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What next for botulism vaccine development?

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Botulism is a severe neuroparalytic disease caused by the toxins produced from several *Clostridium* species. Botulinum neurotoxins (BoNTs) cause flaccid paralysis by inducing a blockade at voluntary motor and autonomic cholinergic junctions that, if not treated, can be fatal. Vaccination to elicit protective circulating antibodies that bind, neutralize and clear toxins before they can be internalized and affect cholinergic neurons remains the most effective form of protection against BoNT. A pentavalent BoNT toxoid vaccine administered in the USA under an Investigational New Drug protocol to at-risk workers was discontinued by the CDC in 2011 due to diminished potency and reactogenic effects. Subsequent research efforts have primarily focused on recombinant protein antigens. This review focuses on the development of a recombinant bivalent vaccine (rBV A/B) composed of purified recombinant BoNT/A and BoNT/B receptor-binding domain proteins, as well as presenting a summary of progress and issues associated with alternative vaccines currently being developed against botulism.

Keywords: botulinum neurotoxin • botulism • mucosal vaccine • subunit vaccine • toxoid vaccine • viral vaccine

Botulism is a neuroparalytic disease caused by the neurotoxins produced primarily by the Gram-positive, spore-forming bacterium *Clostridium botulinum* and infrequently by *Clostridium baratii*, *Clostridium butyricum* and *Clostridium argentinense*. Botulism can result from the ingestion of food containing the preformed toxin (food-borne botulism), a toxicoinfection caused by the ingestion or inhalation of spores or bacteria causing local toxin production (infant botulism), growth of *C. botulinum* spores in contaminated wounds accompanied by *in vivo* toxin production (wound botulism) or inhalation of the preformed toxin (inhalational botulism). Naturally occurring botulism in humans is caused by serotypes A, B and E and less frequently by serotype F, while types C, D and G cause illness in birds, cattle and horses. However, the neurotoxins from all seven serotypes are lethal in laboratory animal models, and presumably in humans as well.

The botulinum neurotoxins (BoNTs) are type III bacterial intracellular exotoxins that are among the most lethal protein toxins known [1]. The BoNTs are initially synthesized as 150-kDa protoxins that are proteolytically cleaved into a 50-kDa light chain (LC) and 100-kDa heavy chain (HC) that remain associated by a single disulfide bond. The HC consists of two structurally unique functional domains: the amino-terminal 50-kDa translocation domain (Hn) and the 50-kDa carboxyl-terminal receptor binding domain (Hc or C-fragment). The intoxication process is a three-step pathway mediated by the individual domains of the neurotoxin. The BoNT Hc binds to ectoreceptors on peripheral cholinergic neurons by binding to both ganglioside and protein components of serotype-specific coreceptors. Structural data [2] suggest that the BoNT/A Hc domain initially interacts with ganglioside GT1b to increase toxin concentrations on the cell surface and facilitate subsequent binding to the SV2 protein [3,4] and/or the FGFR3 [5] coreceptor component. Synaptic vesicle membrane proteins synaptotagamin I and II have been likewise identified as the protein receptors for BoNT serotypes B and G, respectively [6,7].

The receptor-bound toxin is internalized by receptor-mediated endocytosis and once within the endosome, the BoNT Hn undergoes a conformational rearrangement that facilitates the formation of a pore within the endosomal membrane. This conformational change is believed to be brought about by the comparatively acidic pH environment of the maturing endosome [8]. However, more recent studies have suggested the endosomal environment is conducive to reducing the repulsive electrostatic interactions between the translocation domain

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and the membrane, potentially contributing to the formation of a transmembrane pore [9]. The LC is translocated from the endosomal lumen through the endosomal pore formed by the Hn and into the cytosol of the affected neuron. The BoNT LC is a zinc-dependent metalloprotease that cleaves the SNARE proteins that are required to form the SNARE complex which mediates the vesicular trafficking of neurotransmitters. The SNARE protein SNAP-25 is cleaved at different sites by BoNT/A, /C and /E, synaptobrevin (also referred to as VAMP), is cleaved at different sites by BoNT/B, /D, /F and /G, and syntaxin is cleaved by BoNT/C [10]. Proteolytic cleavage of the SNARE proteins prevents docking and fusion of synaptic vesicles to plasma membranes and release of acetylcholine into the synaptic cleft of the neuromuscular junction. This effectively blocks neuromuscular communication, which results in the flaccid paralysis characteristic of BoNT intoxication. The time to onset of symptoms, the severity and the duration of the disease is largely dictated by the route of administration, the exposure dose and the serotype, but usually occurs within 72 h [11,12]. The neuroparalytic effects of botulism initially manifest with cranial nerve palsies followed by an acute, afebrile, descending, bilateral flaccid neuromuscular paralysis. Fatalities are most often associated with respiratory failure, but can also be attributed to secondary infections typically associated with prolonged mechanical ventilation.

Upon confirmation of a diagnosis of botulinum intoxication, the recommended therapeutic intervention consists of the administration of botulinum antitoxin and ventilatory assistance for individuals exhibiting respiratory distress. The antitoxin scavenges the BoNT remaining in the bloodstream, preventing it from precipitating further neuronal damage and therefore limiting the progression and severity of the disease. However, effective administration of the antitoxin is limited to a relatively narrow window of opportunity of time and should be given within 24 h of symptomatic onset of the disease [13,14]. BabyBIG® (Massachusetts Public Health Biologic Laboratories, MA, USA and Cangene Corporation, MB, Canada), human immune globulin intravenous (BIG-IV), is derived from the plasma pooled from adults vaccinated with the pentavalent botulinum toxoid vaccine (against serotypes A–E) that displayed high titers of neutralizing antibodies. Being of human origin, BabyBIG negates both the risk of anaphylaxis and the potential for lifelong hypersensitivity associated with equine plasma-derived products. BabyBIG was approved by the US FDA in 2003 and is currently available through the California Infant Botulism Treatment and Prevention Program for use in treating infant botulism from serotypes A and B. BabyBIG has been shown to significantly reduce the period of hospitalization and decrease treatment costs up to US$70,000 per incident [15]. A new heptavalent botulinum antitoxin (HBAT, Cangene Corp.) composed of <2% intact IgG and ≥90% Fab and F(ab')2 immunoglobulin fragments became available on 1 March 2012 through a CDC-sponsored FDA Investigational New Drug (IND) protocol for the treatment of naturally acquired noninfant botulism [16]. Intravenous Fab/F(ab')2 display faster circulatory clearance than IgG [17], which could potentially have clinically relevant consequences with respect to the administration of the HBAT.

