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Review

The life history of a botulinum toxin molecule

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ABSTRACT

There is an emerging literature describing the absorption, distribution, metabolism and elimination of botulinum toxin. This work reveals that the toxin can be absorbed by both the oral and inhalation routes. The primary mechanism for absorption is binding and transport across epithelial cells. Toxin that enters the body undergoes a distribution phase, which is quite short, and an elimination phase, which is comparatively long. During the distribution phase, botulinum toxin migrates to the peri-neuronal microcompartment in the vicinity of vulnerable cells, such as cholinergic nerve endings. Only these cells have the ability to selectively accumulate the molecule. When the toxin moves from the cell membrane to the cell interior, it undergoes programmed death. This is coincident with release of the catalytically active light chain that paralyzes transmission. Intraneuronal metabolism of light chain is via the ubiquitination-proteasome pathway. Systemic metabolism and elimination is assumed to be via the liver. The analysis of absorption, distribution, metabolism and elimination of the toxin helps to create a life history of the molecule in the body. This has many benefits, including: a) clarifying the mechanisms that underlie the disease botulism, b) providing insights for development of medical countermeasures against the toxin, and c) helping to explain the meaning of a lethal dose of toxin. It is likely that work intended to enhance understanding of the fate of botulinum toxin in the body will intensify. These efforts will include new and powerful analytic tools, such as single molecule–single cell analyses in vitro and real time, 3-dimensional pharmacokinetic studies in vivo.

1. Introduction

There is an impressive body of information describing the actions of botulinum toxin on the surface and in the interior of neuronal cells. Both the nature of this information and the rate at which it was gathered are in large part due to the availability of model systems. Excised tissues, primary cell cultures and immortalized cell lines, many of which exist in genetic variants, have afforded investigators the opportunity to: a) identify each of the major steps in toxin action, b) define the conditions under which these steps can proceed, and c) identify procedures and agents that either facilitate or inhibit these steps (Simpson, 2004).

The advances in our understanding of the cellular, subcellular and even molecular actions of botulinum toxin are impressive, but a thoughtful consideration of these advances brings to light an unintended consequence. The intense desire to achieve a full explanation for toxin action on individual cells, or the substrata of these cells, has caused other and equally important lines of research to go largely unexplored. For example, there is comparatively little information on the disposition of botulinum toxin in the intact body. Processes such as absorption of toxin, distribution of the molecule throughout the body, systemic metabolism of toxin, and elimination of the molecule from the body are not well understood.

One might reasonably argue that there are now compelling reasons to pursue a better understanding of the fate of botulinum toxin in the human body. Perhaps the

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most important reason is the wide array of settings in which the toxin is encountered. The five most important of these are:

a. Recognizing the toxin as an etiologic agent in naturally occurring disease.
b. Contending with the toxin as a potential weapon in acts of bioterrorism and biological warfare.
c. Exploiting the toxin as a pharmacologic tool, particularly in the analysis of neuronal exocytosis.
d. Administering the toxin as a therapeutic agent, or potential therapeutic agent, in an ever-expanding number of clinical disorders.
e. Deconstructing and/or re-designing the toxin for novel uses either as a research tool or as a therapeutic agent.

There is one thing that all of these examples have in common, which is the need to know the fate of the toxin from the moment it enters the body until it is eventually eliminated from the body. In other words, there is a need to have a clear picture of the life history of a botulinum toxin molecule.

2. Entry mechanisms

2.1. Structure–Activity relationships

Botulinum toxin exists in seven different serotypes, designated A to G, and the individual serotypes can exist in multiple subtypes. It is questionable – and some would say doubtful – that all of the botulinum toxin subtypes have been discovered.

The bacteria that manufacture botulinum toxin do not typically release it into surrounding media as homogeneous neurotoxin. Instead, the neurotoxin is ordinarily encountered as one component in a non-covalently associated progenitor complex with other molecules (Sakaguchi, 1983; Minton, 1995). In the context of entry mechanisms, the molecules that have drawn most attention are the hemagglutinins (HA) and a so-called non-toxin, non-hemagglutinin (NTNH). There are three classes of HA, as determined by molecular weight (HA1, ca. 33–35 kDa; HA2, ca. 15–18 kDa; HA3, ca. 70 kDa). There is only one class of NTNH, which has a molecular weight of ca. 130 kDa.

Several hypotheses have been advanced that implicate HA and NTNH as essential for efficient entry of neurotoxin into the body. However, these auxiliary proteins are thought not to be involved in the actions of the toxin after it enters the general circulation. There are two observations that support this premise. First, the pH and salt composition of biological fluids such as blood promote dissociation of the heteromolecular complex (Eisele et al., 2011). Second, HA can bind to receptors on red blood cells (Lamanna, 1948), and this too would be expected to promote dissociation.

The auxiliary proteins are also seen as unnecessary for neurotoxin-induced paralysis. In fact, the observation that auxiliary proteins are not essential for blockade of transmission was made almost immediately after techniques were developed to isolate homogeneous neurotoxin from HA and NTNH (DasGupta and Boroff, 1968; Beers and Reich, 1969). Stated differently, the specific toxicity of homogeneous neurotoxin is greater than that of the progenitor toxin complex.

These various lines of investigation have led to the conclusion that HA and NTNH could play a role in entry mechanisms, and particularly oral absorption, but after absorption the neurotoxin sheds auxiliary proteins and proceeds through the remainder of its life cycle as a homogeneous protein.

2.2. Role of neurotoxin

There are two broad mechanisms for the toxin (or the organisms that make the toxin) to enter the body, the first of which involves penetration of broken cells or disrupted membranes. Examples of this are illicit drug use, such as injection of heroin (MacDonald et al., 1985; Werner et al., 2000; Mitchell and Pons, 2001; Gordon and Lowy, 2005; Kalka-Moll et al., 2007), and surgical or accidental wounds (Dezfulian and Bartlett, 1985; Isacsohn et al., 1985; Hansen and Tolo, 1979). Of a much different and more favorable nature, toxin can be injected through surface membranes and into the immediate vicinity of nerve endings to achieve a host of therapeutic purposes (Brin et al., 2002; Carruthers and Carruthers, 2005; Cooper, 2007).

The other broad category of entry mechanism is passage through intact cells and membranes, which is essential for oral and inhalation poisoning. In both of these cases the toxin must cross epithelial barriers to reach the general circulation, and in both cases the process is described as absorption (Fig. 1).

Research on absorption of botulinum toxin can be divided into two historic phases. The earlier of these occurred before techniques had been developed for isolation of pure neurotoxin. In addition, test systems for analyzing the absorptive process often had inherent flaws. As a result, some of the conclusions reached during the earlier phase are now subject to question. There is, however, one notable exception. It has been known for decades that the oral potency of botulinum toxin in the heteromolecular complex is substantially greater than that of neurotoxin that is free in solution (Bonventre, 1979). There is a consensus among investigators that the auxiliary proteins HA and NTNH protect the neurotoxin from gastric metabolism, thus increasing the amount that reaches the upper small intestine for absorption.

The more recent phase of research on absorption has benefited from several advances, and most notably: a) development of techniques for generation of pure neurotoxin (DasGupta and Boroff, 1968; Beers and Reich, 1969), b) recognition of the conditions that cause the progenitor toxin complex to remain intact or to dissociate (Eisele et al., 2011), c) identification of model systems for studying toxin absorption, and particularly immortalized human gut and airway epithelial cell lines (Maksymowych and Simpson, 1998, 2004), and d) development of sensitive assays for toxin that are suitable for routine use (Stanker et al., 2008; Al-Saleem et al., 2012). The latter advance is truly important, because it has produced a fundamental change in the way that absorption studies can be done. Rather than using

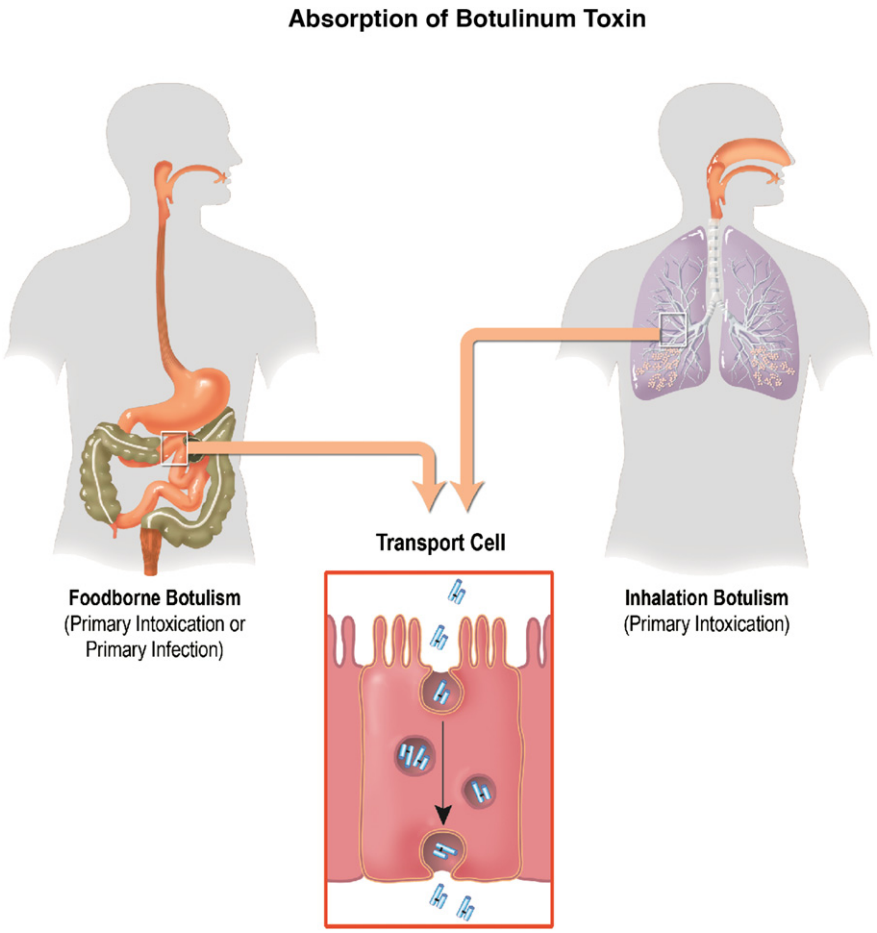

Fig. 1. Botulinum toxin can be absorbed by both the oral route and the inhalation route. In the case of oral poisoning, either the toxin (primary intoxication) or the organisms that make the toxin (primary infection with secondary intoxication) enter the gastrointestinal system. In the case of inhalation poisoning, only primary intoxication has been reported. In both oral and inhalation poisoning, the toxin binds to the apical surface of epithelial cells (transport cells), after which it is carried to the basal surface of cells and released into the general circulation.

the indirect technique of monitoring in vivo toxicity as an indicator of absorption, investigators can now use the direct technique of measuring post-absorption levels of toxin in biological specimens such as serum and plasma.

