
12 Pharmacological Countermeasures for Botulinum Intoxication*

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I. INTRODUCTION

The botulinum neurotoxins (BoNTs)** comprise a family of seven distinct neurotoxic proteins (A–G) produced by immunologically discrete strains of the anaerobic bacterium, *Clostridium botulinum*.^{1,2} These toxins act on peripheral cholinergic

*The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the view of the Department of the Army or the Department of Defense.

**In this chapter, BoNT is used to designate both pure botulinum neurotoxin as well as the neurotoxin complex. Some authors prefer to use BoTx for the latter.

synapses to inhibit spontaneous and impulse-dependent release of acetylcholine (ACh).^{3,4} Intoxication by BoNT results in muscle weakness, which can be fatal when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation.⁵ BoNTs are the most potent substances in nature and ingestion of as little as 1 ng/kg is sufficient to cause human lethality.⁶

The purpose of this chapter is to use the insights gained in our understanding of the mechanism of BoNT action during the past decade to establish a conceptual framework within which to develop effective treatment strategies for intoxication. The chapter is organized into three major topics consisting of (1) an overview of BoNT action, (2) a description of foodborne, wound, and infant botulism, and (3) a discussion of possible treatment options.

From the first description of botulism in 1793 until the mid 1950s, the primary role of BoNT was that of a public health problem due to its association with food poisoning.^{6,7} Although implicated in only a small fraction of all foodborne illnesses (<0.1%), the severity of the clinical syndrome produced by BoNT and the potential for multiple casualties led each outbreak to be considered as a potential health crisis.⁷⁻⁹ During and shortly after World War II, BoNT was developed as a biological weapon, due to its ability to cause extensive morbidity and mortality.¹⁰ BoNT reverted once again to this role with the Iraqi stockpiling of BoNT prior to the Persian Gulf War (1991), and with the rise of terrorist organizations, such as the Japanese Aum Shinrikyo cult, which has not only produced but reportedly deployed biological and chemical agents.¹¹⁻¹³

Systematic research on the mechanism of action of BoNT began with Emile Pierre van Ermengem's historic isolation and characterization of *Clostridium botulinum* following a large outbreak in Ellezelles, Belgium, in 1895 and has continued with increasing interest and excitement to the present time.^{2,7,9} Early work on BoNT intoxication established the existence of multiple serotypes, localized the site of action to peripheral cholinergic synapses, proposed the mechanism of impaired ACh release, and ruled out non-cholinergic, sensory, and central nervous system involvement.¹⁻⁵ Most of these findings were firmly established by 1949.^{14,15}

The remarkable specificity for peripheral cholinergic synapses and long duration of action led to the use of BoNT/A for a growing number of focal dystonias and movement disorders following its approval in 1989 as an "orphan drug" by the Food and Drug Administration for the treatment of strabismus, blepharospasm, and hemifacial spasm.¹⁶ The attributes that render BoNT a dreaded poison also make the neurotoxin an ideal therapeutic agent to treat diseases of muscle hyperactivity. In addition to its original indications, some BoNT serotypes are also used for treatment of spasticity from brain and spinal cord injuries, stroke, multiple sclerosis, cerebral palsy, and numerous other disorders. Expansion and refinement in its clinical use constitute the most active focus of current BoNT research, and a number of excellent reviews have been published recently.¹⁶⁻¹⁸

In the last decade, enormous progress has been made in understanding the action of BoNT at the molecular level. This was spurred by three crucial developments: (1) elucidation of the amino acid sequence leading to recognition of the zinc binding motif, (2) demonstration of zinc metalloprotease activity with identification

of substrates and cleavage sites, and (3) solution of the crystal structure for BoNT/A and /B.^{19–21} These developments opened the possibility for rational studies of mechanism and active-site inhibitor design.

A. CHARACTERISTICS OF BOTULINUM NEUROTOXIN (BoNT) INTOXICATION

The typical manifestation of botulism is a flaccid paralysis that is bilateral and descending, involving primarily skeletal muscle but also structures innervated by autonomic parasympathetic fibers.^{1–9} Human intoxication is caused by serotypes A, B, E, and, to a much lesser extent, by serotype F and is manifested as foodborne, wound, and infant botulism.^{1,2,6} Wound and infant botulism, however, are usually confined to serotypes A and B.^{22,23} Data compiled for foodborne botulism during the past 50 years in the United States indicate that serotype A was responsible for 37.6% of all outbreaks while serotypes B, E, and F accounted for 13.7%, 15.1%, and 0.7%, respectively, of intoxications in which serotype involvement was established.⁷