The potent, extended activity of BoNT in the neuron has made specific toxin serotypes effective drugs for the treatment of neurological disorders, and in 1998 BoNT/A was licensed by the FDA for the treatment of strabismus and blepharospasms. Since then, BoNT/A and BoNT/B have been successfully employed to treat an ever-increasing variety of neuromuscular disorders and there are currently four FDA-licensed BoNT products used in the US based on serotypes A (Botox® [Allergan, CA, USA], Dysport® [Ipsen Biopharm Ltd, Wrexham, UK] and Xeomin® [Merz Pharma, ON, Canada]) and B (Myobloc® [Solstice Neurosciences, Inc., CA, USA]). Unfortunately, the same properties that make BoNTs effective therapeutic drugs also make them a formidable biological offensive weapon. Based on animal studies, BoNT/A has a lethal human dose (LD_{50}), assuming 70 kg weight, of approximately 0.09–0.15 µg by intravenous administration, 0.7–0.9 µg by inhalation and 70 µg by oral administration [11,18]. The extreme potency of the toxin, its persistence within affected neurons, the need for protracted intensive care and the lack of an effective postintoxication therapeutic intervention has resulted in BoNT being classified as a Tier 1 biological threat agent by the CDC.

Toxoid vaccines

There is currently no FDA-licensed prophylactic product against botulism available in the USA. Some of the earliest successful efforts were traditional toxoid-based vaccines and have been extensively reviewed [19,20]. The most widely distributed BoNT toxoid vaccine was developed by the Michigan Department of Public Health (MDPH) in the 1970s. The MDPH initially produced monovalent botulinum toxoids against strains A (Hall), B (Beans strain), C (Onderstepoort C1d), D (Onderstepoort D6f) and E (Dolman) but manufactured a pentavalent formulation under contract from the US Army. The five monovalent toxoid antigens were mixed based on the concentrations that were shown to confer single-dose protective immunity in guinea pigs against a challenge with 10^5 mouse intraperitoneal 50% lethal doses ($MIPLD_{50}$) of the corresponding BoNT toxin. The pentavalent BoNT toxoid (PBT) contained 10–15% protein formulated with 0.022% formaldehyde, 0.01% thimerosal included as a preservative and 70 mg of aluminum phosphate ($AlPO_4$). The PBT was administered subcutaneously in a 0.5-ml dose at 0, 2, 12 and 26 weeks followed with an annual boost. The MDPH PBT vaccine was administered under CDC IND-161 to workers at risk for exposure to botulinum toxins as well as the US Army Office of Surgeon General IND-3723 for use with military personnel at risk during deployment. However, the PBT vaccine began to exhibit declining immunogenicity in the mid-1990s and by 2004 elicited anti-PBT antibodies in less than 15% of vaccinees [19–21]. As a result of the diminished potency, the CDC announced it would no longer provide investigational PBT after 30 November 2011 [21]; however, IND-3723 still remains active and the PBT could still be used to vaccinate military personnel.

Torii *et al.* reported on a formalin-inactivated tetravalent BoNT toxoid (ABEF) formulated with 0.011% thimerosal, 0.23 mg aluminum and 0.0006% formaldehyde that was administered intramuscularly at 0, 4 or 6 and 20 weeks to 15 volunteers [22]. The

vaccinees initially exhibited substantial neutralization titers ranging from 1.1 to 8.9, 0.6 to 0.8, 0.4 to 3.2 and 0.8 to 1.6 IU/ml to toxin serotypes A, B, E and F, respectively. However, they exhibited a 90% decrease in BoNT-specific neutralizing antibody titers by 9 months, with nearly all antitoxin titers less than 0.1 IU/ml at 12 months.

A purified monovalent botulinum type F toxoid with 10 µg of toxoided protein (residual formaldehyde ≤0.01%) formulated with 0.85 mg of aluminum and 0.01% thimerosal was administered either subcutaneously or intramuscularly to 116 volunteers in a Phase II clinical trial [23]. Participants initially displayed a 90% seroconversion rate at 70 days after vaccination but by 6 months were less than 0.010 IU/ml in most individuals. However, 100% of the vaccinated individuals exhibited ≥0.10 IU/ml after a 12-month booster dose, with antitoxin levels persisting for at least 360 days in most subjects. Other laboratories have reported contemporary studies of BoNT toxoids and report passive immunity in animal models [24–26]; however, these products have not been evaluated in humans, and there is currently no BoNT toxoid vaccine in any FDA clinical trials.

While BoNT toxoids have been demonstrated to elicit protective immunity, large-scale production of a polyvalent BoNT toxoid vaccine would necessitate culturing large amounts of each individual strain of *C. botulinum* and handling of the toxin during the purification and toxoiding process. Because *C. botulinum* are spore-formers, the manufacturing of a botulinum toxoid would require a dedicated, licensed facility and would be cost prohibitive. However, rapid advances in recombinant DNA technology have largely supplanted BoNT toxoid vaccines in favor of recombinant nontoxic protein antigens produced in microbial expression platforms that are amenable to existing large-scale manufacturing technologies.

