
- Smith LA (2009) Botulism and vaccines for its prevention. *Vaccine* **27**:D33–D39.
- Stanker LH, Merrill P, Scotcher MC, and Cheng LW (2008) Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type A and their use in analysis of milk by sandwich ELISA. *J Immunol Methods* **336**:1–8.
- Takahashi T, Joshi SG, Al-Saleem F, Ancharski D, Singh A, Nasser Z, and Simpson LL (2009) Localization of the sites and characterization of the mechanisms by which anti-light chain antibodies neutralize the actions of the botulinum holotoxin. *Vaccine* **27**:2616–2624.

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