Clostridium botulinum spores are widely distributed in soils, sea sediments, decaying vegetation, animal carcasses, and sewage.⁷ The intestinal tracts of birds, mammals, and fish may also acquire *Clostridium botulinum* as a transient member of their intestinal flora.^{6,7} The hosts do not exhibit botulism since growth of *Clostridium botulinum* is suppressed when there is competition from other organisms and a functional immune system.^{7–9} The resistance of clostridial spores to harsh environmental conditions enables their dissemination by air currents and dust particles leading to surface contamination of exposed food products.^{7–9} Botulism is not contagious, however, and contact with spores does not usually lead to disease except in young infants or following germination in wounds (see Sections IIB and IIC).^{7–9}

B. SYMPTOMOLOGY

The clinical syndrome of botulism reflects toxin-induced blockade of ACh release from neuromuscular and neuroeffector junctions.^{1,2} The basic syndrome of BoNT intoxication is similar for foodborne, infant, and wound botulism and does not vary appreciably among toxin serotypes.^{7,9} The earliest symptoms generally include visual disturbances (diplopia, blurred vision) and xerostomia.⁷ With low-level exposure these symptoms may gradually resolve, even in the absence of medical intervention.⁷ In more severe cases, the initial symptoms are followed by dysphasia, dysphonia, and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action.^{1,2} A descending generalized skeletal muscle weakness may then develop, progressing from the upper to the lower extremities.^{7,9,17} Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death unless appropriate supportive care is provided.^{7,17} Although motor function is severely impaired, there is little or no sensory alteration or central nervous system (CNS) involvement in botulinum intoxication.^{2,5,7}

Symptoms are usually observed 12–36 h after exposure, although onset times as short as 4 h or as long as 8 days have been reported.⁷ The preponderance of

symptoms, including the potentially lethal respiratory collapse, stems from inhibition of neuromuscular transmission.^{2,5,14,15} Parasympathetic dysfunction is responsible for blurred vision, xerostomia, constipation, and urinary retention.^{5,7,24} The absence of more extensive autonomic involvement in BoNT intoxication reflects a lower overall sensitivity of the autonomic nervous system to the actions of the neurotoxin.²⁵

C. FUNCTIONAL DOMAINS OF BoNT

BoNT are synthesized as ~150 kDa single-chain protoxins (range, 140–167 kDa). They are proteolytically activated (nicked) to form dichain molecules consisting of an ~50 kDa light chain (L-chain) and an ~100 kDa heavy chain (H-chain).^{1,2} The two chains are coupled by a single disulfide bond and by non-covalent forces.

In addition to the neurotoxin, all serotypes of *Clostridium botulinum* synthesize a group of non-toxic proteins, designated as neurotoxin-associated proteins (NAP); some of these possess hemagglutinin activity. These proteins associate with BoNT in the bacterial culture medium by noncovalent interactions and protect the neurotoxin from proteolytic and low pH-mediated inactivation. They have also been suggested to facilitate absorption of BoNT from the gastrointestinal tract into the bloodstream.²⁶ The ability of BoNT to manifest oral toxicity has generally been attributed to the presence of these proteins; conversely, the inability of tetanus neurotoxin (TeNT) to produce foodborne intoxication has been ascribed to the absence of such NAP.²⁷ Recent data from Maksymowych et al.²⁸ has raised some questions of the importance of NAP in BoNT toxicity, especially with regard to their role in transcytosis of the neurotoxin. These investigators demonstrated that pure BoNT/A lacking NAP was still toxic to mice, although to a lesser extent than the toxin complex. When examined at elevated concentrations, the differences in potency between pure and NAP-containing neurotoxin were progressively reduced. These results indicate that pure neurotoxin does not require accessory proteins for absorption. Moreover, even though the NAP are clearly protective, sufficient pure neurotoxin can survive the inhospitable environment of the gastrointestinal tract to produce lethality.

In conformity with the sequential processing of bacterial protein toxins such as diphtheria or cholera toxin, the action of BoNT involves multiple discrete steps: binding to surface receptors, internalization via receptor-mediated endocytosis, transport from endosome to cytosol, and cleavage of target proteins in the cytosol.^{1,2,17,19} Binding and internalization are mediated by the C- and N-terminal domains of the BoNT H-chain, respectively.^{1,2} The L-chains have zinc metalloprotease activity, targeted selectively to one of three proteins that are required for the docking and fusion of synaptic vesicles with active zones at the cytoplasmic surface of the nerve terminal.^{16–20}

Serotypes B, D, F, and G cleave different sites on the synaptic vesicle protein, synaptobrevin (VAMP), whereas serotypes A and E cleave the presynaptic membrane-associated protein SNAP-25.¹⁷ Serotype C1 is unique in that it cleaves two cytoplasmic proteins, syntaxin and SNAP-25.²⁹ Interaction of these proteins (designated as SNARE) on the surface of synaptic vesicles and active zone membranes is required for voltage- and Ca^{2+} -dependent release of neurotransmitter; cleavage by

BoNT inhibits this process, leading to muscle weakness and paralysis.³⁰ Cleavage of SNARE proteins appears to be sufficient to account for all actions of the BoNT. The SNARE hypothesis has received near-universal acceptance since its introduction in the early 1990s; an alternative hypothesis has been advanced based on results obtained in PC12 pheochromocytoma cells,³¹ however, its generality has not yet been established.