Recombinant protein antigen Hc vaccines

Some of the first successful attempts at producing a recombinant protein vaccine antigen against botulism were based on the information derived from the development of the structurally similar tetanus toxin (TeNT) Hc vaccines. Fairweather *et al.* reported that a recombinant 50-kDa C-terminal TeNT protein produced in *Escherichia coli* conferred protective immunity in mice against ten mouse LD_{50} of TeNT [27]. Subsequent efforts using codon-optimized genes to address A/T-rich tracts that were potential transcription termination signals, as well as eliminating rare codons, dramatically improved the yield of the recombinant protein to 12.1 g/l in a *Pichia pastoris* yeast expression platform [28].

Dertzbaugh and West described the expression and purification of ten overlapping gene fragments representative of the entire *BoNT/A* gene in *E. coli* [29]. Protective immunity was assessed by inoculating mice four times with 10 µg of the recombinant proteins with a monophosphoryl lipid A (MPL) adjuvant followed by a low-level (two $MIPLD_{99}$) BoNT/A challenge. Only proteins derived from HC fragments ($H^{455–661}$ and $H^{1150–1289}$) provided protection. Recombinant proteins encoding the complete BoNT/A Hc domain and the individual 25-kDa Hc-N and Hc-C subdomains purified from *E. coli* were evaluated [30]. Mice given a single inoculation of 10 µg of BoNT/A Hc were completely protected against 10^3 $MIPLD_{50}$ of BoNT/A, while the Hc-N and Hc-C antigens, individually or when combined, provided no immunity. However, three inoculations of 10 µg of Hc-C and Hc-N provided 100 and 30% protection, respectively, against a challenge of 10^5 $MIPLD_{50}$ of BoNT/A. These studies suggested that higher levels of protective immunity might be afforded by recombinant antigens bearing structurally accurate antigenic determinants found in complete protein domains. This was confirmed in a 1995 study that reported that three inoculations of 1 µg of a purified recombinant BoNT/A Hc produced in *E. coli,* adjuvanted with alum and delivered subcutaneously, completely protected mice against an intraperitoneal challenge of 10^6 mouse LD_{50} of BoNT/A [31].

Codon-optimized genes encoding the BoNT Hc domains for serotypes A–F have been expressed in *P. pastoris* and the highly purified recombinant antigens elicited significant protection against lethal botulinum toxin challenges in both murine (Table 1) [19,20] and nonhuman primate models [32,33]. Three doses of 1 µg of a recombinant BoNT/B Hc adsorbed to 0.2% Alhydrogel® and administered intramuscularly to rhesus monkeys at 0, 4 and 8 weeks provided complete protection against an aerosol challenge of approximately 61–63 LD_{50} [32]. A combination vaccine consisting of four separate recombinant protein antigens for anthrax (rPA), botulism (5 µg BoNT/A Hc), plague (rF1-V) and staphylococcal toxic shock (STEBVax) was administered to rhesus macaques at 0, 4 and 8 weeks [33]. The inoculations were administered just beneath the skin using specially designed microneedles designed to prevent mixing of the antigens. The BoNT/A Hc antigen component of the vaccine was sufficient to elicit complete protective immunity against an aerosol challenge of 50 LD_{50} of the homologous toxin.

Recombinant bivalent BoNT/A1 & B1 Hc vaccine

A recombinant bivalent Hc vaccine against serotypes A1 and B1 (rBV A/B) produced in *P. pastoris* was granted a Fast Track designation by the FDA in 2004. A preclinical safety assessment was performed to determine if the route of administration, dosage and method of administration of the rBV A/B vaccine adsorbed to 0.2% Alhydrogel would be reasonably safe in humans [34]. The study, conducted on CD-1 mice and New Zealand white rabbits, indicated the vaccine was well tolerated and produced no apparent systemic effects. A specialized behavioral neurotoxicity study to address potential safety concerns that the Hc antigen could bind neurons and exert neurological effects indicated no adverse events attributed to the vaccine. The safety and immunogenicity of rBV A/B was evaluated in a Phase I clinical study conducted by Dynport Vaccine Company (MD, USA) under contract from the Department of Defense Joint Vaccine Acquisition Program [35]. Equal proportions of BoNT/A and /B Hc antigens adsorbed to 0.2% (w/v) Alhydrogel were administered to 33 human volunteers in three ascending doses (10, 20 and 40 µg) at 0 and 28 days. The two highest doses were sufficient to elicit sustained BoNT/A and /B neutralizing antibody concentrations (NAC) above the lower

Table 1. Protective efficacy and immunological response of recombinant botulinum neurotoxin Hc antigens in mice.

Antigen	Doses (n)†	% survival against challenge‡ (10^5 $MIPLD_{50}$)	Serum neutralization titers (IU/ml)
BoNT/A Hc	2	100	21.0
	3	100	79.2
BoNT/B Hc	2	100	28.7
	3	100	28.7
BoNT/C1 Hc	2	100	284
	3	100	451
BoNT/D Hc	3	100	ND
BoNT/E Hc	2	80	1.4
	3	100	48.2
BoNT/F Hc	2	100	23.7
	3	100	70.7

†All groups received 1 µg of the Hc vaccine except BoNT/D Hc, which received 5 µg of the Hc vaccine.
‡Ten mice per group.
BoNT: Botulinum neurotoxin; $MIPLD_{50}$: Mouse intraperitoneal 50% lethal dose; ND: Not determined.
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measurement limit. Geometric mean antibody concentrations, as well as the proportion of volunteers seroconverting, increased for more than 3 months after the second vaccine injection [36]. The Phase I clinical study also included an assessment of the immunogenicity and safety of the Alhydrogel adjuvant [37]. Volunteers were inoculated intramuscularly with 0.5 ml of the rBV A/B vaccine containing 40 µg of the protein antigen formulated in buffer with or without 0.2% Alhydrogel adjuvant on days 0 and 28. The rBV A/B vaccine was safe and well tolerated when either adsorbed or devoid of adjuvant.