The modern era of research on absorption has produced a number of informative observations and insights, including:

a. *Absorption of botulinum toxin across epithelial cells (transcytosis) is an active process.* This has been confirmed by kinetic analyses (Maksymowych and Simpson, 1998, 2004; Couesnon et al., 2008, 2009), visualization experiments (Ahsan et al., 2005), and drug inhibitor experiments (Maksymowych and Simpson, 1998; Couesnon et al., 2008). The process involves binding of toxin to receptors on the apical surface of epithelial cells, receptor-mediated endocytosis and transcytosis, and release of toxin from the basal surface of epithelial cells. It is true epithelial cells rather than specialized M-cells that mediate the transport process.

b. *The botulinum toxin molecule by itself can bind and penetrate epithelial barriers* (Maksymowych and Simpson, 1998, 2004; Couesnon et al., 2008). The toxin does not require auxiliary proteins such as HA and NTNH for this process. Both preclinical data and clinical observation support this conclusion. For example, dose–response curves for progenitor toxin complex and for pure neurotoxin, when administered directly into the upper small intestine or into the airway, indicate that the toxin with auxiliary proteins and toxin free of these proteins are almost identical in potency (Maksymowych et al., 1999; Al-Saleem et al., 2012). Furthermore, airway administration of equimolar amounts of progenitor complex and free neurotoxin produce equivalent circulating titers of toxin (Al-Saleem et al., 2012). In the clinical realm it has been observed that serotypes E and F are active by the oral route in humans (Dolman and Iida, 1963; Shapiro et al., 1998; Caya et al., 2004; Sobel et al., 2004). Neither of these serotypes manufactures HA (Popoff, 1995; Raffestin et al., 2004), making it impossible for HA to be seen as essential for absorption.

c. *The minimum essential domain for binding and transcytosis is located in the carboxyterminal half of the toxin*

heavy chain (Maksymowych and Simpson, 1998, 2004). Removal of the light chain does not hinder the ability of residual heavy chain to cross epithelial barriers. Recombinant truncation mutants (deletion from the N-terminus) representing ca. 90% (HC90), 80% (HC80), 66% (HC66), and 50% (HC50) of the heavy chain all retain efficacy for transcytosis.

d. *The binding of botulinum toxin by epithelial cells and by neuronal cells is not identical.* It has been known for many years that the initial binding sites on nerve cells for most of the toxin serotypes are polysialogangliosides (Schiavo et al., 2000; Montecucco et al., 2004). Recent work on site-directed mutants to generate toxin variants that do not bind to gangliosides or to nerve endings indicates that these polypeptides retain the ability to bind and cross epithelial cells (Elias et al., 2011). Apparently the initial binding sites for oral or inhalation absorption are different from those for neuronal binding and uptake.

e. *The cellular handling of botulinum toxin by epithelial cells and by neuronal cells is fundamentally different, and these differences are related to cellular fate of the toxin* (Maksymowych and Simpson, 1998; Couesnon et al., 2008, 2009; Elias et al., 2011). Neuronal cells bind and endocytose the toxin, after which acidification of the vesicle lumen leads to conformational changes in toxin structure that facilitate translocation across membranes and into the cytosol. Epithelial cells bind and endocytose toxin, after which transport vesicles carry the toxin across the cell length and release it into the general circulation. In this case there is no toxin delivery to the cytosol. Another difference relates to toxin structure. During the process of membrane translocation in neuronal cells, the disulfide bond between the heavy and light chains is broken and the two chains eventually separate (Fischer and Montal, 2007a; Montal, 2010). During transcytosis in epithelial cells, the structure of the toxin remains intact. Thus, it is the dichain holotoxin that is delivered to the circulation (Maksymowych and Simpson, 1998; Couesnon et al., 2008).

2.3. Role of auxiliary proteins

2.3.1. Gastric metabolism

The original observation that auxiliary proteins protect neurotoxin from inactivation by gastric proteases was made decades ago, and there is now universal acceptance of the idea that HA and NTNH increase oral potency of toxin by decreasing proteolysis (Bonventre, 1979). In the intervening years there have been only a limited number of attempts to clarify how auxiliary proteins protect the neurotoxin, and these efforts have not been completely successful. An examination of three different lines of work can serve to highlight some of the issues that remain to be resolved.

In a study intended to detail the structure of a progenitor toxin complex (serotype D), Hasegawa et al. (2007) used transmission electron microscopy to quantify the number of components in a progenitor complex, as well as to determine the spatial relationships among these components. Their work led them to conclude that the neurotoxin is associated with NTNH in a 1:1 stoichiometry. The HA components had no direct contact with neurotoxin, but instead formed three arms that projected outward from NTNH. The precise composition of each arm was proposed to be one HA3 linked to one HA2, which in turn was coordinated with two HA1.

Surely one of the most perplexing questions to arise from this work pertains to protection. In a model in which auxiliary proteins project outward from the neurotoxin, it is not clear how the former could be providing protection to the latter. With the exception of the contact surface between the neurotoxin and NTNH, the entirety of the toxin is exposed. To the extent that this model is correct, it would suggest that a large proportion of the neurotoxin is itself resistant to proteolysis, and it is only the contact region between the neurotoxin and NTNH that is vulnerable and therefore needs protection.

In another line of work, Marks and his colleagues have attempted to map the number and location of epitopes in the neurotoxin (serotype A). In one of their studies they used antibodies as probes to determine the accessibility of different domains within the toxin when it was free and when it was in complex with auxiliary proteins (Chen et al., 1997). Their work demonstrated that portions of the neurotoxin, including parts that reside within the light chain, were accessible (i.e., could associate with antibody) when the progenitor complex was intact. A similar observation has been made by Zhao et al. (2012), who used a different panel of anti-light chain monoclonal antibodies. Once again, the data suggest that there are parts of the neurotoxin that are exposed in the complex. This in turn suggests that there are parts of the neurotoxin that are inherently resistant to proteolysis, and that auxiliary proteins do not entirely embrace the neurotoxin, but merely associate with those surface areas that are presumably vulnerable to proteolysis.

The most recent contribution to this line of research is also the most sophisticated. Jin and his collaborators have examined the crystal structure of NTNH both when it is free in solution and when it is in complex with neurotoxin (serotype A; Gu et al., 2012). They made the fascinating observation that the conformational structure of NTNH bears a surprising similarity to that of the neurotoxin. In addition, they provided a model for interaction between toxin and NTNH that is far more detailed than that provided by any previous group of investigators. In contrast to the work on serotype D, which suggested an extended relationship between toxin and NTNH, the work on serotype A suggested an intertwined relationship.

Analysis of the crystal structure of serotype A neurotoxin and NTNH gave rise to the concept that NTNH is a "bioshield" that physically protects the neurotoxin from gastric metabolism. At the same time, the model envisions that there are significant parts of the neurotoxin that are not physically protected. This could mean that the HA components shield the remainder of the neurotoxin, but the experiments with antibody probes suggest that this could not be true (see above). At most, the HA components can provide only partial protection.

The work that has been done to date seems to indicate that there are two phenomena that contribute to toxin

survival in the gut. The first is the one that is widely appreciated: Auxiliary proteins provide a physical barrier that protects portions of the neurotoxin from proteolysis. The second phenomenon is one that is not as widely appreciated and is rarely mentioned: The neurotoxin itself possesses regions that are inherently resistant to proteolysis, and these regions do not require a bioshield.

2.3.2. Metabolic activation versus metabolic degradation

There is at least one situation in which the absence of auxiliary proteins may promote the biological activity of botulinum toxin, and this is the phenomenon of proteolytic activation.

Botulinum toxin is manufactured as a single chain polypeptide that possesses little ability to paralyze neuronal transmission. This single chain polypeptide must undergo post-translational modification by trypsin or a trypsin-like protease, during which the polypeptide is cleaved ("nicked") to produce an activated dichain molecule (DasGupta and Sugiyama, 1972). Some clostridia possess the protease that is necessary to nick the toxin, so it is the activated molecule that is found in culture media. As an illustration, botulinum toxin type A is ordinarily released from bacteria as the fully active toxin. Other clostridia do not have the ability to nick toxin, so it is the inactive protoxin that is released. Botulinum toxin type E is the most well studied of the naturally occurring, unnicked molecules.

When unnicked botulinum toxin type E is added to isolated tissues, such as the phrenic nerve-hemidiaphragm preparation, it is between one and two orders of magnitude less potent than the nicked variant (Simpson and DasGupta, 1983). Similarly, intravenous administration of the unnicked serotype E is far less potent than the nicked variant.

Given that the unnicked toxin is so weakly active, and given that the serotype E toxin does not occur in a protective complex with HA, one might assume that it would be unlikely to cause oral disease. However, this is not true. Serotype E is definitely associated with oral poisoning, although the incidence is low.

The explanation for this paradox appears to be the competing outcomes of two forms of gastric proteolysis. On the one hand, the absence of a complete progenitor complex may make the toxin more susceptible to metabolic degradation. But on the other hand, the susceptibility of the toxin to proteolysis means that the unnicked toxin can be converted to nicked toxin. The quantitative impact of activation appears to be greater than the impact of degradation, and thus serotype E can cause oral poisoning.

2.3.3. Absorption

In the recent past there have been a number of publications that have presented an alternative view of the role of auxiliary proteins in the absorptive process. This view begins with the premise that the free neurotoxin can be absorbed across epithelial barriers, but adds the premise that HA can enhance this baseline rate of absorption. In the initial version, it was hypothesized that HA serves as a carrier molecule to transport toxin across epithelial cells (Fujinaga et al., 1997, 2000; Fujinaga, 2006). In a subsequent and strikingly different version, it was hypothesized that HA crosses epithelial barriers and acts on the basal side of cells to disrupt tight junctions (Matsumura et al., 2008; Fujinaga et al., 2009; Jin et al., 2009). According to this view of the process, the toxin passively diffuses between cells rather than being actively transported across cells. Both of these hypotheses arose from studies on isolated oral epithelial cells.