For each BoNT serotype, the dichain form constitutes the active configuration of the neurotoxin; the isolated L- and H-chains are devoid of systemic toxicity. The absence of toxicity is consistent with findings that the L-chain cannot ordinarily gain access to the cytosol unless it is coupled to the H-chain and that, on its own, the H-chain lacks the ability to inhibit neurotransmitter release.^{1,2} The isolated L-chain does, however, remain enzymatically active as evidenced by its ability to cleave SNARE proteins in cell-free assays,³² and by its capacity to inhibit ACh release in skeletal muscle when delivered by liposomes.³³ It is not clear whether any portion of the H-chain is internalized along with the L-chain and, if so, whether it exerts a role in enhancing the catalytic activity or stability of the L-chain.

All BoNT serotypes suppress ACh release, show high specificity for cholinergic synapses, and share the same overall mode of action; they differ, however, in potency and in duration of action. Type A neurotoxin exhibits the highest potency,⁶ and types A and C1 produce the longest intoxication times.^{34,35} Other differences include targeting of different functional surface receptors at the motor nerve terminal³⁶ and cleaving unique peptide bonds in the appropriate SNARE proteins.¹⁷

II. MANIFESTATIONS OF BOTULISM

In adults, botulinum intoxication generally results from ingestion of preformed toxin elaborated in contaminated foods (foodborne), or from colonization by *Clostridium botulinum* of deep wounds with subsequent production of toxin (wound botulism).^{8,37} A third form, termed infant botulism, is observed in young infants and originates from colonization of the large intestine by *Clostridium botulinum* with subsequent production and absorption of toxin.^{22,23} Rarely, adults also exhibit a syndrome resembling infant botulism and some authors regard this as the fourth manifestation of botulism.²³

A. FOODBORNE BOTULISM

Elaboration of BoNT in foods requires contact with *Clostridium botulinum* spores under conditions that allow bacterial cell proliferation and toxin production. These consist of an anaerobic environment, temperatures between 4 and 40°C, pH above 4.6, water activity greater than 0.94 (<10% NaCl), and lack of adequate preservatives.^{7-9,38} The requirements for growth of *Clostridium botulinum* are stringent, especially anaerobiosis, making outbreaks relatively rare; nevertheless, episodes of foodborne botulism constitute a persistent public health threat.⁹ In fact, food-related botulism outbreaks in the United States have shown no significant reduction during the past century, with an average of approximately 24 cases per year.⁷ The primary vehicle for foodborne botulism presently and during most of the 20th century has

been improperly prepared home-preserved food products, often involving vegetables with a low acid content.⁷⁻⁹ Other sources are restaurants that use unsafe food handling procedures and contaminated commercially canned food products; the latter has become rare since the introduction of modern methods.^{7,39}

Although the numbers of outbreaks have been relatively constant, the case-to-fatality ratio has improved markedly. From 1899 to 1950, foodborne botulism was associated with 60% mortality; from 1950 to 1996, the average annual mortality fell to 15.5%, and decreased even further to under 10% during the last decade.⁷ These advances in survival have come primarily from improvements in critical care.^{7,39} Further reductions in morbidity and mortality from botulinum intoxication will require better methods for detection of BoNT and availability of specific pharmacological treatments.^{11,12}

B. WOUND BOTULISM

Wound botulism is relatively rare, accounting for only 5% of all outbreaks. The majority of these are caused by serotype A, and the remainder by serotype B.⁷ The neurological symptoms of wound botulism differ little from that of foodborne botulism except for the general absence of gastrointestinal symptoms. Historically, this form of botulism was so uncommon that it was not even recognized until the last half of the 20th century. From its discovery in 1943 until 1985, only 33 incidents of wound botulism were documented.^{7,37} An examination of these cases indicated that wounds susceptible to *Clostridium botulinum* are generally deep, with avascular areas, but need not appear obviously infected or necrotic. Additional risk factors include compound fractures and extensive crush injuries.^{7,37} Contamination of wounds with *Clostridium botulinum* spores leads to germination and colonization at the injection site. Localized weakness results from production of toxin at the wound site, and systemic botulism can occur from toxin transmitted via the bloodstream to distant targets.³⁷