However, administration of the vaccine without an adjuvant elicited a poor immunogenic response, particularly for the BoNT/B Hc component. The rBV A/B formulated with Alhydrogel stimulated the production of serotype-specific neutralizing antibodies in the majority of the volunteers. An increase in both the geometric mean NACs as well as the percentage of the recipients seroconverting was observed for at least 3 months after the second inoculation.

The protective capacity of human BoNT/A1 and BoNT/B1 neutralizing antibodies from clinical volunteers given the rBV A/B vaccine was determined in a guinea pig passive vaccination model [38]. Purified human immunoglobin from volunteers was used to passively vaccinate guinea pigs intraperitoneally to NAC levels of 0.06 U/ml for BoNT/A and 0.014 U/ml for BoNT/B, the lowest NAC levels that can be accurately determined by the mouse neutralization assay. All of the guinea pigs survived a challenge with 10^2 $MIPLD_{50}$ of BoNT/A1 or BoNT/B1 and displayed no clinical signs of botulism 2 weeks postchallenge, demonstrating neutralizing antibody as a surrogate marker for clinical efficacy. A nonhuman primate animal model was developed for the evaluation of the rBV A/B efficacy studies to understand and document the pathophysiology and dose–response relationships after BoNT aerosol exposures [39]. This study determined the inhaled LD_{50} and 50% lethal exposure concentrations relative to time (LCt_{50}) for BoNT/A1 and BoNT/B1 in rhesus macaques using well-characterized challenge material. The rhesus macaque aerosol challenge model will be used for future evaluations of rBV A/B efficacy against inhalational BoNT/A1 and BoNT/B1 intoxication.

The rBV A/B Phase II clinical trial was a randomized, double-blind study to evaluate the safety and immunogenicity and antibody kinetics of the vaccine [101]. Approximately 40 µg of the rBV A/B vaccine was administered to 165 human volunteers at either 0, 28 and 182 days or 0, 56 and 182 days. The volunteers were monitored for up to 12 months after the final vaccination and while the study was completed in December 2010, the final data analysis is not yet completed [35].

Multiple domain BoNT vaccines

The success of the Hc vaccines has resulted in the production and assessment of recombinant protein antigens representing other BoNT domains as well as enzymatically neutralized holoproteins to explore any potential additive or synergistic increases in protective immunity. The contribution of the individual domains was clarified in a series of studies that characterized the recognition profiles of anti-BoNT antibodies from cervical distonia patients who had become immunoresistant to BoNT/A and /B therapy [40–43]. Antisera from these patients were analyzed for their ability to bind to a comprehensive set of synthetic peptides that collectively represent the entire neurotoxin protein. Blocking antibodies were found on both the LC and HC and this information could potentially serve as a means for both epitope-specific management of the antibody responses and for the design of synthetic peptide vaccines. Although there have been reports of potent, high-affinity neutralizing antibodies against the BoNT LC regions with binding affinities in the picomolar range [44], the catalytic domain has not been found to be a potent immunogen. Recombinant catalytic domains of BoNT/A, /B and /C have been evaluated as potential vaccine candidates and found to provide lower levels of protective immunity than other domains of the toxin [45–48].

Recombinant catalytic and translocation domain (LC–Hn) proteins have also been evaluated as potential vaccine candidates [46,48,49]. Single-dose potency assays with a BoNT/A1 LC–Hn protein indicated an ED_{50} of 89 ng against BoNT/A1 toxin, 209 ng against BoNT/A2 subtype (FRI-Honey strain) and 192 µg against BoNT/A3 (Loch Maree strain) after a single-dose vaccination (Table 2) [48]. A combination of the BoNT/A1 LC–Hn and Hc vaccine antigens displayed an additive effect that provided ED_{50} values of 14, 254, 739 ng against BoNT/A1, /A2 and /A3, respectively (Table 2). Shone *et al.* reported that a recombinant BoNT/A1 LC–Hn provided ED_{50} values of 49 ng against BoNT/A1, 280 ng for BoNT/A2 and 2.2 µg for BoNT/A3 [49]. Shone *et al.* also reported that a single inoculation of BoNT/B1 LC–Hn provided protection against BoNT/B1 (ED_{50} 140 ng) and BoNT/B4 (ED_{50} 360 ng). While both /A1 and /B1

LC–Hn protein antigens were adsorbed to Alhydrogel adjuvant, one BoNT/A LC–Hn study required formaldehyde crosslinking for full protective immunity [49].

Recombinant BoNT HC proteins, comprised of the Hn and Hc domains, have been proposed as a potential vehicle for the intracellular targeting of therapeutics [50,51], but have proven difficult to produce due to their highly insoluble nature [52,53] and often require an additional moiety to promote solubility [50,51]. This would make downstream manufacturing processes difficult and expensive to develop. Despite difficulties reported with yield and solubility, recombinant HC protein antigens produced in *E. coli*, purified using a polyhistidine tag and adjuvanted onto Alhydrogel, have been demonstrated to effectively elicit protective immunity. A 2005 study reported that mice inoculated intraperitoneally with 2.5 µg of rBoNT/A1, /A2 or /E1 adsorbed to Alhydrogel were fully protected against an intraperitoneal challenge of up to 10^5 LD_{50} of the parental toxin [54]. While the subtype vaccines were fully cross protective against both /A1 and /A2 toxins, no cross-serotype protection was observed. Henkel *et al.* reported that mice given three intraperitoneal inoculations with 1 µg of rBoNT/A1, /A2, /A3 or /A4 HC were completely protected from a challenge of 10^3 $MIPLD_{50}$ of BoNT/A1, /A2 or /A3 and a subsequent challenge of 10^4 $MIPLD_{50}$ of the same toxins given 4 days later [55]. The same study reported that mice given two inoculations of 1.0 µg of the rBoNT/A1 or /A2 were largely protected against a 10^3 intraperitoneal challenge of BoNT/A1 or /A2 while the same dosing schedule of 0.1 µg provided only partial protection to the same challenge. Mice given three intraperitoneal inoculations of a recombinant heptavalent (BoNT/A-G) HC vaccine containing 1 µg of each seven antigens, followed by a boost without adjuvant, provided complete protection from an intraperitoneal challenge of 10^3 LD_{50} of the homologous toxin [56]. Survivors were protected from a 10^5 challenge of the homologous toxin.