There are a number of reasons to be cautious about the hypothesis that HA disrupts tight junctions to permit passive flux of toxin into the circulation. To begin with, data that purportedly support this model come from studies on immortalized gut epithelial cells, and the conditions that were used are not equivalent to those found in the human body. The duration of experiments was much longer than the amount of time needed for a bolus of food to pass through the upper small intestine, and the pH of solutions was more acidic than that found in the upper small intestine. Another problem is that the concentration of HA needed to produce significant disruption of tight junctions was higher than one would expect to obtain in vivo on the basal side of epithelial barriers.

One particularly troubling aspect of the HA disruption model is that a key finding actually contradicts the model. According to the authors of the model, HA acts weakly and slowly on the apical side of epithelial cells to disrupt tight junctions, but more strongly and rapidly on the basal side to produce the same effect (Matsumura et al., 2008; Jin et al., 2009). This led to the hypothesis that the principal site of HA action is on the basal side of epithelial cells. However, if this were a true reflection of the absorptive process, one would expect to see a complex phenomenon when HA is added to the apical surface of cells. There should be an initial and slow rate of disruption, but as HA reaches the basal surface of cells there should be an increasingly rapid rate of disruption. Curiously, this was not the outcome that was observed. Addition of HA to the apical side of cells produced a slow and monotonic rate of disruption, raising the question whether meaningful amounts of HA actually reached the basal side of cells.

In an attempt to clarify whether HA can facilitate absorption of neurotoxin, Al-Saleem et al. (2012) conducted a series of experiments on inhalation absorption in live animals. The inhalation route was selected to bypass issues associated with gastric metabolism. Two types of experiments were done. In the first, progenitor toxin complex and free neurotoxin, in equimolar amounts relative to toxin, were administered intranasally to mice, and in vivo toxicity was monitored. In the second experiment, equimolar amounts of toxin complex and free neurotoxin were again administered intranasally to animals, and this time the circulating titers of toxin were measured. Both experiments yielded the same result. The presence of HA in the toxin complex did not significantly enhance in vivo potency of toxin, nor did it enhance in vivo absorption of toxin recovered in serum or plasma.

When all of the studies that have been published to date are taken into account, the data on botulinum toxin absorption support the following conclusions:

a. Auxiliary proteins protect the neurotoxin from metabolism in the stomach, which increases the delivery of active toxin to the site of its absorption in the upper small intestine.
b. Free neurotoxin has the inherent ability to cross epithelial barriers in the gut, including the upper small intestine. The presence of HA is not an absolute requirement for absorption.
c. There is an hypothesis that HA can enhance oral absorption of toxin by disrupting epithelial tight junctions. This hypothesis arises from studies on epithelial cells in culture, but there are no confirmatory studies on an in vivo animal model. The work that has been done to support this hypothesis cannot yet be seen as convincing.
d. Free neurotoxin has the inherent ability to cross airway epithelial monolayers in vitro and airway epithelial barriers in vivo. HA is not essential for, nor does it significantly enhance, neurotoxin passage across airway epithelial cells.

3. Distribution

3.1. Extracellular fluid compartment

Toxin that reaches the circulation is distributed throughout extracellular fluid compartments in the body, with the exception of those in the central nervous system. Toxin in the periphery is distributed between two major fluid compartments: the vascular compartment, and the extravascular, extracellular compartment. The latter is the fluid compartment through which toxin must pass to reach vulnerable cells, such as peripheral cholinergic nerve endings.

The pharmacokinetics of botulinum toxin after its direct administration into the vascular compartment of mice and rats has been analyzed (Ravichandran et al., 2006; Al-Saleem et al., 2008). The data show that there are two processes that occur (Fig. 2). There is an initial and rapid process, with a $t½$ in minutes, that represents toxin distribution throughout the general circulation. When a pseudo-steady state has been reached, there is a second and much slower process, with a $t½$ in hours, that represents toxin elimination.

It is interesting that the rate constants for these two processes are independent of dose, which can probably be attributed to the low concentrations of toxin in the body (Al-Saleem et al., 2011). When toxin is administered at a high dose (5 ng per 20 g mouse; ca. 1000 MLD50), the peak plasma concentration is only about 10 pM (i.e., ca. 1.5 ng/ml; ca. 300 MLD50/ml). At lower doses the resulting plasma concentrations would be even less. It is highly unlikely that concentrations this low would saturate an uptake mechanism, a metabolic system, or any other actively-mediated process. This, as well as other considerations that will be presented shortly, help to explain why the characteristics of distribution and elimination are little affected by dose.

During the time that the toxin is present in blood it is highly stable. In vivo studies on rodents and ex vivo studies on rodent blood have shown that the toxin molecule remains intact for extended periods of time (i.e., there is an absence of metabolites; Ravichandran et al., 2006; Al-Saleem et al., 2008). One clinical study has provided rather dramatic evidence for stability of the toxin in the

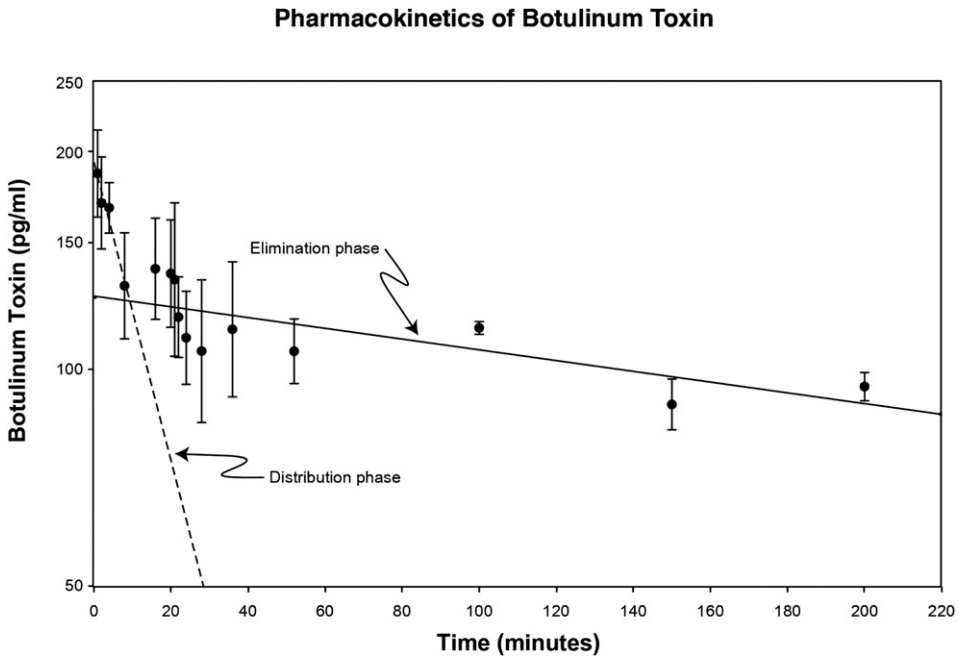

Fig. 2. Intravenous administration of botulinum toxin (500 pg) to mice reveals that the disposition of the molecule can be divided into two phases. There is an initial and rapid distribution phase, and a subsequent and much slower elimination phase. In mice, the $t½$ for elimination is several hours, but in humans it may be as much as several days.

human body. Fagan et al. (2009) performed toxicity assays on archived serum samples from patients diagnosed with type A oral botulism. They were able to detect active toxin in the circulation of patients as much as 11 days after ingestion of tainted food. Another clinical study provided even more dramatic evidence for stability. Sheth et al. (2008) reported the presence of active toxin in the serum of a patient 25 days after onset of illness.

These human and laboratory animal studies on toxin stability have prompted the concept that the general circulation should be seen as a "holding compartment" (Al-Saleem et al., 2008). Toxin that enters the circulation remains there in a structurally intact and biologically active form until it is delivered to target cells, or until it is eliminated from the body.

In terms of cellular uptake, it has long been known that most cells and organs in the body do not accumulate toxin (Black and Dolly, 1986). The clear exceptions are target cells for toxin action, such as cholinergic nerve endings. As will be explained more fully below, for any given multilethal dose of toxin, only a small fraction of the total body burden is actually associated with paralysis of target cell function. Therefore, the concentration of toxin in the fluid compartments of the body can have a dramatic effect on cellular uptake, whereas cellular uptake may have little effect on the pharmacokinetics of toxin in the general circulation (A more complete and quantitative basis for this statement is presented in Section 5.2).

3.2. Neuronal compartment

Toxin that is distributed to target cells and enters the cytosol undergoes an immediate metabolic transformation. During the process of translocation across endosome membranes, the disulfide bond linking the heavy and light chains is broken, releasing the light chain to express its catalytic activity in the cytosol (Koriazova and Montal, 2003; Fischer and Montal, 2007a). In terms of a life history model, this marks the end of the intracellular existence of the intact and biologically active molecule. Even if the light chain or heavy chain were to be exported from the cell, neither by itself can poison cell function. Only the holotoxin possesses the ability to progress through the multiple steps that culminate in blockade of transmission.

4. Metabolism and elimination

Following administration of a multilethal dose of toxin, only a minority of the total body burden enters nerve cells while the remaining and larger portion remains in the extracellular compartment. There does not yet exist a full understanding of the mechanisms for metabolism and elimination of toxin in either the extracellular or intracellular compartments.

4.1. Systemic handling

There are several ways in which the body might be expected to dispose of a foreign protein. To begin with, the general circulation is a large and complex compartment in which there are candidate mechanisms for inactivating foreign substances. There are circulating cells that can engulf these substances, and there are circulating proteases that can degrade these substances. However, these candidate mechanisms play little if any role in disposition of botulinum toxin. As explained earlier, the toxin is very stable in the circulation. There is simply no evidence for cellular uptake or proteolysis.

Another possibility is renal clearance and excretion, but this too is not likely to play a role. The molecular weight cut-off for renal filtration in mammals is ca. 50,000, whereas the molecular weight of botulinum toxin is ca. 150,000. This is a strong indication that patients with normal renal function will not eliminate meaningful amounts of intact toxin in urine.