From 1980 to the present time, wound botulism has been observed predominantly in illicit drug users following repeated subcutaneous administration of narcotics or in individuals with nasal or sinus lesions from chronic cocaine abuse.^{7-9,37} Recent increases in subcutaneous and intranasal routes for drug abuse have led to a greater incidence of wound botulism. During the last decade alone, wound botulism in the above population has exceeded the total reported in the preceding 40 years by a factor of almost three.⁷

For reasons that are not completely understood, wounds are much more likely to be contaminated by *Clostridium tetani* than with *Clostridium botulinum*. Although an aggressive vaccination program has nearly eliminated tetanus in developed nations, the absence of universal tetanus vaccination in many "third world" countries results in over 300,000 cases annually.⁸ A large number of these occur in neonates, often by infection of the umbilical stump.⁸

C. INFANT BOTULISM

Infant botulism is a consequence of intoxication by BoNT following ingestion or inhalation of clostridial spores that colonize the large intestine; young infants,

especially those between 2 and 4 months of age, are susceptible to this form of botulism.^{22,23} Germination of spores and growth of vegetative cells leads to production of BoNT; the neurotoxin thus elaborated crosses the intestinal wall and reaches susceptible targets such as skeletal muscle via the bloodstream.²³ The characteristic symptoms are poor sucking, constipation, generalized weakness, and respiratory insufficiency. The risk factors are not completely understood, but the incidence drops off sharply after 28 weeks of age, which is likely to be related to development of a more diversified intestinal flora. The latter has been shown to suppress germination and growth of *Clostridium botulinum* spores in mice.⁴⁰ Of all food products that may be contaminated with *Clostridium botulinum* spores, honey has been the one most often implicated in infant botulism; it is therefore recommended that honey not be given to young infants.⁴¹

Although infant botulism was not recognized until a large outbreak occurred in California in 1976,²² it is currently the most prevalent form of botulism in the United States, accounting for approximately 70% of all cases.^{7,23} Because infant botulism results from a continual elaboration of BoNT, it is more effectively treated by antitoxin than is foodborne botulism. Recently concluded clinical trials carried out with a human botulinum immune globulin (BIG) has revealed a greater than two-fold reduction in the mean duration of hospitalization in infants treated with BIG; treatment was effective even when infusion was initiated several days after the onset of symptoms (Arnon, personal communication).

Under rare conditions, adults may manifest a syndrome similar to infant botulism. Such cases generally occur in hospitalized patients treated with a long course of multiple antibiotics that eliminate the normally suppressive intestinal flora; other predisposing factors include inflammatory bowel disease and surgical alterations of the bowel.²³

III. TREATMENT OPTIONS

BoNT are the most potent toxins known to mankind and exposure to as little as 1 ng/kg by ingestion or 3 ng/kg by inhalation can result in human fatality.⁶ Treatment consists of intensive care and infusion of trivalent equine antitoxin.⁷ Over 80% of adults with botulism receive antitoxin, although this passive immunization is only effective if administered early during the course of illness.⁷ This temporal limitation of antitoxin treatment was appreciated as early as 1929.⁴² Recovery, especially from type A intoxication, is slow and residual symptoms may persist for years after exposure. Vaccination provides a high degree of protection and is commonly administered to laboratory investigators who are at risk of exposure. The current vaccine is pentavalent (A–E), and has been available from the Centers for Disease Control and Prevention (CDC) as an Investigational New Drug (IND) for the past 40 years.⁴³ The vaccine is administered at 0, 2, and 12 weeks and requires a booster at 1 year to generate long-term protection. A heptavalent vaccine (A–G), consisting of conventional formaldehyde-treated toxin but of higher purity than the IND product, is currently under development by the United States Army.^{10,11} In addition, a vaccine made from the recombinant binding domain of BoNT H-chain (C-fragment) is also under development.¹¹

Although these vaccines are highly effective, all require multiple injections and as much as 1 year from onset to generate adequate protection. In addition, since the BoNT antibodies remain elevated for an unknown period of time after the 1-year booster, vaccinated individuals may be precluded from use of local BoNT administration for treatment of spasticity or movement disorders that may develop during their lifetime.¹⁷ These limitations argue strongly in favor of a supplementary pharmacological approach for the management of botulism.