Catalytically inactive BoNTs

Catalytically inactive recombinant BoNT holoproteins (ciBoNTs) have also been promoted as neuronal targeting vehicles for delivering intracellular payloads of therapeutic countermeasures [57–59]; however, these proteins have also been shown to be effective vaccine candidates in unrelated studies [45,48]. A recombinant ciBoNT/C1 with LC active site mutations $H^{229}G$, $E^{230}T$ and $H^{223}N$ was expressed in *E. coli* and purified using a 6X histidine tag [45]. Approximately 4 µg of the recombinant protein was administered to mice either per os (p.o.; using an intragastric feeding needle) at 0, 14, 28 and 42 days and also by a parallel subcutaneous vaccination schedule using 2 µg of the antigen. Both routes of administration provided complete protection against 10^4 $MIPLD_{50}$ of BoNT/C1. Pier *et al.* reported the production of a recombinant inactive BoNT/A1 with $R^{363}A$ and $Y^{365}F$ amino acid mutations produced in *C. botulinum* strain LNT01 and purified with a 6X histidine tag [60]. Mice inoculated at 0, 14 and 28 days with 1 µg of the recombinant protein adsorbed with an equal amount of Alhydrogel were completely protected against a challenge of 10^4 $MIPLD_{50}$ of BoNT/A1. While these results indicated efficacy against homologous toxin challenges, neither of these studies reported challenges with any BoNT subtypes. Webb *et al.* reported that a single inoculation of a ciBoNT/A with three mutations in the LC active site ($H^{223}A$, E^{224} A and $H^{227}A$) produced in *P. pastoris* and purified to homogeneity provided protection not only against the homologous BoNT/A1 toxin, but against dissimilar subtypes A2 and A3 as well (Table 2) [48]. Furthermore, the ciBoNT/A1 was significantly more potent than the Hc antigen against the /A2 or /A3 toxin subtypes (Table 2). The ciBoNT/A HP was also found to be significantly more potent against the A3 subtype than either the LC–Hn or Hc vaccine. The /A1 and /A3 toxins display an 84.6% heterogeneity at the amino acid level and so the combined antibody response afforded from all three domains of the ciBoNT/A may account for its increased levels of protection against the A3 subtype. While a single dose of ciBoNT/A antigen elicited higher protective immunity when compared with the BoNT/A Hc antigen, comparable protective immunity was observed when multiple vaccinations were administered in a mouse efficacy study [48]. Mice given three intramuscular inoculations of 1 µg of ciBoNT/A1 or /A1 Hc at 0, 4 and 8 weeks showed similar protection against a challenge

Table 2. Single-dose potency bioassay results showing the ED_{50} values and the associated 95% fiducial limits against a 10^3 $MIPLD_{50}$ challenge of the indicated toxin.

Antigen	BoNT/A1	BoNT/A2	BoNT/A3	Ref.
ciBoNT/A1	18 ng	132 ng	144 ng	[48]
	14–23 ng†	75–220 ng†	2 ng–1 µg†	
BoNT/A1 Hc	52 ng	6 µg	18 µg	[48]
	28–66 ng†	4–11 ng†	5–36 µg†	
BoNT/A1 LC–Hn	89 ng	209 ng	192 ng†	[48]
	46–163 ng†	39–803 ng†		
	49 ng‡	280 ng‡	2.2 µg‡	[49]
	ND†	ND†	ND†	
BoNT/A1 LC–Hn+Hc	14 ng	254 ng	739 ng	[48]
	3–26 µg†	60 ng–1 µg†	87 ng–38 µg†	
Antigen	**BoNT/B1**	**BoNT/B4**		**Ref.**
BoNT/B1 LC–Hn	140 ng‡	360 ng‡		[49]
	ND†	ND†		

†95% fiducial limits.
‡Indicates that the toxin challenge was performed 28 days postvaccination. All other challenges were carried out 21 days postvaccination.
BoNT: Botulinum neurotoxin; ciBoNT: Catalytically inactive recombinant botulinum neurotoxin holoprotein; ED_{50}: Median effective dose; $MIPLD_{50}$: Mouse intraperitoneal 50% lethal dose; ND: Not determined.
Adapted with permission from [48].

of 10^5 $MIPLD_{50}$ of BoNT/A1 or A2 toxins or 10^4 $MIPLD_{50}$ of A3 toxin (lower production yields of BoNT/A3 would not permit a challenge level of 10^5 MLD_{50}).

The ciBoNT vaccines could also be used to address two potential issues with the current Hc vaccines: LC–Hn domains with engineered alternative targeting domains and BoNT subtypes with significant amino acid heterogeneity. BoNT LC–Hn with alternative receptor binding domains have been suggested for a variety of medical therapeutic applications [61–63]. Chemically conjugated alternative receptor binding domains have been shown to retarget the LC–Hn to different types of cells and interfere with vesicular trafficking [64–66]. Most recently, a recombinant genetic fusion consisting of a BoNT/D LC–Hn with a GH-releasing hormone peptide ligand domain produced in *E. coli* was shown to target pituitary somatotrophic cells and reduce circulating levels of IGF-1 [67]. While these chimeras may represent a new class of therapeutic drugs, the lack of a native receptor-binding domain would render the current Hc-based vaccines ineffective in the event that the retargeted LC–Hn proteins were ever employed for malicious purposes. A recombinant ciBoNT holoprotein vaccine would have the advantage of eliciting protective antibodies from all three of the native domains, and so would most likely be an effective vaccine in such a scenario. However, the converse argument is that new medical therapies based on the delivery of an active LC–Hn domain by an alternative receptor binding domain would not be negatively impacted by vaccination with BoNT Hc-based protein antigens.