The third and most plausible mechanism for metabolism and elimination is hepatic biotransformation. Cleavage reactions, and perhaps conjugation reactions as well, could lead to transformation of the toxin into inactive metabolites, and these reactions could be a prelude to elimination. It is disappointing to acknowledge that although these mechanisms seem plausible, there is no body of evidence to demonstrate their involvement. It is even more disappointing to report that in the entire modern era of botulinum toxin research, there does not exist a single publication that specifically addresses hepatic biotransformation of the toxin or the products that are generated.

Whatever may be the involvement of the liver, there is one point on which most investigators would agree. The very long systemic half-life for botulinum toxin in the human body can probably be attributed to the fact that, even at multilethal doses, the circulating titer of toxin is too low to be easily or efficiently detected by the liver or any other metabolic cell or system. To invoke a popular expression, the toxin may persist for long periods of time because it is "flying below the radar".

4.2. Target cell handling

No aspect of botulinum toxin has been more thoroughly studied than its ability to enter vulnerable nerve endings and cause blockade of exocytosis. The ability of the toxin to undergo binding, internalization, and intracellular expression of neurotoxicity has been reviewed many times, beginning in 1981 (Simpson, 1981) and continuing to the recent past (for representative reviews, see Schiavo et al., 2000; Meunier et al., 2002; Coffield, 2003; Simpson, 2004; Brunger et al., 2008; Rossetto and Montecucco, 2008; Simpson, 2009; Montal, 2010; Swaminathan, 2011). The purpose of the current review is not to revisit the extensive literature on blockade of transmitter release, which culminated in the ground-breaking discovery that clostridial neurotoxins are metalloendoproteases (Schiavo et al., 1992a,b; Blasi et al., 1993). Instead, the goal is to analyze the phenomena that relate to distribution and metabolic fate of the toxin molecule.

4.2.1. Peri-neuronal microcompartment

The extravascular, extracellular fluid compartment is the largest fluid compartment to which the toxin is distributed. The most important microdomain of this

compartment is the extracellular space that is immediately juxtaposed to the non-myelinated vicinity of vulnerable nerve endings, such as cholinergic nerve endings that impinge on voluntary muscle. Whatever may be the route of toxin administration (e.g., oral, inhalation, injection), the molecule itself must reach this microdomain before it can begin the sequence of events that culminate in paralysis of transmission.

To date, no evidence has been presented to show that botulinum toxin is selectively distributed to the peri-neuronal microcompartment. Presumably the concentration of toxin in the fluid compartment bathing nerve endings is no different from that of fluid compartments bathing other cells. The critical distinction is that target cells can efficiently extract toxin from these microcompartments, whereas other cells cannot.

Pharmacokinetic experiments on isolated neuromuscular preparations have shown that the *t½* for toxin extraction from the peri-neuronal microcompartment is relatively rapid (i.e., *t½* for binding, ca. 12 min; *t½* for internalization, ca. 5 min; Simpson, 1980). This is the same order of magnitude of the *t½* for toxin distribution in vivo (see above). This suggests that, when pseudo-steady state has been reached, the rate of toxin extraction from the peri-neuronal microcompartment would not be significantly limited by the rate of toxin distribution to this compartment. However, this conclusion must be tempered by one proviso. The rate constant for toxin distribution in vivo is an aggregate number that sums rates for distribution to all microcompartments. It cannot be stated with certainty that delivery of the molecule to the peri-neuronal microcompartment is the same as that to all other microcompartments. Even so, it is well established that intravenous administration of large doses of toxin to mice can cause death in 20–30 min. This observation suggests that there are no significant obstacles to toxin distribution to nerve endings.

Assuming that delivery to nerves is rapid, and given that binding and internalization are rapid, one might reasonably ask why the *t½* for toxin elimination from the body is so long. There are several reasons for this, three of which relate specifically to nerve endings. First, there is no reason to believe that toxin uptake into nerve endings is the only mechanism, or even the most important mechanism, underlying whole body elimination. Second, the peri-neuronal microcompartment is miniscule compared to the total fluid volume of the vascular and extravascular, extracellular compartments. Therefore, efficient extraction of toxin from this microdomain has only a minimal influence on the *t½* for toxin elimination from the total fluid compartment. Third, efficient extraction is a waning process. As toxin enters nerves and blocks exocytosis, it indirectly blocks the process of endocytosis (Henkel et al., 1996). With the passage of time, the fractional uptake of toxin by nerve endings that are being poisoned is ever diminishing.

4.2.2. Vulnerable nerve endings

The author was the first to propose a multi-step model for toxin action that included binding, internalization, and intracellular expression of toxicity (Simpson, 1980, 1981). Prior to this formulation there was a prevailing belief that the toxin, being a large protein, would have to act at the cell surface. One widely held view was that the toxin could in some way block calcium channels. By blocking depolarization-dependent calcium entry into nerve endings, the toxin could indirectly block exocytosis. This and all other hypotheses based on a cell surface model of toxin action have now fallen by the wayside, and the multi-step model is universally accepted.

The first step in toxin action is binding to receptors. The concept that gangliosides could serve as neuronal receptors was put forward some time ago (Simpson and Rapport, 1971a,b). In the intervening years the weight of evidence showing that gangliosides are receptors has grown substantially (Bullens et al., 2002; Yowler et al., 2002; Rummel et al., 2004), but so has the complexity of the hypotheses about receptor binding. It is now envisioned that gangliosides are initial binding sites that bring the toxin out of the fluid phase and into the plane of the membrane (Montecucco, 1986). This means that it brings the toxin out of a comparatively large 3-dimensional space and into a much smaller 2-dimensional space (Montecucco et al., 2004). This in turn facilitates toxin binding to a protein receptor (Brunger et al., 2008). This combined binding is essentially irreversible, so toxin really is extracted from the peri-neuronal microdomain. But at the same time, the toxin is still substantially exposed and therefore accessible to certain neutralizing antibodies.

The process of internalization involves two membrane penetrating events: receptor-mediated endocytosis to cross the plasma membrane, and pH-induced translocation to cross the endosome membrane. High affinity receptor binding, combined with delivery of any residual endosomal components to lysosomes, makes return of the toxin or any of its components to the cell exterior unlikely. In addition, the conformational changes associated with pH-induced light chain translocation to the cytosol create a “trap” that makes retro-translocation into the endosome impossible (Montal, 2010).

The fact that there is a pH-dependent translocation step was deduced from the finding that agents that prevent or reverse acidification of endosomes are antagonists of toxin action (Simpson, 1983; Simpson et al., 1993b). This was followed by the observation that acidification of membranes in which the toxin was embedded produce discrete forms of porosity, as opposed to non-discrete rupture of membranes (Hoch et al., 1985; Montecucco et al., 1989). This has culminated in work that lays out a sequence of toxin–membrane interactions that permit the light chain to escape the lumen of the endosome and reach the cytosol (Fischer and Montal, 2007b; Montal, 2010).

The passage of the light chain into the cytosol is accompanied by three notable structural changes: a) the disulfide bond linking the light chain and the heavy chain is broken, b) the belt domain that arises from the amino-terminus of the heavy chain and that covers the substrate groove in the light chain is loosened, and c) the light chain and the heavy chain separate. These three structural changes, which probably take only seconds to occur, mark the boundaries of two opposing events. They signal death of the intraneuronal population of intact botulinum toxin

molecules, and they initiate the events that culminate in intracellular poisoning.

It should be mentioned that there is another fascinating life cycle phenomenon that is associated with translocation. The structurally intact holotoxin has a chelation site that holds zinc (Binz et al., 1990; Schiavo et al., 1992a,b). The light chain is a metalloendoprotease that requires zinc for catalytic activity. Interestingly, the zinc that is held by the catalytically inactive holotoxin is probably not the same zinc that is held by catalytically active light chain (Simpson et al., 1993a, 2001). Acidification of the endosome will likely cause protonation of sites that are essential for zinc binding. In addition, translocation requires denaturation of light chain, and loss of conformation will also mean loss of structural integrity of the chelation site. In combination, protonation and loss of structural integrity would cause dissociation of bound zinc. The light chain cannot regain its chelation site until it regains charge distribution and conformation in the cytosol. When this occurs, the zinc that is captured is not that originally held by the holotoxin, but instead that which is part of the ambient pool of intraneuronal zinc (Simpson et al., 1993a, 2001). The exploitative ability of the toxin to capture zinc in target cells means that its long journey from the media of bacterial cultures to the interior of vulnerable cells – culminating in programmed death and onset of toxicity – can be completed.

The metabolic fate of the heavy chain after translocation of the light chain is unknown; indeed, the fate of the heavy chain has received almost no attention. By contrast, there have been attempts to determine the fate of the light chain.

One can conceive of three possible pathways that could lead to disappearance or loss of activity of light chain:

Export. Botulinum toxin undergoes high affinity binding and transmembrane transport to reach the cell interior. At least speculatively, one might propose that the light chain can undergo binding and transport to be returned to the cell exterior. Interestingly, there is work showing that the light chain of one serotype does bind to the internal face of the plasma membrane (Fernandez-Salas et al., 2004), but this work does not envision membrane binding as a prelude to export. Quite the contrary, binding is seen as a sequestration process that localizes the light chain near its site of action on the membrane while simultaneously hindering its movement toward potential sites of metabolism in the cytosol.

Diffusion. The volume of nerve endings in peripheral motor nerves is small compared to the total volume of these nerves. This could mean that at least a portion of the light chain that reaches the cytosol could simply diffuse up the axon to the cell body. This passive removal of light chain from its site of action would effectively terminate its local neuroparalytic effects. On the one hand, the possibility of passive diffusion appears appealing, in part due to its simplicity; but on the other hand, the anatomy and dynamics of the junctional region between nerve endings and the axon may not be conducive to significant diffusion. It would be worthwhile to determine whether, and if so to what extent, there can be passive retrograde diffusion of light chain in vivo. The observation by Lawrence et al. (2012) that there may be retrograde axoplasmic flow of toxin in rat sympathetic neurons in vitro is strong encouragement to conduct analogous experiments in vivo.

Proteolysis. Nerve cells possess two major mechanisms for proteolytic degradation: lysosomes and proteasomes. It is known with certainty that acid-dependent lysosomal endoproteases can degrade light chain in broken cell experiments, but this is not compelling evidence that lysosomal proteases play a metabolic role in intact neurons. No work has been done to show how lysosomes would accumulate the low levels of light chain that would be found in the cytosol.