A. PHARMACOLOGICAL INTERVENTION

From the time that inhibition of ACh release was established as the mechanism of BoNT action, attempts were made to antagonize the neurotoxin by measures that enhance ACh release. Until recently, however, development of a treatment for BoNT intoxication had low priority, in part because early efforts were generally unsuccessful and in part because an effective vaccine and antitoxin were already available. Currently, there is an increased impetus to develop pharmacological treatments following recognition of the potential for overdose with the expanding clinical use of BoNT.¹⁷ In addition, the experience gained in preparation for a potential BoNT threat during the Persian Gulf War made it clear that delays in generating adequate protection by the BoNT vaccine were incompatible with the requirement for rapid deployment of military personnel.^{10–12}

Some of the earliest putative BoNT antagonists were cholinesterase inhibitors, selected for their ability to prolong the actions of ACh. Carbamate anticholinesterase agents such as neostigmine and physostigmine were investigated as early as 1924 in animals⁴⁴ and in 1947 on nerve muscle preparations,¹⁴ but they were unable to antagonize the effect of BoNT. More recent studies have tended to confirm earlier findings,⁴⁵ although there have been occasional reports of human botulism responding to the short-acting cholinesterase inhibitor, edrophonium.⁴⁶

Other potential antagonists of BoNT such as elevated calcium, calcium ionophores, lanthanum, black widow spider venom, 2,4-dinitrophenol, and agents that raise cyclic AMP levels were examined for their ability to reverse BoNT toxicity. Addition of the above compounds to BoNT-intoxicated preparations led to increases in the frequency of spontaneous miniature endplate potentials (MEPP) but resulted in little or no enhancement in the amplitude of evoked endplate potentials (EPP).^{47,48} Since these compounds generally increased spontaneous but not evoked activity, they were not considered to be of practical value for treatment of BoNT intoxication.

1. Potassium Channel Blockers

Potassium channel blockers were found to be more effective in antagonizing the paralytic action of BoNT than were the former group of compounds. Their higher efficacy comes from their ability to prolong the duration of the presynaptic action potential, leading to a greater influx of calcium during nerve stimulation. Coupling of increased calcium influx to nerve impulses enables the potassium blockers to

produce striking increases in the amplitude of EPP and of nerve-evoked twitch tensions.⁴⁹

A number of potassium channel blockers have been evaluated for their ability to antagonize the actions of BoNT including guanidine, 4-aminopyridine, 3,4-diaminopyridine (3,4-DAP), and tetraethylammonium.^{50–52} Of these, the most promising candidate was 3,4-DAP; 4-aminopyridine exhibited undesirable CNS side effects, and tetraethylammonium caused a marked postsynaptic depression of end-plate potentials and muscle contractions that actually exacerbated BoNT-mediated inhibition.^{45–49}

When added to nerve-muscle preparations prior to BoNT, 3,4-DAP produced a marked delay in the time-to-block of nerve-evoked muscle contractions.⁵² When applied after BoNT paralysis, 3,4-DAP was able to restore tensions to near control values.^{45,50–53} Unlike many candidate antagonists, 3,4-DAP could restore tension even several days after total paralysis was established.⁵³ In spite of these successes with 3,4-DAP, two fundamental limitations were noted: its efficacy was largely limited to serotype A,^{50–52} and it had a brief *in vivo* lifetime relative to that of BoNT.⁵³ Of the two, the latter is less critical since the short lifetime can be compensated by use of an infusion delivery as shown recently by Adler et al.⁵⁴

The basis for the lack of response to 3,4-DAP by the other serotypes is not well understood. Recent identification of the cleavage sites suggests that some characteristic of the target proteins may provide an answer: serotypes B, D, F, and G cleave synaptobrevin on the synaptic vesicle membrane, while serotype A cleaves SNAP-25 at the active zone.¹⁷ However, serotype E also cleaves SNAP-25, but muscles intoxicated by BoNT/E do not respond appreciably better to 3,4-DAP than do those exposed to serotypes that cleave synaptobrevin.^{52,53}

At a functional level, serotype A-intoxicated neuromuscular junctions undergo an attenuated but synchronous release of ACh following stimulation; preparations intoxicated by serotypes B, D, and F produce asynchronous release where the ACh quanta are dispersed and cannot summate to produce suprathreshold EPP.^{48,50,51} It is readily apparent that the lack of synchrony would prevent 3,4-DAP from restoring transmitter release, however, the precise relationship between cleavage of synaptobrevin and loss of synchrony is not readily apparent.

An additional concern with potassium blockers comes from human case reports. These indicate that while the potassium blockers guanidine and 3,4-DAP produced a moderate increase in muscle strength, their use did not lead to the return of spontaneous ventilation in BoNT-intoxicated individuals.^{55,56} It is not clear if human diaphragm or intercostal muscles are refractory to the potassium blockers or whether the doses used were insufficient to reverse muscle paralysis.^{55,56} The latter may be the case, since BoNT/A-paralyzed rat or mouse diaphragm muscles respond vigorously to the actions of 3,4-DAP.^{45,52} Higher doses of 3,4-DAP were not attempted in these patients to avoid the risk of seizures and other potential side-effects. At the present time, the potassium blockers hold promise as potential therapeutic agents, but development of more selective compounds or targeting of the inhibitors to neuromuscular synapses will be required to exploit their full potential.

