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Vaccines against botulism

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The clostridial neurotoxins (CNTs) are the most toxic proteins for humans and include botulinum neurotoxins (BoNT) and tetanus neurotoxin (TeNT). CNT neurotropism is based upon the preferred binding and entry into neurons and specific cleavage of neuronal SNARE proteins. While chemically inactive TeNT toxoid remains an effect vaccine, the current pentavalent vaccine against botulism is in limited supply. Recent advances have facilitated the development of the next generation of BoNT vaccines, utilizing non-catalytic full-length BoNT or a subunit vaccine composed of the receptor binding domain of BoNT as immunogens. This review describes the issues and progress towards the production of a vaccine against botulism that will be effective against natural BoNT variants.

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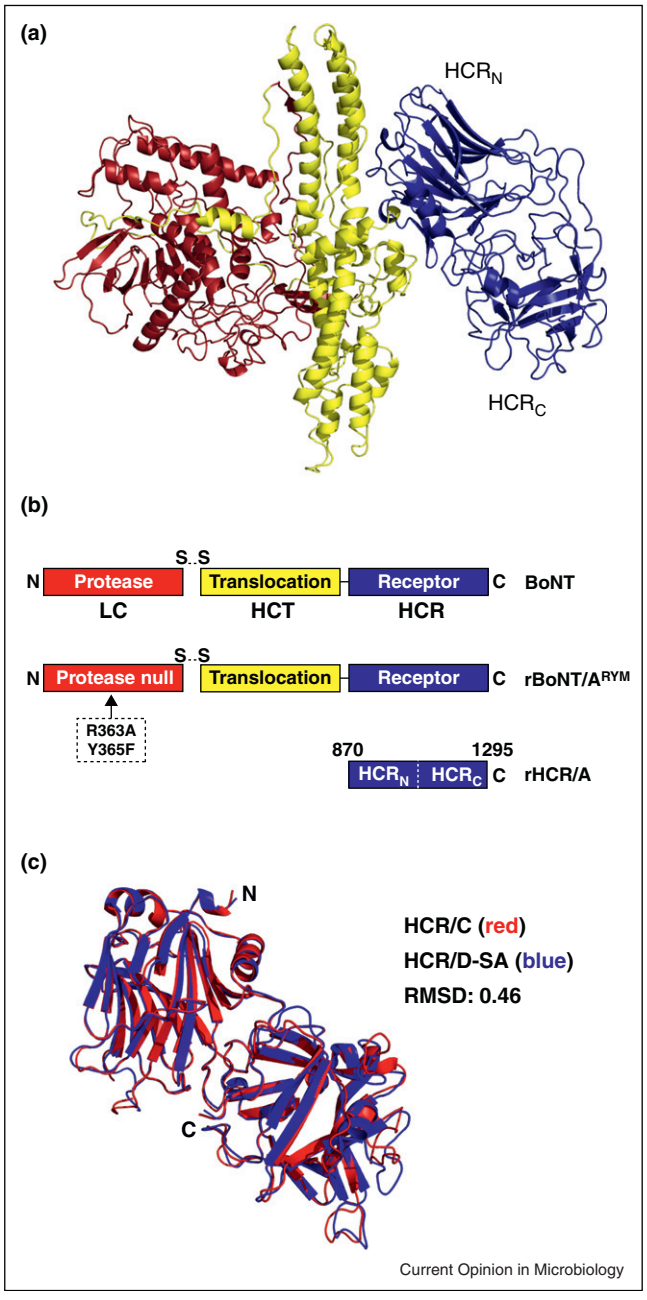
Introduction

Botulism is a flaccid, paralytic disease due to the action of botulinum neurotoxin (BoNT) that is produced by anaerobic bacteria of the genus Clostridium. By contrast, tetanus is a spastic, neurologic disease due to the action of tetanus neurotoxin (TeNT). While TeNT and BoNTs share structure–function properties, the unique paralyses elicited by these two neurotoxins are due to differential neurotoxin trafficking within the host nervous system. BoNTs are categorized into seven serotypes (A–G) based on serotype-specific immunological cross protection (1). BoNT/A,/B,/E and/F elicit human botulism, but all serotypes are toxic to non-human primates. Progression of botulism is linked to the consumption of food contaminated with BoNT or the ingestion of spores of clostridia that germinate in the gastrointestinal tract and subsequently produce BoNT. The mechanism of neurotoxin action has been subjected to considerable research due to their extreme potency in humans and their public health impact cause by food poisoning, therapeutic application, and potential utilization as a bioterrorism agent [1]. This review presents our current understanding of the structure–function properties of BoNT and describes strategies being developed to prevent botulism, emphasizing vaccines based on recombinant BoNT-derivatives [2,3].