The recombinant BoNT Hc antigens have repeatedly been demonstrated to elicit protective immunity against significant challenges with parental toxins. However, there have been an increasing number of unique BoNT subtypes with small to significant variations in the amino acid sequence reported in the literature [68–71]. The variations observed in the amino acid sequence have been demonstrated to impact the binding and protection of neutralizing antibodies [67] and so raises concerns that they may prove problematic to the development of both vaccines and therapeutic treatments against dissimilar subtypes.

Although the ciBoNTs are excellent vaccine candidates against heterologous toxin subtypes, the development of this class of vaccines within the USA has been hampered by select agent regulatory constraints. In response to a petition from investigators at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) to obtain an exemption for the production of ciBoNT/A in a biosafety level-2 laboratory, the CDC Division of Select Agents and Toxins (DSAT) ruled in May 2010 that while the recombinant proteins might be used without restriction, the DNA encoding the ciBoNTs was to be considered a biological select agent or toxin (BSAT) and subject to select agent restrictions [72,102].

Virally vectored vaccines

The use of replication-deficient viral systems to produce vaccine protein immunogens *in vivo* was first documented almost 30 years ago when a recombinant vaccinia virus (VACV) expressing a hepatitis B surface antigen was shown to produce protective immunity to hepatitis B in chimpanzees [73]. Despite an encouraging start and substantial efforts at development, there are very few licensed viral vector vaccines due to the difficulty of licensure, and issues associated with large-scale production and stability. Because the BoNT Hc has been produced as a stable recombinant protein and shown to be an excellent immunogen, it has been assessed in a number of different promising viral delivery platforms (Table 3).

A human codon-optimized BoNT/C Hc in a recombinant attenuated human serotype 5 adenoviral (AV) vector under the control of a human cytomegalovirus promoter was used to vaccinate mice intramuscularly with either 10^5, 10^6 or 2×10^7 PFU [74]. The parental inoculations were sufficient to provide 12, 75 and 100% protection, respectively, against a challenge of 10^2 $MIPLD_{50}$ of the homologous toxin. In addition, it was shown that mice infected intranasal (in.) with 2×10^7 wild-type human AV and subsequently inoculated intramuscularly with 2×10^7 PFU of the AV vectored BoNT/C Hc vaccine were afforded the same level of protection, indicating pre-existing immunity to the AV vector did not diminish the efficacy of the vaccine. A follow-up study reported that mice given a single in. inoculation with 10^5, 10^6 or 2×10^7 PFU of the adenoviral BoNT/C Hc vaccine elicited a robust IgG, IgG1 and IgG2 response and were protected at 50, 92 and 100%, respectively, against a challenge of 10^2 $MIPLD_{50}$ of the homolgous toxin [75]. A single in. inoculation of 2×10^7 PFU provided protection against a challenge of 10^4 but the protection dropped to 17% against a 10^5 challenge. The protective immunity was shown to persist for at least 27 weeks, as indicated by mice surviving a subsequent challenge of 100 $MIPLD_{50}$.

Yu *et al.* compared the BoNT/A Hc antigen delivered as a Semliki Forest virus (SFV) viral DNA replicon or a conventional DNA vaccine [76]. Mice given four intramuscular inoculations with 30 µg of the SFV viral replicon at 0, 2, 4 and 6 weeks were protected against a challenge of 10^3 $MIPLD_{50}$, while the same dosing schedule with the DNA vaccine alone provided only 50% protection at the same challenge level. A follow-up study compared both BoNT/B Hc and a bivalent A/B Hc when delivered as a conventional DNA vaccine, a SFV DNA replicon or recombinant SFV viral replicon particles (VRPs) [77]. Four intramuscular doses of 10 µg of the SFV DNA replicon provided complete protection against a 10^4 $MIPLD_{50}$ challenge of BoNT/B, while the DNA vaccine alone provided 50% protection with a 10^3 $MIPLD_{50}$ challenge. The same dosing schedule of the dual expression bivalent A/B DNA replicon afforded 87% protection against a challenge of 10^4 $MIPLD_{50}$ of a mixture of BoNT/A and /B toxins. Three subcutaneous inoculations of 5×10^6 PFU of the recombinant bivalent VRP provided 75% protection against 10^4 $MIPLD_{50}$ of the same mixed toxins. Mustafa *et al.* reported that mice given two intramuscular inoculations of 10 µg of a β-propiolactone inactivated rabies virus (RABV) virions expressing a serotype A Hc were protected against a challenge of 10^3 $MIPLD_{50}$ of BoNT/A [78].

Mucosal vaccines

Ravichandran *et al.* reported that 20 µg each of recombinant BoNT/A, /B and /E Hc antigens produced in *E. coli* and admixed with a 5% w/v vitamin E TPGS (D-α tocopheryl polyethylene