It is also known that the light chain is vulnerable to ubiquitinylation and proteasome-mediated degradation, both in broken cell and in intact cell experiments (Shi et al., 2009; Kuo et al., 2011). The fact that this phenomenon has been observed in functioning cells lends credibility to the hypothesis that this could be the true metabolic process governing intraneuronal levels of light chain.

There is a very intriguing line of work that adds weight to the proteasome hypothesis (for review, see Shoemaker and Oyler, 2013). The different serotypes of botulinum toxin are known to have different durations of action (Eleopra et al., 1998; Keller et al., 1999; Raciborska and Charlton, 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003; Keller, 2006). Serotype A has a rather long duration of action, whereas serotype E has a much shorter duration of action. Experiments designed to test the relative susceptibility of type A light chain and type E light chain to the ubiquitinylation-proteasome pathway revealed cellular mechanisms (de-ubiquitinylating enzymes) that tend to conserve and extend the half-life of type A light chain but not that of type E light chain (Tsai et al., 2010). These results support the hypothesis that proteasomes are central players in intraneuronal metabolism of light chain.

4.2.3. Central nervous system (CNS)

Tetanus toxin, which is closely related in structure and function to botulinum toxin, is known to act in the CNS. Therefore, tetanus toxin is often used as a comparator when seeking to determine whether botulinum toxin acts centrally.

There are two analogies that are routinely drawn when comparing the actions of botulinum toxin with those of tetanus toxin. The first and more widely used might be called the “natural disease” analogy. According to this scheme, each toxin relies on a series of two cells to produce its pathophysiologic effects. For botulinum toxin, there is a transport cell that carries the toxin into the body, and this would ordinarily be an oral or airway epithelial cell. The toxin binds to the apical surface of these cells, is endocytosed into transport vesicles, and is carried the length of cells to be released at the basal membrane. Transcytosed toxin that reaches the general circulation can then bind, enter and poison target cells, such as peripheral cholinergic motor neurons. The scheme is somewhat different for tetanus toxin, because the disease tetanus begins when organisms that manufacture the toxin have penetrated

barriers and are already in the body. Tetanus toxin made by these organisms diffuses into the circulation to reach transport cells, such motor or autonomic fibers. The toxin binds, is internalized, and is carried the length of transport cells (i.e., transcytosis, but more commonly called retrograde axonal transport), then released as the intact holotoxin into the synaptic space. This toxin can bind and be internalized by target cells, such as inhibitory neurons, where it progresses through the same multi-step sequence described above for botulinum toxin.

The second scheme for comparing the two toxins might be called the "high dose" analogy. In this case it is envisioned that unnaturally high local concentrations of each toxin can mimic the behavior of naturally low concentrations of the other toxin. Thus, high local concentrations of tetanus toxin can lead to binding to peripheral cholinergic nerve endings, receptor-mediated endocytosis, local pH-induced translocation, and blockade of peripheral transmitter release. Similarly, high local concentrations of botulinum toxin can lead to binding to peripheral nerve endings, receptor-mediated endocytosis into transport vesicles, retrograde axonal transport, and release of holotoxin into the vicinity of central inhibitory neurons.

Throughout the history of botulinum toxin research there has been concern that this molecule, like tetanus toxin, could be transported into the CNS and cause adverse neurologic effects. In the context of disease, in which the toxin is distributed throughout the vascular and extravascular, extracellular compartments, any cell that has nerve endings peripherally and cell bodies centrally might be considered a potential conduit. In the context of therapeutics, in which toxin is injected in the vicinity of target cells, it would be only these cells or their near neighbors that could potentially be conduits.

Although there are studies that purportedly show central transport of botulinum toxin, many of them have methodologic or interpretative flaws. Nevertheless, there is still a residual body of evidence indicating that the toxin can reach the CNS. Unfortunately, this literature does little to address three key questions:

- For any given potential conduit, what is the minimum essential concentration in the peri-neuronal microcompartment that will actually lead to significant transport of botulinum toxin into the CNS?
- For any given neuronal conduit that does transport toxin centrally, what are the CNS cells or systems that would likely be affected, and what would be the likely adverse outcomes?
- To what extent does the clinical literature (either poisoning due to toxin or treatment with toxin) provide evidence that there are adverse outcomes consistent with the conduits that would be involved, and with the CNS cells and systems that would be affected?

In the absence of answers to questions like these, it is impossible to be rigorous in describing or quantifying phenomena such as fractional delivery of toxin to the CNS, persistence of intraneuronal toxin molecules or toxin activity, or the universality of models for light chain metabolism or elimination. One is obliged to conclude that this is an area of investigation in which there is much work to be done.

5. Implications of the life history model

Understanding mechanisms for absorption, distribution, metabolism and elimination of botulinum toxin contributes greatly to an understanding of botulism. This is true both for disease that is naturally occurring and for disease that is the product of malice (i.e., bioterrorism and biological warfare). However, adoption of the life history model can have implications that extend to other areas. When there is a sufficient body of pharmacokinetic data, this information can be used as a powerful tool to analyse problems beyond merely describing disposition of the toxin molecule. Several examples that may help to illuminate the power of pharmacokinetic data arise from the timely efforts to find agents that will block or reverse toxin action.

5.1. Medical countermeasures against botulinum toxin

5.1.1. Vaccine development and antibody action

Until the relatively recent past, the overwhelming majority of vaccine studies conformed to two widely held beliefs. First, it was thought that an efficacious vaccine would have to be administered by an injection route. Second, it was believed that the most important mechanism of antibody action was to associate with toxin in a way that blocked binding to nerve endings. The life history approach to studying botulinum toxin teaches that neither of these beliefs is entirely correct.

Research on botulinum toxin absorption demonstrates that the best strategy for antigen presentation is not an injection route but rather a mucosal route (Kiyatkin et al., 1997; Park and Simpson, 2003; Ravichandran et al., 2007). The neurotoxin can be absorbed by both the oral and inhalation routes, which suggests that these could also be the routes for antigen administration. One possibility would be to give a genetically altered and inactive form of the holotoxin (Kiyatkin et al., 1997), but this may not be necessary. As discussed earlier, the carboxyterminal half of the heavy chain (HC50) has the ability to bind and cross mucosal barriers (Maksymowych and Simpson, 2004). In addition, this polypeptide has more epitopes than the rest of the toxin molecule combined (Chen et al., 1997). Therefore, one might predict that this polypeptide could be an efficacious oral and inhalation vaccine.

This concept has been tested by administering the HC50 domain of serotypes A, B, and E to mice by the inhalation route, either individually or in combination (Ravichandran et al., 2007). Individual antigens and the combination of all three antigens evoked resistance to poisoning by the parent toxins. The observation that mucosal administration of the HC50 polypeptides produced protection against poisoning was important in its own right, but this observation also served as the basis for new insights into mechanisms of antibody action.

One of the things to emerge from the mucosal vaccine work was the finding that inhalation administration of the antigen evoked a secretory IgA response in the airway (Ravichandran et al., 2007). These secretory antibodies possessed the ability to associate with the toxin and block its binding and transcytosis across epithelial barriers. In other words, secretory IgA blocked absorption of the toxin. Without doubt, blocking toxin entry into the body is the single most important thing an antibody can do.

There was yet another observation to arise from this work. When botulinum toxin was administered to vaccinated mice and plasma samples were collected to perform pharmacokinetic experiments, the circulating titer of free toxin was far less than that in control (non-vaccinated) mice (Ravichandran et al., 2007; Sepulveda et al., 2010; Al-Saleem et al., 2012). This was evident when samples were collected during the distribution phase and also during the elimination phase. In a typical experiment with well vaccinated mice, the circulating titer was approximately two orders of magnitude lower than that in control animals, and these levels remained depressed throughout testing.

In companion experiments, various organs were harvested from vaccinated and non-vaccinated mice 30–60 min after toxin administration. For most organs the levels of toxin were comparable in the two groups of animals, but there were large differences in liver and spleen. In vaccinated mice the levels of toxin in these two organs were 5–10-fold higher than in control mice.

When taken collectively, the data show that neutralizing antibodies can evoke the phenomenon of clearance. Antibodies decorate the surface of antigens, after which the antibody–antigen complexes are marked for removal from blood. This constitutes a highly efficient mechanism for protecting against botulism. The fact that most of the body burden of toxin is rapidly removed from the general circulation means that it cannot reach nerve endings and therefore cannot cause paralysis. Another aspect of the work is that it applies not only to active immunization but also to passive immunization (see Section 5.1.2). Therapeutic antibodies can evoke clearance when they are in the circulation before toxin, and they can also evoke clearance when added to the circulation after toxin. Finally, the work contributes to a fuller and more accurate appreciation of the mechanism of action of neutralizing antibodies (Fig. 3). Following mucosal vaccination, secretory IgA can act at mucosal surfaces to block toxin entry into the body. This constitutes a first layer of protection. If some fraction of toxin overcomes this immune barrier and enters the body, circulating IgG will trigger clearance. This constitutes a second layer of protection.

When animals are robustly immunized, it is not likely that an oral or inhalation dose of toxin could surmount the first and second layers of protection. However, if this should occur, antibodies could exert the action that was alluded to earlier. They can associate with the toxin in a way that prevents binding and/or internalization at nerve endings. This represents the third layer of protection. When all three layers of protection are operative, they pose a formidable barrier to poisoning by botulinum toxin.

5.1.2. Therapeutic antibodies and the window of opportunity

Active immunization evokes a high level of protection against poisoning, but it is questionable whether this protection will have a widespread impact. Vaccination is currently being used to protect specific populations that are at high risk, but at the present time there is no plan to adopt universal vaccination (Arnon et al., 2001). Thus, if there were to be a bioweapons event that targeted civilian populations, most if not all of those populations would be vulnerable. This would leave therapeutic antibodies and pharmacologic antagonists as the putative agents of intervention in a post-incident scenario.

Research on pharmacologic antagonists has yet to identify drugs that have a high likelihood of being effective in a human population in a post-incident scenario, but research on therapeutic antibodies has shown more immediate promise. One formulation has already received regulatory approval for use in infants who succumb to natural poisoning by serotypes A and B (Arnon et al., 2006; Arnon, 2007), and other formulations seem destined for regulatory review for use in adult populations that are victims of a bioweapons event (Amersdorfer et al., 1997; Nowakowski et al., 2002).