Early studies determined that botulism was due to the action of BoNTs on neurotransmitter function. Specifically, acetylcholine synthesis was unaffected, but the release of acetylcholine from neurons was impaired [4,5]. Subsequently, the neurotoxins were shown to cleave SNARE proteins that inhibited synaptic vesicle fusion to the cell membrane and neurotransmitter release (reviewed in [6]). The modular nature of BoNT was initially described by DasGupta and Sugiyama who showed that trypsin cleaved BoNT into two fragments, a light chain (LC) and a heavy chain (HC) that were joined by a disulfide bond [7]. An early model described BoNT action in three discrete steps: initial binding of the neurotoxin to cell surface receptors, internalization of the neurotoxin into target cells and finally, the blockade of neurotransmitter release [8]. Subsequent structural studies of BoNT revealed an **AB** structure–function organization where the three steps of intoxication were ascribed to one of three, independent functional domains (Figure 1A and 1B) [9–11]. The LC is globular protein with Zn^{2+}-metalloprotease activity (**A** domain). LC cleaves neuronal SNARE proteins that contribute to neuronal tropism. LC is joined to the HC (**B** domain) by disulfide bond and a loop from the HC, 'the belt,' which wraps around the LC. The HC domain possesses two, structurally unique domains: the N-terminal translocation domain (HCT) and the C-terminal receptor binding domain (HCR). The HCT possesses long α-helices that are proposed to form a channel in the endosome membrane that facilitates passage of the LC into the cytosol [12,13]. The HCR drives the initial association of BoNT with target cells. The HCR is essentially a fusion of two lectin-like protein folds (Figure 1**C**). The N terminus of the HCR (HCR_N) assumes a jelly-roll fold that is defined by a series of anti-parallel beta strands that are connected by short loops to form two β-sheets. The jelly-roll fold is common to legume seed lectin-like structures (L-type lectins) [14]. To date, HCR_N has not been assigned a function, but appear to coordinate with the HCRc for efficient neurotoxin entry [15]. The C terminus of the HCR (HCR_C) comprises the receptor binding domain that contributes to the neuronal tropism of BoNT and assumes a β-trefoil fold that is common to ricin-type lectins (R-type) [14,16]. Each of the three

Figure 1



BoNT structure–function properties. **(A)** Ribbon diagram of the BoNT/A1 crystal structure (PDB:3BTA). The three functional domains are colored independently: the light chain (LC, red) encodes the protease activity, the translocation domain (HCT, yellow), and the receptor binding domain (HCR, blue) are required to deliver the LC to the cytosol of target cells. The HCR N-terminal and C-terminal subdomains are referenced. **(B)** BoNT domain organization is shown (BoNT, top). Recombinant proteins used as subunit vaccines against botulism are shown (middle and bottom). One approach utilizes a catalytically inactive BoNT/A construct (rBoNT/ARYM) where the introduction of two point mutations Arg363 to Ala (R363A) and Tyr365 to Ala (Y365A) into the LC inhibits LC/A cleavage of SNAP25. These mutations to the LC do not disrupt protein structure. Another approach utilizes the HCR (rHCR/A) that elicits a protective immune response to challenge by the homologous BoNT serotype. HCR can be divided into two domains: the N terminus (HCRN) and the C terminus (HCRC) Adapted from Baldwin, MR, *et al.*, 2008. **(C)** Crystal structures of HCR/C (red) and HCR/D-SA (blue). Proteins assume jelly-roll and β-trefoil folds in the N terminus and C terminus, respectively, which is characteristic for each of the seven BoNT-HCRs. The root mean square deviation (RMSD) for HCR/C and HCR/D-SA is 0.46, which indicates the structures are similar. Subtle structural divergence is observed in the two loops at the extreme C terminus of the HCRs. Adapted from Karalewitz, AP, *et al.* Biochemistry, 2010.

domains (LC, HCT, and HCR) contributes to motor-neuron intoxication.

Recent studies have begun to define the molecular events performed by the CNTs, including the recognition of host-cell receptors by the HCR and of the mechanism of SNARE proteins cleavage by the LC; by comparison, the mechanism of translocation is less well understood. The initial recognition of neurons is accomplished by the HCR domain binding dual receptors: a complex ganglioside and a synaptic vesicle (SV) protein [17–23]. Surface bound BoNT is internalized by SV cycling. Once internalized, the HCT directs LC translocation upon acidification of the endosome. The LC appears to escape the endosome through a channel formed by the HCT [24]. Once in the cytosol, LC targets and cleaves either vesicle or plasma membrane SNARE proteins that are required for neurotransmitter release effectively blocking neuron-muscle communication, which results in the flaccid-paralysis characteristic of BoNT intoxication [1,25,26].