glycol 1000 succinate) excipient administered in. at 0, 2 and 4 weeks was sufficient to provide protection for at least 1 week against either an in. or intraperitoneal 10^4 $MIPLD_{50}$ challenge with both the parental toxin and with a polyvalent challenge of 3×10^4 $MIPLD_{50}$ cumulative dose of all three parental toxins [79]. However, the same study reported survival of greater than 1 week against the same challenge level using a parental inoculation of a trivalent A/B/E Hc (20 µg each) adsorbed with alum and administered intramuscularly followed by two in. inoculations coadministered with vitamin E TPGS. A recombinant adenovirus fiber 2 (AdF2) mucosal targeting factor fused with a BoNT/A Hc trefoil domain consisting of amino acids 864–1295 (AHcβtre) produced in *P. pastoris* was made in an effort to enhance binding in the mucosal epithelium [80]. Four in. inoculations of 50 µg of the AdF2–AHcβtre or 25 µg of the AHcβtre alone, each formulated with 2 µg of cholera toxin (CT) immunostimulant, provided complete protection against 2×10^5 $MIPLD_{50}$ of BoNT/A. However, three in. inoculations of the same amounts of the AdF2–AHcβtre and AHcβtre antigens delivered without the CT failed to provide any protection against a lower challenge of 2×10^3 $MIPLD_{50}$. In a follow-up study, the BoNT/A AdF2–AHcβtre fusion protein described above was administered by sublingual inoculation route as an alternative mucosal administration route [81]. Mice were given five sublingual inoculations with either 25 µg of the AHcβtre or 50 µg of AdF2–AHcβtre, both coadministered with 2 µg of CT, and challenged with 10^3 or 5×10^3 $MIPLD_{50}$ of BoNT/A. Mice inoculated with AdF2–AHcβtre + CT were afforded 100% protection against the higher challenge dose while the AHcβtre + CT provided only 50% protection against the lower challenge dose. However, the AdF2–Hcβtre administered without CT protected only 20% of the mice at the 10^3 $MIPLD_{50}$ challenge dose, indicating the CT is required for the BoNT antigen to elicit protective immunity in this vaccine model. A separate study was performed using the same dosing regimen but with a fusion consisting of the AdF2 and a BoNT/B Hcβtre consisting of amino acid residues 786–1291 [82]. Mice covaccinated in. with the AdF2–BHcβtre plus CT showed a significant increase in sIgA in the nasal mucosa as well as serum IgG1 and IgG2a compared with mice inoculated without the CT. However, no direct toxin challenges were performed and the serum neutralization studies showed protection against only a low-level toxin challenge of 2 $MIPLD_{50}$ of BoNT/B.

Yuki *et al.* reported that four inoculations of 500 µg of a recombinant BoNT/A Hc produced in *E. coli* and delivered in. to cynomolgus macaques conferred protection against a systemic challenge of 25 µg/kg (2.7×10^6 LD_{50} as calculated from the neutralizing capacity of serum from vaccinated macaques) [83]. This study also utilized *in vivo* quantitative imaging and whole-body imaging of nasally administered ^{18}F-BoNTHc/A in mice as well as full cranial imaging of macaques using a combination of MRI and PET scanning technologies. Despite the differences in the surface area of the nasal cavity containing the olfactory epithelium, 70–80% in mice and approximately 10% in nonhuman primates, neither animal model showed evidence of deposition of the ^{18}F-BoNTHc/A in the cerebrum or olfactory bulb, indicating no perfusion into the CNS occurred via in. administration. However, the total amount of antigen delivered was 2 mg, over 130–600-times the amount used to elicit protection in other nonhuman primate studies when the Hc antigens was administered intramuscularly [32,33].

Transgenic rice, made with RNAi knockouts of endogenous seed storage proteins to allow for greater accumulation of recombinant proteins, were used to express BoNT/A Hc, denoted MucoRice-BoHc, for use as a mucosal vaccine [84]. Mice were administered three in. inoculations with 100 µg of purified MucoRice-BoHc, BoNT/A Hc produced in *E. coli*, 25 µg of MucoRice-BoHc, with or without CT, or MucoRice-BoHc with 10 µg of mCTA/LTB (mutant CT E112K subunit A with *E. coli* heat-labile enterotoxin pentameric B subunit). Mice inoculated with 100 µg of MucoRice-BoHc or *E. coli* BoNT/A Hc provided 80 and 60% protection against a 1.1×10^4 $MIPLD_{50}$ of BoNT/A. Mice inoculated with MucoRice–BoHc with either CT or mCTA/LTB all survived a 5.5×10^4 $MIPLD_{50}$ challenge while 25 µg of MucoRice–BoHc without an immunostimulatory agent provided no protection.

Table 3. Botulinum neurotoxin vaccine viral vaccine platforms.

Antigen	Viral platform	ROI/dose	Challenge†/survival (%)	Ref.
BoNT/C Hc	HAV	im. 2×10^7 PFU	10^2/100	[74]
BoNT/C Hc	HAV	in. 2×10^7 PFU	10^4/100	[75]
BoNT/A Hc	SFV replicon	im. 30 µg	10^3/100	[76]
BoNT/B Hc	SFV replicon	im. 10 µg	10^4/100	[77]
BoNT/A-/B	SFV replicon	im. 10 µg	$10^{4‡}$/100	[77]
BoNT/A Hc	SFV VRP	sc. 5×10^6 PFU	10^3/100	[77]
BoNT/B Hc	SFV VRP	sc. 5×10^6 PFU	10^3/100	[77]
BoNT/A-/B Hc	SFV VRP	sc. 5×10^6 PFU	10^4/87	[77]
BoNT/A Hc	RABV virions	im. 10 µg	10^3/100	[78]

†Toxin challenge is given in $MIPLD_{50}$ of homologous toxin.
‡Toxin challenge performed using combination of equal amounts of BoNT/A and BoNT/B toxin.
BoNT: Botulinum neurotoxin; HAV: Human adenovirus; im.: Intramuscular; in.: Intranasal; $MIPLD_{50}$: Mouse intraperitoneal 50% lethal dose; RABV: Rabies virus; ROI: Route of administration; sc.: Subcutaneous; SFV: Semliki Forest virus; VRP: Viral replicon particle.