2. Specific Strategies for Therapeutic Intervention

The above examples of treatment strategies are based on antagonizing the actions of BoNT after the neurotoxin has undergone internalization and subsequent cleavage of some or all of its target protein. In addition, since these approaches were developed before the intracellular targets were identified, they do not specifically address the basic mechanism of toxin action. Rather, they act by elevating intracellular calcium levels in an attempt to compensate for the toxin-mediated inhibition of ACh release. The discrete stages of clostridial neurotoxin action of binding, internalization, and catalysis suggest that there are multiple sites for direct pharmacological intervention. These stages are mediated by different domains of BoNT and, in principle, each can be specifically inhibited.^{1,2} Three areas where significant progress has been made are discussed below.

a. Inhibitors of binding

A reasonable starting point for developing pharmacological countermeasures for BoNT intoxication is the use of receptor antagonists to reduce or prevent the binding of toxin to the nerve terminal. Complications with this approach are that each serotype may have a unique receptor and that the receptor appears to be a complex of a polysialoganglioside and protein.⁵⁷ Evidence for involvement of gangliosides is extensive.^{58–60} However, as pointed out by Middlebrook, there are numerous findings that are at variance with the proposal that gangliosides constitute productive BoNT receptors.⁶¹ The most obvious is that polysialogangliosides are distributed throughout the nervous system, while the neurotoxins show specificity for peripheral cholinergic nerve terminals.⁶²

The protein component of the BoNT receptor is less well characterized. In recent years, the synaptic vesicle protein, synaptotagmin has been suggested as a receptor candidate for BoNT serotypes A, B, and E.^{63,64} Sharing of a single protein receptor by these serotypes is inconsistent, however, with the absence of significant competition among them for binding to motor nerve terminals.⁶⁵ A possible explanation for these apparent inconsistencies is that the ganglioside and protein receptor components may be arranged in distinct geometric patterns on the cholinergic nerve terminal, and that each serotype recognizes a unique arrangement. Alternatively, instead of a dual receptor with polysialoganglioside and protein components, the receptor may be a serotype-selective glycoprotein in which the common feature is a sialic acid in the carbohydrate moiety.^{61,66}

Regardless of the actual identity of the BoNT receptor, pronounced antagonism of neurotoxin binding has been achieved with lectins from *Triticum vulgaris* (TVL) and *Limax flavus*.⁶⁶ Pretreatment by these lectins led to a concentration-dependent inhibition in the binding of BoNT/B and TeNT to preparations of rat brain membranes, approaching total inhibition at the highest concentration. The most effective lectins were those that had an affinity for N-acetyl- α -sialic acid; six lectins with specificities for other carbohydrates were ineffective.⁶⁶ In complementary experiments on mouse phrenic nerve-hemidiaphragm preparations, TVL delayed the time-to-block of nerve-elicited muscle contractions with all BoNT serotypes examined.

If one defines the time-to-block in the presence and absence of BoNT antagonist as a protective index, the values for the different serotypes ranged from 1.3 to 1.9. Although the physiological actions of TVL appear less striking than its antagonism of binding, it must be borne in mind that a 10-fold decrease in bound neurotoxin can only be expected to produce a 2-fold slowing in the time-to-block.⁶⁶

The major advantage of the lectins is that they are effective against all clostridial toxin serotypes, although to different degrees. Disadvantages of lectins include (1) they must be administered as pretreatments, and (2) they only slow the time course but do not prevent muscle paralysis. The first limitation is inherent in the basic mechanism of BoNT action; thus no antagonist of surface receptor binding would be expected to be protective once BoNT is internalized and symptoms are manifested.

b. Inhibitors of internalization

Following binding of the clostridial neurotoxins to surface receptors on cholinergic nerve terminals, the toxins undergo internalization prior to reaching their ultimate intracellular targets.^{1,2} Internalization is thought to involve endocytosis of the BoNT-receptor complex, acidification of the resulting endocytotic vesicle, dissociation of the L- and H-chains, and release of the L-chain into the cytosol.^{1,2} The most direct evidence for internalization comes from experiments in which colloidal gold-BoNT conjugates have been visualized inside cholinergic motor axon terminals⁶² and torpedo electric organ synaptosomes.⁶⁷

Internalization affords the next opportunity to ameliorate the toxic actions of BoNT. A number of pharmacological agents have been examined for inhibition of this process with various degrees of success. Simpson demonstrated that pretreatment of phrenic-nerve hemidiaphragm preparations with the lysosomotropic agents ammonium chloride or methylamine hydrochloride delayed the time-to-block of nerve-evoked contractions after exposure to BoNT serotypes A, B, C1, and TeNT.⁶⁷ The maximum protective index was approximately 2, making this strategy somewhat more effective than use of putative receptor blockers.⁶⁶

Incubation of nerve-muscle preparations with ammonium chloride and methylamine hydrochloride was effective if applied before, concurrently, or 10–20 min after toxin exposure. The efficacy of the lysosomotropic agents was reduced rapidly with further delays in addition, such that no effect was observed if they were administered 30–35 min after toxin exposure.