The molecular mechanism of HCR-dual receptor engagement has been characterized to atomic resolution. Gangliosides are glycosphingolipids that are enriched in the plasma membrane of neurons and serve as functional BoNT receptors [27]. The details of ganglioside binding were initially determined in TeNT and later determined that BoNT utilizes a similar binding mechanism [28–31]. A conserved ganglioside binding motif was identified and defined by the residues H. . .SXWY. The ganglioside binding motif maps to a conserved region of the HCR known as the Ganglioside Binding Pocket (GBP). The H and W of the GBP directly contact the ganglioside. The second receptor is a resident SV protein that becomes accessible to BoNT following neuron stimulation. A region on the extreme C terminus of the HCR, adjacent to the GBP, mediates binding to the SV protein receptor [32,33].

History of vaccines against botulism

The success of formalized diphtheria toxin and tetanus toxin as a means to develop a toxoid-based vaccine that protected against diphtheria and tetanus, respectively, prompted research into the feasibility of producing a vaccine against botulism utilizing a similar approach [34,35]. Initial attempts to produce a vaccine against botulism utilized formalin-treated crude *C. botulinum*

extracts. Liquid filtrates treated with formalin retained immunogenicity, and eliminated toxicity. Animals that received repeated doses of formalin-treated neurotoxin were resistant to lethal challenge by the homologous BoNT serotype [35]. An additional step of toxin purification utilized alum precipitation that increased toxoid purity, while retaining the capacity to elicit a protective immune response in animals [36]. Repeated immunization with formalized, alum-precipitated toxoid established immunity. Although local and systemic reaction to the initial vaccination with this BoNT toxoid was relatively mild, the incidence and intensity of systemic reaction increased in individuals who received a second inoculum [37], which stimulated the development of several approaches to reduce reactivity. One procedure that utilized acid, sodium chloride, and cold ethanol precipitation of crude culture supernatant resulted in a pure, crystalline form of BoNT toxoid that elicited a measurable neutralizing immune response in animals [38,39]. A pentavelent formulation of BoNT/A,/B,/C,/D and/E was effective against the homologous serotypes with minimal reactivity following immunization [40] however, the supply of this vaccine is limited. Advances in molecular biological approaches provide tools to dissect the molecular mechanism of BoNT neutralization and to develop a new generation of BoNT subunit vaccines with improved efficacy and safety.

Future vaccines and therapies against botulism

Molecular techniques have led to the development of therapeutics that may replace traditional chemically detoxified vaccines. Identification of non-toxic domains of TeNT produced in *Escherichia coli* that protected against neurotoxin challenge provided the foundation for contemporary BoNT vaccines [34,41••]. An extension of these pioneering studies showed that the HCR domain (Figure 1B) was an effective subunit toxoid vaccine [3,42,43••,44]. Derivatives of this approach showed the production of this subunit toxoid in *Pichia pastoris* [45] what was secreted from yeast as a glycosylated HCR. Glycosylation circumvented by expressing the HCR in the host cytoplasm. The intracellular HCR was more immunogenic than secreted HCRs [44] and protected against challenge by the homologous BoNT serotype when administered by the intranasal route [46]. Subsequent studies showed that the BoNT HCRS could be efficiently produced in *E. coli*, which lead to the development of a subunit HCR-derived vaccine that protected against the seven BoNT serotypes (Table 1). Characterization of the HCRs allowed the resolution of a mechanism for vaccine-derived BoNT neutralization. These studies implicated protective epitopes within the HCR_N and the HCR_C subdomains [47,48] and showed that sera from mice vaccinated with a hepta-serotype HCR vaccine blocked the ability of the HCR to bind ganglioside, providing a molecular basis of neutralization [43••]. Currently unknown is whether neutralizing antibody binds directly to the ganglioside binding pocket or neutralizing antibody bind an epitope distal to the GBP but sufficient to disrupt association of the HCR with ganglioside. Another approach to generate an effective BoNT vaccine utilizes full-length BoNT rendered catalytically inactive (nontoxic) that is produced in clostridia. Introduction of point-mutations at multiple amino acids of the LC of BoNT/A eliminates SNAP25 cleavage and toxicity, but retains immunogenicity (Figure 1B) [49]. Similar work was subsequently repeated using catalytically inactive BoNT/A produced in *P. pastoris* [50].