Immune-targeted vaccines

Coadminstration or genetic fusions consisting of the agent-specific protein antigen and a costimulatory molecule designed to target and enhance a specific immunological response has been shown to provide an enhanced protective immunity in some vaccine models [85–87]. Li *et al.* described the construction of a SFV plasmid DNA replicon expressing a BoNT/A Hc gene fusion with a murine GM-CSF designed to increase the numbers and activity of APCs to elicit an enhanced T-cell response [88]. Mice given three

intramuscular inoculations of 30 µg of the BoNT/A Hc-GM-SCF with an $AlPO_4$ adjuvant displayed 75% protection against a challenge of 10^4 $MIPLD_{50}$, while mice inoculated without adjuvant were afforded only 50% protection. White *et al.* described the expression of a codon-optimized BoNT/A Hc expressed as fusion with a murine IgG2a Fc region that binds Fcγ receptors on the surface of dendritic cells in an effort to facilitate antigen processing and B-cell response [89]. The recombinant protein was secreted in a baculovirus expression platform and purified using a polyhistidine affinity tag. An *in vitro* T-cell proliferation assay demonstrated a 3.8-fold-higher T-cell proliferation than was seen when APCs were loaded with Hc antigen alone. Serum from mice given a single subcutaneous inoculation of 7.4 pmol of the recombinant protein with a monophosphoryl lipid A adjuvant was sufficient to completely neutralize 0.5 pM BoNT/A in a rat spinal cord cell SNAP-25 cleavage assay.

Expert commentary

The nefarious use of BoNT as a biowarfare or bioterrorist weapon has been a government concern for many decades. Significant investments have been made by the Department of Defense to develop safe and efficacious vaccines against the toxins with the goal of FDA licensure. Although widespread use of a botulism vaccine is not envisioned due to the therapeutic utility of the toxin for treating numerous debilitating neurological disorders, the need for a vaccine exists to protect at-risk workers from occupational exposure to the toxin and for a select population of the military who may require it. To this end, chemically inactivated toxoids represented the first successful attempt at eliciting protective immunity against botulinum toxins. However, the high cost of manufacturing chemically inactivated BoNT toxoids prompted investigations into alternative approaches. Recombinant protein antigens representing one or more of the three individual BoNT toxin domains have been evaluated as potential vaccine candidates and the Hc domain has emerged as the lead candidate for several reasons. The recombinant BoNT Hc protein antigens can be produced in high quantities using existing mammalian or microbial expression platforms, can be purified to apparent homogeneity by using conventional chromatography (e.g., ion-exchange and hydrophobic resins), can be combined with other serotype Hc antigens in a stable polyvalent formulation, are safe and well tolerated in animal models and humans and can elicit protective immunity against significant toxin challenges after two to three vaccinations.

The success of the recombinant BoNT Hc-based vaccine prompted their incorporation into a variety of other vaccine production and delivery platforms. DNA and viral-vectored BoNT Hc vaccines have been used to elicit protective immunity in mice, but have not been assessed in other animal models. Historically, DNA vaccines initially showed great promise in small animal studies but were poorly immunogenic in humans and displayed a low T-cell response [90]. Second-generation DNA vaccines have incorporated changes in antigen design, formulation, molecular adjuvants and delivery methods in an effort to enhance the immune response, but these vaccines are still largely in development and testing phases [90]. The use of attenuated viral production and delivery systems has been shown to elicit significant protective immunity against BoNTs, both as viral replicons and VRPs when delivered intramuscularly and/or in. However, there are few available studies and this work has been carried out exclusively in murine models. Mucosal vaccines have likewise elicited protective immunity against BoNT, but many of the published studies require CT as a costimulate. There could be associated safety concerns as CT has been shown to cross the blood–brain barrier, target the olfactory nerves and epithelium and be transported to the olfactory bulb, potentially promoting uptake of vaccine proteins into olfactory neurons [91]. Botulism is a toxemia and parenteral vaccination elicits protective immunity against an aerosol exposure to BoNT. Mucosal vaccination is not required for prevention of botulism resulting from exposure of the toxin through the respiratory system.

Recombinant BoNT protein antigens having properly folded discontinuous epitopes are the critical determinants for eliciting superior levels of toxin-neutralizing antibodies, emphasizing the importance of the B-cell response in adaptive immunity. Antibodies elicited from vaccination with BoNT antigens are accepted surrogate markers for clinical efficacy, enabling FDA licensure under the 'Animal Rule'. Ongoing and future evaluation of recombinant BoNT subunit and ciBoNT vaccine candidates must include extensive research, development, test and evaluation necessary to comply with regulatory expectations and requirements for a specified biotechnology drug product and adequate production yields must be attainable.

Five-year view

The rBV A/B (*P. pastoris*) will enter Phase III clinical trials in 2013. Pivotal animal efficacy studies will be conducted in 2013 and the manufacturing process will have completed its final validation. FDA licensure of the bivalent A/B vaccine is anticipated in 2016. The next milestone to be achieved, and certainly attainable within the next 5 years with appropriate resourcing, is the cGMP-manufacturing and clinical evaluation of a trivalent vaccine to increase coverage against toxin serotypes C, E and F.

Disclosure

The views and opinions expressed in this paper are those of the author(s) and do not reflect official policy or position of the Department of the Army, Department of Defense or the US Government.

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Key issues

- Botulinum neurotoxins (BoNTs) are Tier 1 threat agents that have the potential to be employed as a biowarfare or bioterrorist weapon.
- A pentavalent BoNT toxoid vaccine manufactured in the 1970s and administered under CDC Investigational New Drug (IND)-161 has been discontinued by the CDC due to diminished potency and an increase in moderate local reactions related to annual booster doses.
- DNA and viral-vectored BoNT Hc vaccines have been used to elicit protective immunity in mice but have not been assessed in other animal models.
- Mucosal vaccines have shown promise but their success is often observed only when coadministered with cholera toxin as an immunostimulatory. This raises concerns about the transport of the vaccine antigen into neural tissues. Mucosal immunity is not required for protection against an aerosol exposure to botulinum toxin.
- Immune-targeted BoNT vaccines are a relatively new technology and have not been well evaluated.
- The recombinant bivalent vaccine (rBV A/B; *Pichia pastoris*) vaccine completed Phase II clinical trials in December 2010 and will enter Phase III clinical trials in 2013. FDA licensure is anticipated in 2016.
- A cGMP lot of a trivalent C, E and F Hc vaccine requires manufacturing and evaluation in a Phase I clinical trial.

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