Other candidates examined for inhibiting BoNT-mediated internalization were the antimalarial agents chloroquine and hydroxychloroquine.⁶⁹ These drugs were selected on the basis of interfering with the actions of a large group of peptide hormones and protein toxins that exert their actions following internalization.⁷⁰ The maximal efficacies of the above 4-aminoquinolines were similar to those of ammonium chloride and methylamine hydrochloride, and both groups exhibited a comparable therapeutic window. They differed in that effective concentrations of the 4-aminoquinolines also produced a reversible depression of neuromuscular transmission.

Work on antimalarial agents was extended by Deshpande et al.⁷¹ to identify candidates that did not block neuromuscular transmission, had a longer therapeutic

window, and could delay the time-to-block to a greater degree. These investigators examined a large group of 4- and 8-aminoquinoline compounds as well as analogous acridines for their efficacy against BoNT in mouse diaphragm preparations. The most effective compounds were quinacrine, amodiaquine, and chloroquine; 8-aminoquinolines such as primaquine were ineffective. The highest protective index, 3.9, was obtained with 20 μ M amodiaquine. This was achieved with no deleterious effects on neuromuscular transmission, and thus defines the present limit for inhibitors of internalization. Unfortunately, the therapeutic window could not be extended; no protection was observed if the antimalarial agents were added ≥ 40 min after exposure to BoNT/A or /B.

A somewhat different approach for attempting to prevent or reduce the internalization of BoNT was to treat nerve-muscle preparations with the proton ionophores monensin and nigericin.^{72,73} These ionophores act by depleting vesicular pH gradients, thereby interfering with several stages in the delivery of active L-chain in the cytosol. These ionophores were found approximately as effective as the other inhibitors of internalization. They were more toxic, however, and high concentrations led to a depression of neuromuscular transmission.^{72,73} Toxicity is difficult to avoid with this group of agents since proton gradients are required for a number of cellular reactions such as the synthesis of ATP and filling of synaptic vesicles.

c. Inhibitors of metalloprotease activity

The third area for therapeutic intervention is inhibition of the metalloprotease activity of the BoNT light chains. This approach is potentially the most promising, especially since the crystal structures of BoNT/A²¹ and /B⁷⁴ have been solved, and studies on crystallographic data for the other serotypes are currently in progress. The presence of a zinc binding motif in the L-chain of all clostridial neurotoxins and findings that zinc is required for neurotoxin-mediated proteolysis of SNARE proteins^{17–20} suggest that two classes of potential inhibitors may be effective in antagonizing the toxic actions of BoNT L-chain: transition metal chelators and zinc metalloprotease inhibitors. Simpson et al.⁷⁵ demonstrated that the zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) caused a marked slowing in the time-to-block of nerve evoked muscle contractions when added to phrenic nerve-hemidiaphragm preparations prior to BoNT. The maximum efficacy was equivalent to or greater than that achieved with TVL, ammonium chloride, methylamine hydrochloride, or the antimalarial agents. In common with the above inhibitors, TPEN was effective against all BoNT serotypes examined. In addition, when co-applied with TVL or the lysosomotropic agents, the protection observed with TPEN was approximately additive with that of the former compounds. These results are encouraging since they demonstrate that, in principle, concerted inhibition of the different stages in the production of toxicity is a viable strategy for managing BoNT intoxication.

Sheridan and Deshpande⁷⁶ examined a number of additional chelators on nerve-evoked twitch tensions and concluded that both a high affinity for zinc and membrane permeability are required for antagonism of BoNT. Interestingly, although TPEN works by an entirely different mechanism, it was subject to the same brief

therapeutic window as were the lectins and lysosomotropic agents. This was not entirely unexpected since the deficit in ACh release resulting from BoNT-mediated proteolysis of SNARE proteins cannot be immediately overcome by inhibition of BoNT.

If zinc chelators are administered after substrate proteolysis is complete, functional recovery will involve removal of the cleaved fragments followed by incorporation of intact SNARE, both of which are unlikely to occur during the course of an acute *in vitro* experiment. Moreover, inhibition of BoNT would have to be sustained for perhaps several weeks in order to prevent cleavage of newly synthesized and incorporated SNARE proteins.³⁵

The results with TPEN in the isolated nerve-muscle preparations were sufficiently encouraging to test this chelator for protection against challenge by BoNT; unfortunately, these results were less encouraging. First, TPEN was found to be highly toxic *in vivo*, producing rapid lethality at doses above 20 mg/kg in mice.⁷⁷ Second, at the highest tolerated dose, TPEN only increased survival by 2–3 h following a 20 LD₅₀ challenge of BoNT/A or /B. Toxicity of TPEN was also observed with primary and clonal cells in culture. TPEN concentrations $\geq 10 \mu\text{M}$ produced morphological damage with characteristics of apoptosis.^{78,79}

Studies with ion replacement indicated that chelation of zinc was the proximal cause of cytotoxicity, and examination of a variety of chelators suggested that those with high membrane permeability were especially apt to produce cell death.⁷⁸ Based on these findings, metal chelators may have a limited use in the therapy of botulinum intoxication since the requirements for efficacy against BoNT are the same ones that promote cellular toxicity.