Given the progress that is being made in therapeutic antibody development, one is justified in shifting attention to practical matters. One of these matters can be stated as a question: When and under what circumstances can therapeutic antibodies be beneficial in a post-incident scenario? This straightforward question can be re-stated in a way that captures its practical essence: How many minutes or hours after exposure to botulinum toxin will administration of therapeutic antibodies be useful?

The interval of time within which therapeutic antibodies exert a beneficial effect is called the “window of opportunity”. If antibodies are administered very shortly after exposure to toxin, there may be complete protection against poisoning. If the interval is longer there will not be complete protection, but the severity or duration of illness may be diminished. And if the interval is too long, there will be no protection or benefit of any kind.

In a recent study using an animal model (mouse), experiments were done to quantify the window of opportunity for therapeutic antibody administration as a function of dose and route of toxin administration (Al-Saleem et al., 2011). Both the survival of animals and the circulating titers of toxin were measured. One of the major findings of the study was that the window of opportunity within which antibodies could provide protection increased as the challenge dose of toxin decreased.

It would be useful to know the precise mechanisms that underlie this outcome, and once again the life history model can be informative. As explained earlier, it has been demonstrated that the half-life for distribution and the half-life for elimination of toxin do not vary significantly as a function of dose. Therefore, one can immediately rule out these pharmacokinetic parameters, as well as any other phenomenon that does not vary with dose, as the major factor that governs the window of opportunity. There must be one or more other factors involved.

A careful analysis of the window of opportunity reveals that there are a number of factors acting in concert:

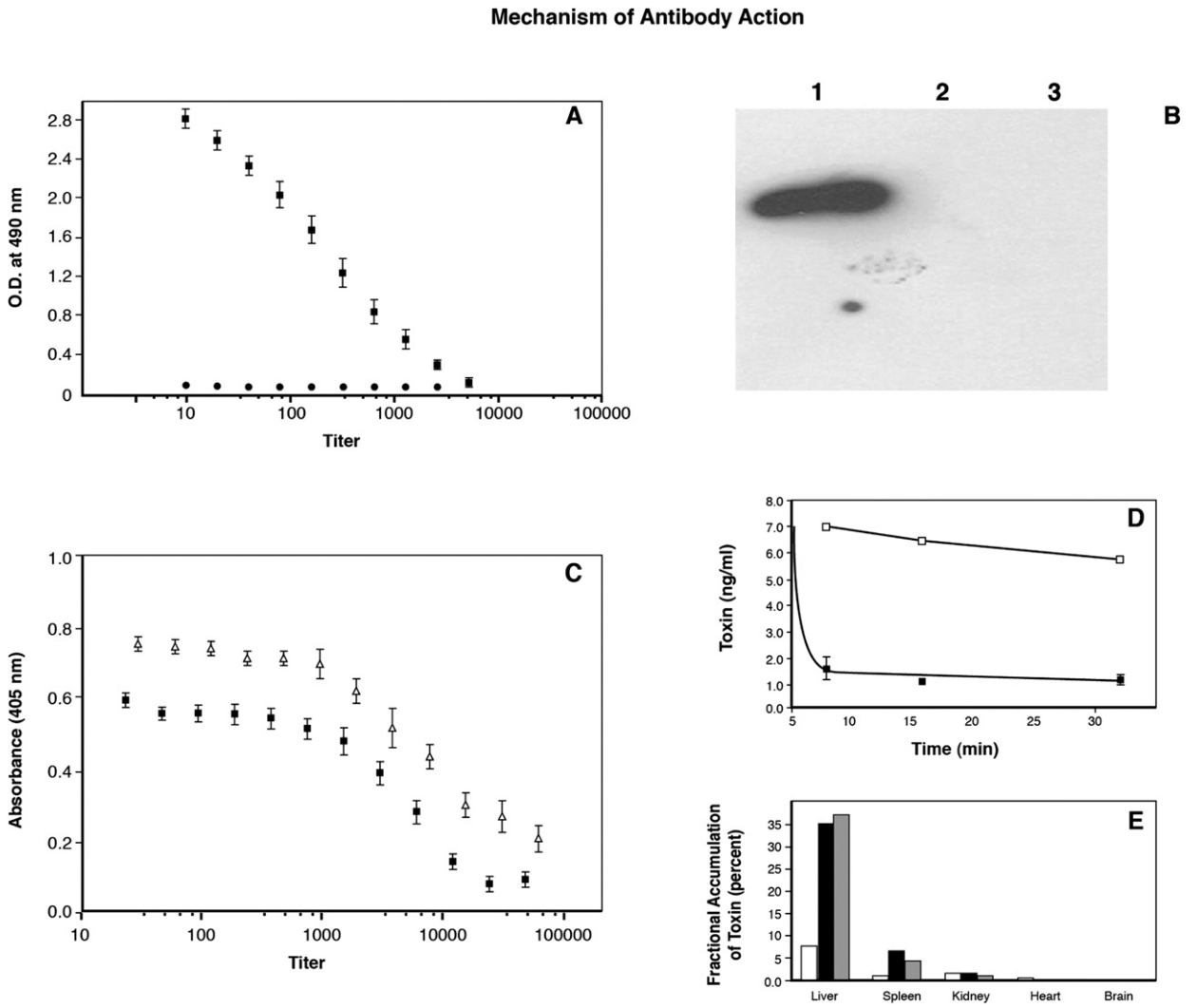

Fig. 3. Vaccination of mice with a recombinant, non-toxic polypeptide from the heavy chain evokes several phenomena that contribute to immunization. A. Naïve mice do not possess anti-toxin antibodies in the airway (●), but intranasal vaccination evokes a substantial secretory IgA response (■; ELISA dilution titer). B. When botulinum toxin is added to the apical side of epithelial cells, it undergoes transcytosis and accumulates on the basal side, where it can be detected by Western blot analysis (1). If neutralizing antiserum (positive control) is added to the apical side of epithelial cells before toxin, the antibodies block transcytosis (absorption; 2). Secretory IgA evoked by intranasal vaccination also blocks toxin absorption (3). C. Intranasal vaccination elicits an airway IgA response (see Part A above), and it also evokes a circulating IgG response (△) and IgA response (■). D. In control mice, botulinum toxin has a long elimination half-life (□; and see Fig. 2). In vaccinated mice, there is a rapid and dramatic decrease in the circulating titers of toxin (clearance; ■). E. In control mice, there is relatively little toxin accumulation in various body organs (open bars). In actively vaccinated (black bars) and passively vaccinated (hatched bars) mice, clearance causes a substantial increase in toxin levels in liver and spleen.

First, botulinum toxin possesses a highly efficient mechanism for binding and entering nerve cells, whereas neutralizing antibodies do not. This means that antibodies can act only if they locate and associate with toxin before the latter enters nerve cells.

Second, the amount of toxin that is necessary to produce a lethal effect is fixed. Perhaps the best known example is the LD50 for botulinum toxin type A, which in a 20–25 g mouse is about 5 pg.

Third, the rate constant for botulinum toxin delivery to nerve endings is fixed, and as noted above this rate constant does not vary with dose.

The fact that the fractional rate of toxin delivery to nerve endings is constant across doses means that the absolute amount delivered to nerves within a fixed interval of time will vary with dose. For any given interval of time, the amount of toxin presented to nerves will increase as dose increases, and it will decrease as dose decreases. Thus, the time needed for a lethal quantum of toxin to enter nerves will vary inversely with dose: the larger the dose, the shorter the interval of time needed to poison nerves; and the lower the dose, the longer the interval of time needed to poison nerves. By extension, one can deduce that the window of opportunity for antibodies to locate and

neutralize extracellular toxin will increase as the challenge dose of toxin decreases (Al-Saleem et al., 2011).

Analyzing the pharmacokinetic data leads to identification of the factors that account for the length of the window of opportunity. The data also bring to light an unexpected conclusion. They strongly suggest that no therapeutic antibody preparation will prove superior to any other antibody preparation in terms of post-challenge efficacy. Assuming that each preparation is administered in a way that leads to equivalent levels of neutralizing antibodies in the circulation, the window of opportunity for providing benefit will be the same for all. This is due to the fact that the window of opportunity is not governed by the inherent characteristics of any particular neutralizing antibody preparation, but instead by the behavior of the toxin. The critical determinant is the time needed for a lethal amount of toxin to reach and enter vulnerable nerves.

To state the obvious, the power of pharmacokinetics should not be misunderstood to mean that quantitative outcomes obtained in one creature, such as a mouse, can be generalized to all creatures. Windows of opportunity for antibody administration will vary as a function of many factors, including toxin serotype, dose and route of toxin administration, dose and route of antibody administration, and the creature being studied. It is within the immediate realm of possibility to conduct laboratory animal studies, such as those described above, that will quantify the window of opportunity in a mouse model. It will have to remain as a future goal to acquire the information that is needed to quantify windows of opportunity in human populations.

5.1.3. The search for pharmacologic antagonists

There is an acknowledged urgency to developing clinically useful pharmacologic antagonists of botulinum toxin. This urgency is due in part to a serious limitation on the use of antibodies as a post-incident medical countermeasure. Unlike botulinum toxin, natural antibodies do not enter nerve endings and cannot counter the effects of toxin that has been internalized. The principal benefit of a pharmacologic antagonist is that it might enter the nerve cytosol and block light chain catalytic activity.

There is a considerable historical record of identifying pharmacologic agents that can block toxin activity, but little of this earlier work was intended to discover efficacious medical countermeasures. Instead, the work was aimed at unraveling the mechanism of action of botulinum toxin. Sadly, little of this historic work can be translated into drugs that are likely to have clinical utility. The shift in perspective from using drugs as pharmacologic tools to analyze toxin action to the administration of drugs as countermeasures to prevent or reverse poisoning is enormous. It brings to light a long list of obstacles that must be overcome, almost all of which can be expressed as counterpoints to the absorption, distribution, metabolism and elimination of botulinum toxin. Some representative examples are:

a. *Botulinum toxin is active by the oral and inhalation routes.* Most pharmacologic antagonists under consideration as countermeasures have little or no ability to cross mucosal surfaces to reach the general circulation. In addition, most would survive the gastric system only poorly, if at all. This means that either formulations must be created that provide protection and promote absorption, which will be a time-consuming endeavor, or antagonists will have to be administered by non-ideal routes, such as injection, which would be a logistic problem when coping with large bioweapons events.
b. *Botulinum toxin has a long biologic half-life in the general circulation.* Almost all pharmacologic countermeasures now being studied would be rapidly eliminated from the body, at least in part by renal clearance. This means that either long-acting formulations must be created (viz., depot formulations), or antagonists would have to be administered repeatedly, perhaps at short intervals.
c. *During its tenure in the general circulation, botulinum toxin remains fully active and available for distribution to vulnerable nerve endings.* There is reason to be cautious in expecting that a clinically useful, extracellularly acting pharmacologic antagonist will be found. There are many reasons for this, some of which are mechanistic, such as: a) an inhibitor must have such a high affinity for toxin as to assure that no toxin will ever be in the free state (e.g., available to poison nerve endings), b) certain types of inhibitors, including those that act to block catalytic activity, may have limited access to the catalytic groove that is covered by the belt domain in the native toxin, and c) extracellularly acting antagonists may offer little advantage over extracellularly acting antibodies.