A more promising approach is the development of metalloprotease inhibitors to target the catalytic activity of BoNT L-chain. This endeavor was made possible by the discovery of the zinc metalloprotease activity of the clostridial toxins that began in 1989 when the HEXXH signature sequence of zinc binding proteins was noted in the TeNT L-chain by Jongeneel et al.⁸⁰ Their finding suggested that clostridial neurotoxins possessed zinc-dependent protease activity. During the next 4 years, the SNARE protein substrates and serotype-specific cleavage sites were identified and correlated with intoxication in a series of elegant studies carried out primarily by Montecucco, Schiavo, and colleagues.^{81,82} It is noteworthy that an enzymatic activity for BoNT/A was suspected as far back as 1947 by Guyton and Marshall¹⁴ in their seminal study on botulinum intoxication:

. . . this minute quantity of toxin necessary to produce poisoning, the duration of poisoning and the physical properties of the toxin all tend to characterize the toxin as a destructive enzyme . . .

Early work with zinc metalloprotease inhibitors focused on the well-characterized agents captopril and phosphoramidon. These were found to have little or no inhibitory activity against any BoNT serotype.^{72,83} Phosphoramidon analogs in which Leu-Trp was replaced by Phe-Glu to resemble the cleavage site of synaptobrevin exhibited little increase in inhibitory activity; one analog was slightly more potent and two were significantly less potent than the parent compound.

Using a somewhat different strategy, Schmidt and colleagues made substitutions near the cleavage site of a 17-mer SNAP-25 peptide that was slowly cleaved by BoNT/A.⁸⁴ Substitution of Cys in the P1 or P2 position of the peptide transformed it from substrate to competitive inhibitor. The best compounds had K_i values of 2 μ M. These peptide inhibitors cannot immediately be used as therapeutic agents since they would be unstable *in vivo* and would have difficulty gaining access to the nerve terminal cytosol to inhibit internalized BoNT L-chain. They can serve, however, as templates for synthesis of organic drug candidates.

Adler et al.³² tested a series of isocoumarin compounds that were originally designed as elastase inhibitors. Molecular modeling studies suggested that these compounds may interact favorably with the BoNT/B active site, and several candidates were able to inhibit BoNT/B L-chain activity. The most effective compound in this series was 7-N-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin (ICD 1578), which had an IC_{50} of 28 μ M when tested in a cleavage assay using a 50-mer synaptobrevin peptide. Although the potency of the isocoumarins is somewhat low, they have the advantage of greater stability and higher lipid solubility relative to the peptide inhibitors.

IV. CONCLUSIONS AND FUTURE RESEARCH

Efforts to develop pharmacological inhibitors of BoNT have increased substantially during the last decade. The major focus of the current research is the design and synthesis of specific metalloprotease inhibitors. Most of the ongoing drug discovery efforts were initiated prior to publication of the crystal structure for BoNT and will be aided enormously by the availability of precise structural information. The crystal structure of BoNT/A has been available since 1998²¹ and that of BoNT/B was recently described;⁷⁴ it is reasonable to expect the crystal structures of all BoNT serotypes to be resolved during the next few years.

Results to date indicate that a number of low molecular weight inhibitors and small peptides are effective against BoNT in cell-free *in vitro* systems. Development of safe and effective metalloprotease inhibitors with *in vivo* efficacy will no doubt be difficult. Some of the challenges involve targeting of drugs to the nerve terminal, ensuring their access to the intracellular compartment, and increasing the bioavailability of the drugs to match the duration of the toxin. In addition, different inhibitors may be needed for each serotype, requiring at least four parallel efforts. A more complete characterization of the BoNT receptor and a better understanding of the internalization process will aid in accomplishing these objectives by refining the drug delivery methodologies.

In addition, it may be necessary to accelerate the removal of cleavage products from the nerve terminal⁸⁵ and to introduce non-cleavable SNARE analogs for a more rapid recovery.⁸⁶ The latter is especially relevant for treatment of persistent serotypes such as BoNT/A.³⁵ The progress in understanding the mechanism of action of the BoNT and detailed structural information gained during the last decade suggest that pharmacological treatments for BoNT intoxication will soon be a reality.

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