The last point is especially important. Antibodies do possess a property that makes them superior to pharmacologic antagonists. They can mark the toxin for rapid clearance from the circulation, rendering the molecule completely incapable of reaching or poisoning nerve endings. This property of antibodies has led to the reasonable suggestion that the best choice for a post-incident intervention would be a cocktail of neutralizing antibodies, which can both promote clearance and block toxin binding to nerve endings, and a pharmacologic antagonist, which can reach the neuronal cytosol to block catalytic activity.

d. *Botulinum toxin is highly selective in binding to target cells.* It will be difficult to create a class of toxin enzyme inhibitors that enter only those nerve endings associated with poisoning. Indeed, there are two challenges here: identifying a high affinity antagonist that can cross any membrane, and then attempting to target that antagonist to cross peripheral cholinergic membranes. Any agent that is non-selective and crosses all membranes carries the potential for adverse drug reactions or interactions. Any agent that crosses the blood–brain barrier would face considerable regulatory scrutiny.
e. *Intraneuronal botulinum toxin displays a persistence of activity that may range from several weeks to more than a year.* A good understanding of all the factors that play into this duration of action is not yet available, and therefore it is not clear what will be the exact nature and

magnitude of effect of an antagonist, or what will be the issues surrounding administration of that antagonist.

These five representative concerns must be superimposed on what would be described as the primary goal of this line of work, which is to find an antagonist of sufficient potency and specificity to warrant evaluation as an authentic countermeasure. When one takes all of these matters into account, it is hard to escape the conclusion that investigators who are committed to finding a clinically useful antagonist of botulinum toxin must possess certain elements of heroism!

5.2. Analyzing the meaning of a lethal dose

The science of toxicology has defined the meaning and significance of an LD50 dose, and these concepts have been embraced by those who study botulinum toxin. The LD50 doses for several serotypes of botulinum toxin, when administered to different animals by different routes, have been reported, although much of this work would benefit from greater experimental rigor. With the advent of techniques for measuring botulinum toxin in biological specimens, it is now possible to correlate an LD50 dose, or multiples of an LD50 dose, with actual titers of toxin in different body compartments.

These relatively straightforward concepts and measurements give rise to some rather provocative questions. Perhaps the most obvious question is this: Of the total body burden of toxin that constitutes a lethal dose, what fraction actually participates in, or is closely associated with, a lethal outcome?

There are different ways to conceptualize this question, and therefore different ways to conduct experiments that could produce a satisfactory answer. One of these is linked intimately to the life history approach for studying botulinum toxin action, and more precisely to the techniques for quantifying the window of opportunity for antibody action.

By definition, a neutralizing dose of antibody will block completely the actions of a challenge dose of toxin, assuming the antibody is administered prior to or simultaneously with the toxin. If administration of antibody is significantly delayed (i.e., the window of opportunity is exceeded; see Section 5.1.2), challenged animals will die. By systematically increasing the interval between administration of a lethal dose of toxin and administration of a neutralizing dose of antibody, one can determine the precise point in time when the ability of antibody to afford protection against a lethal outcome is lost. Data of this nature for three doses of botulinum toxin type A are presented in Fig. 4.

A careful analysis of the data in this Figure leads to an interesting deduction. A dose of 500 pg of toxin can be used to make the point. When this dose of toxin is administered intravenously to mice, a neutralizing dose of antibody must be administered within 20 min of the challenge dose of toxin. If administered after this interval of time, 100% of the animals will succumb. In other words, within slightly more than 20 min a lethal quantum of toxin has already entered nerves, and therefore intervention with antibodies cannot produce survival.

Using mice as a model, the $t½$ for botulinum toxin type A elimination is almost 300 min (e.g., Fig. 2). This means that at the 20–30 min time point following toxin administration, the residual body burden is still more than 95% of that at time zero. In other words, less than 5% of the original body burden is essential for a fatal outcome.

This small fraction of the original body burden would be divided among several compartments, the largest of which would be the extracellular compartment associated with blood, lymph, and interstitial fluid. The extracellular fluid bathing peripheral cholinergic nerve endings is the microcompartment through which the toxin must pass to gain entry into vulnerable nerves. The actual size of the microcompartments surrounding phrenic nerve endings and intercostal nerve endings (i.e., the nerve endings that control respiration, and thus the nerve endings whose paralysis of function can produce a fatal outcome) is miniscule compared to the size of the total extracellular fluid compartment in the body. By extrapolation, one can deduce that the amount of toxin that is extracted from perineuronal microcompartments by phrenic and intercostal nerves is only a tiny fraction of the total amount of toxin in the extracellular fluid compartment. Stated differently, only a tiny fraction of the 5% of the total body burden that is associated with a lethal outcome actually reaches and enters key nerve endings. In all likelihood it is orders of magnitude less than 5%.

As one would expect, increasing the administered dose of toxin means that a smaller proportion of that dose is essential for a fatal outcome, while diminishing the administered dose means that a larger proportion of the total body burden is essential for a fatal outcome. The underlying arguments to support these statements are analogous to those that explain the relationship between administered dose of toxin and window of opportunity for antibody administration.

To safeguard against possible confusion, there is one further point that should be made about toxin that is distributed throughout the various fluid compartments. Although only a small amount of a lethal dose of toxin may be needed to enter and paralyze nerves, the remaining amounts of toxin should not be viewed as unimportant. To the contrary, the pseudo-steady state concentration of toxin in exchangeable fluid compartments will influence whether there is a lethal outcome. This concentration must be sufficiently close to the dissociation constant that governs the interaction between toxin and neuronal receptors to permit a lethal quantum of toxin to bind and enter nerve endings.

6. Future directions

There remain a considerable number of gaps in our understanding of the disposition of botulinum toxin in the body. To cast the matter in more positive terms, there are fertile grounds for research for those who believe that examining the life history of a botulinum toxin molecule can be rewarding. Of the many areas that might be pursued, there are two that would surely attract wide attention. The first pertains to the coalescing of in vitro and in vivo findings. The second, which is an extension of the first, relates

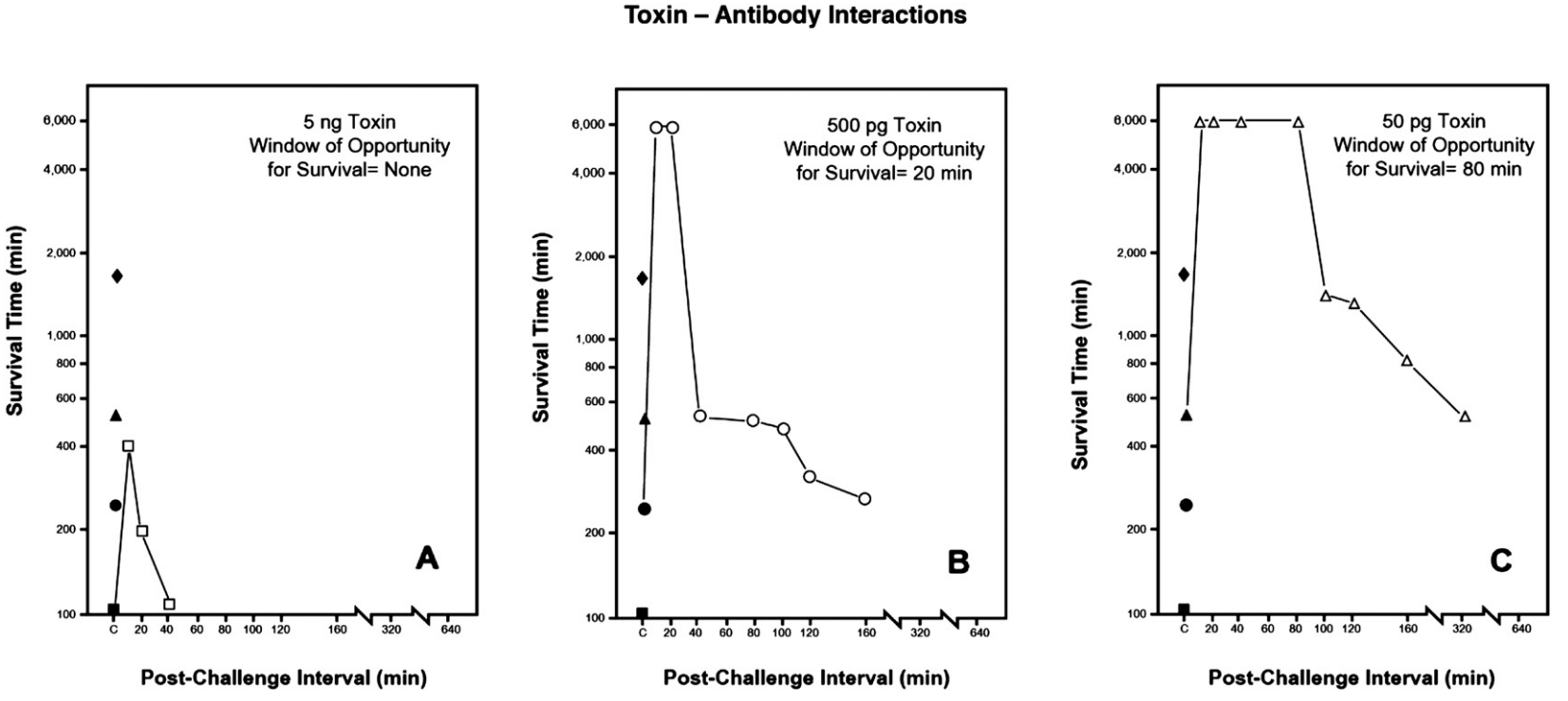

Fig. 4. Intravenous administration of increasing doses of botulinum toxin to mice produces dose-dependent decreases in survival times (5 ng, ■; 500 pg, ●; 50 pg, ▲; 10 pg, ◆). A. Intravenous administration of neutralizing doses of antibody at various times (5 min–40 min) after intravenous botulinum toxin (5 ng; 1000 MLD50) shows that antibody can prolong survival, but it cannot provide complete protection (□). B. Intravenous administration of antibody at various times (5 min–160 min) after botulinum toxin (500 pg; 100 MLD50) shows that the window of opportunity for providing full protection is ca. 20 min (○). C. Intravenous administration of antibody at various times (5 min–320 min) after botulinum toxin (50 pg; 10 MLD50) shows that the window of opportunity to provide full protection is ca. 80 min (△).

to the development of methods that will allow visualization of toxin distribution and action in vitro and in vivo.

6.1. The final common pathway

Enormous strides have been made in deciphering the cellular and subcellular actions of botulinum toxin, due mainly to the availability of isolated cell assays, broken cell assays, and cell free assays. Generally speaking, these test systems can be manipulated in ways and to extents that are difficult or impossible to achieve in the intact body, and these manipulations are often key to analyzing some particular aspect of toxin action. Conversely, the intact body presents pathways for efficient and realistic delivery (e.g., vasculature) and removal (e.g., biotransformation and elimination) of toxin that are not typically integrated into – or even considered – in most in vitro studies. Clearly, the ultimate goal should be to ensure that the results of in vitro studies and in vivo studies are a close approximation of one another, or at least closely supportive of one another.

There may be no better example of this than the analysis of toxin action on vulnerable nerves. For instance, one would expect that the Kd that describes toxin interaction with its receptors, which is a quantitative measure that ordinarily comes from in vitro work, to bear some meaningful relationship to the circulating titers of toxin in the extracellular fluid compartments of animals receiving sublethal, lethal, and supralethal doses of toxin, which is a measure that could only arise from in vivo studies. Similarly, the intraneuronal concentration of toxin that produces half-maximal blockade of transmitter release in an isolated neuronal preparation should be close to, or even identical to, that which produces half-maximal blockade of transmitter release in the same nerves while still in the body of a poisoned animal.

Another area in which there needs to be better coalescence of in vitro and in vivo work relates to identification of pharmacologic antagonists of botulinum toxin. There certainly is an emerging literature in which investigators have examined one or another class of putative antagonists. The preponderance of this work has been done on in vitro preparations, which is an altogether proper starting point, but far too little of the work has been extended to in vivo models and realistic biothreat scenarios. Unless and until in vitro work is complimented with in vivo studies on a realistic model, this work will not be fully meaningful.

There is an implied responsibility in calling for better melding of in vitro and in vivo work, and that responsibility is for investigators in each camp to venture into the adjoining realm. Hopefully, the prospect of obtaining an integrated picture of toxin distribution and action will encourage these efforts.

6.2. To see most clearly

Of the many ways in which research is conducted, there is one that seems to hold universal appeal. Investigators of all stripes take great satisfaction in being able to see a process, especially in real time and in 3-dimensions. And there is reason to be optimistic that this approach to studying botulinum toxin is almost within reach.

Over the past decade there have been a host of reports describing techniques for seeing the movements of individual molecules within individual cells. There is no doubt that these techniques can be applied to research on botulinum toxin. With an appropriately labeled toxin molecule, one should be able to visualize the steps of binding, internalization, and accumulation at local sites of action. More ambitiously, one could attach suitable and discrete labels to the light chain and heavy chain, and afterward track the separate paths these polypeptides follow in nerve endings. This would mean that the precise moment of programmed death of the intact toxin molecule could be determined, and the precise moment of onset of toxicity could also be set. In a similar vein, one could monitor the lifetime of a single light chain in the neuronal cytosol, and even compose an intraneuronal travelogue that reveals whether individual light chains are sedentary or prone to wandering.

The techniques for single molecule–single cell analyses are evolving so quickly that it is all but certain that one or more of these methods will be applied to botulinum toxin and nerve cells. This will ensure that a full understanding of the intraneuronal fate of the molecule will become available. Unfortunately, there will be a longer wait to adopt equally powerful techniques to follow the fate of populations of toxin molecules in the body. The toxin is an exquisitely potent substance, and as yet there are no visualization techniques that are adequate to study biologically relevant concentrations of the molecule.

Although the methods for visualizing active toxin movements in real time and in three dimensions are not at hand, there are ways to gain insight into how such methods could be used. One of these is to exploit a recombinant, non-toxic fragment derived from the toxin. An obvious choice would be the carboxyterminal half of the heavy chain, which is being evaluated as a potential vaccine, and which could be administered at doses that are orders of magnitude higher than an LD50 of toxin.

In Fig. 5, representative data are presented from a recent study in which the vaccine candidate was administered intravenously to rodents. Unlike botulinum toxin, the vaccine candidate is small enough to undergo partial renal clearance. This was obvious in the collected images, in which the polypeptide was highly localized in the kidney, and shortly thereafter in the bladder, before being eliminated.

It is impossible to know when visualization techniques will become sufficiently sensitive to allow similar types of studies on small doses of active toxin. However, when that day does come, one can conceive of the ultimate coalescence of in vivo and in vitro work. The in vivo methods will permit tracking of the toxin throughout the body, including those molecules that are extracted from the peri-neuronal microcompartment to begin their journey to the cell interior. At this point, the single molecule–single cell approach will afford the opportunity to track the destiny of every toxin molecule that participates in paralysis of neurotransmission. When investigators have accomplished this remarkable coalescence of in vivo and in vitro analyses, there truly will be a fully written life history of botulinum toxin in the body.

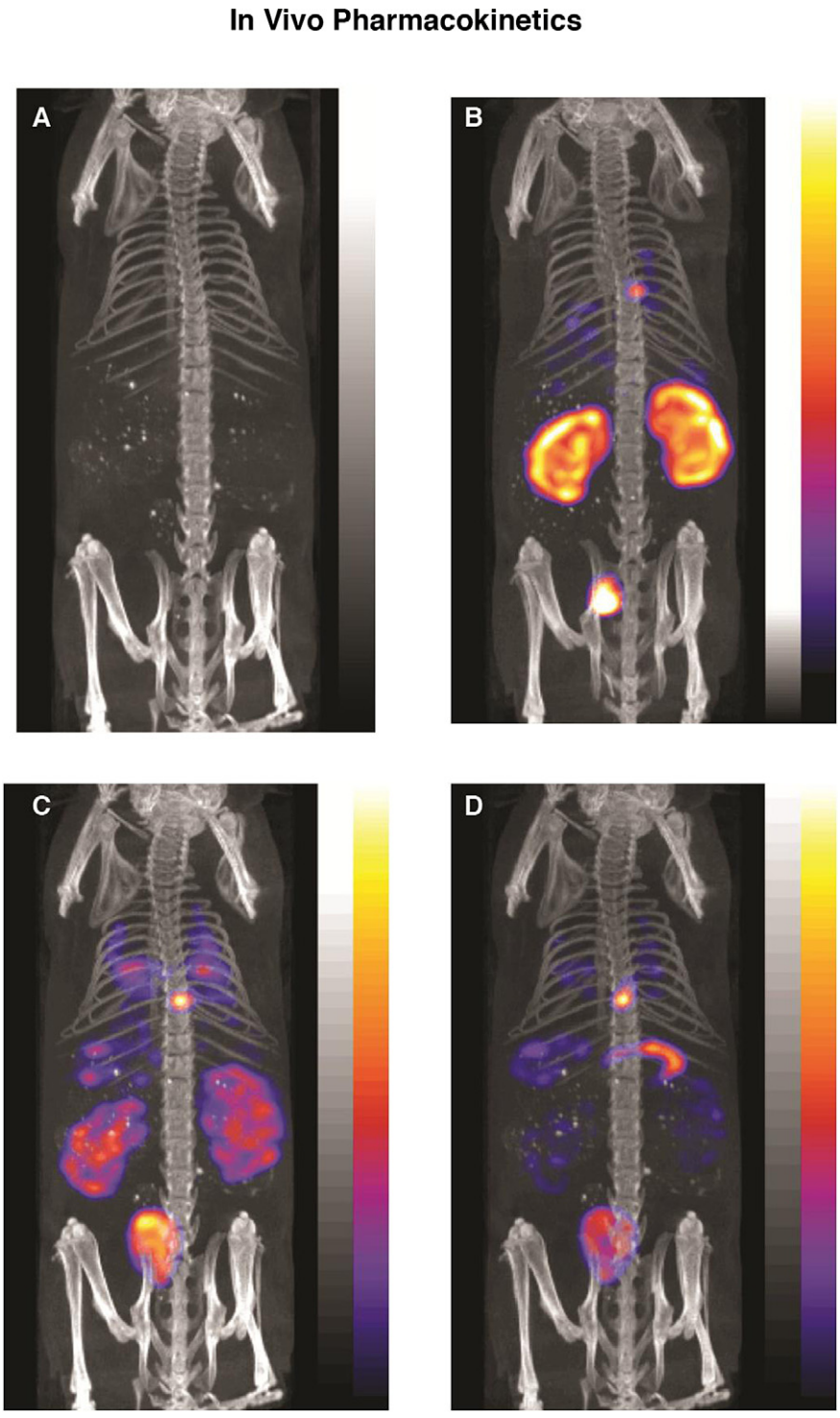

Fig. 5. Real time, 3-dimensional pharmacokinetic studies on the carboxyterminal half of the heavy chain were performed in mice. An initial series of CT scans were performed to obtain anatomical landmarks (A). Labeled polypeptide was administered intravenously, and single photon emission computer assisted tomography images were captured at 30 min (B), 60 min (C) and 120 min (D). The local concentrations of polypeptide were translated into pseudo-color (right bars on parts B to D). Each image is a single snapshot of a real time, 3-dimensional movie. Note that the labeled polypeptide accumulated in the kidney (B), then disappeared from kidney and accumulated in the bladder (C). At the 120 min time point, most of the administered dose had been eliminated (D).

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Conflicts of interest

The author declares that there are no conflicts of interest.

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