Table 1

Hepta-serotype subunit HCR vaccine protects against challenge by the seven BoNT serotypes[a]

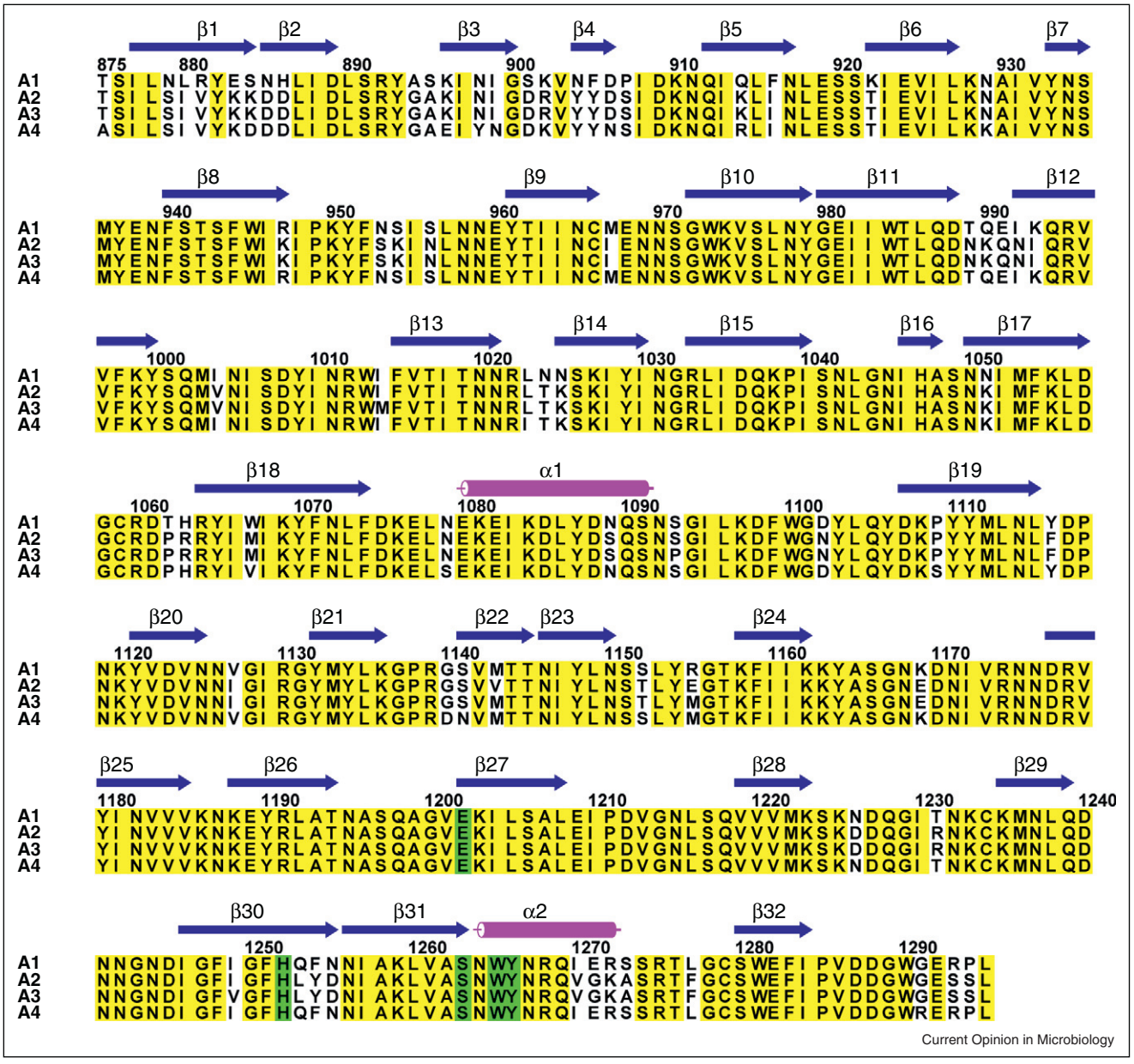
Serotype	Number of surviving mice/total number challenges with BoNT serotype at:	
	1000 LD_{50}	10 000 LD_{50}
A	5/5	5/5
B	5/5	5/5
C	4/5	4/4
D	5/5	5/5
E	5/5	5/5
F	5/5	5/5
G	5/5	5/5

[a] Mice were immunized with 4 doses of 1 μg of each HCR (serotypes A through G) and then challenged with the indicated BoNT serotype (1000 LD_{50}). After 3 days, survivors were scored and survivors were challenged with 10 000 LD50 of the indicated BoNT serotype and scored for survival at 4 days. Control experiments showed that 5/5 mice vaccinated with adjuvant alone did not survive challenge by 100 LD_{50} of BoNT/A. Adapted from Baldwin, MR, *et al.* (2008).

To define the neutralizing epitopes along the entire neurotoxin, Atassi and colleagues measured the specificity to linear epitopes of antibodies from cervical dystonia patients who were refractive to BoNT therapy. These studies identified multiple immunoreactive epitopes within each BoNT domain, and showed that the immunoreactive epitopes are not conserved between BoNT/A and BoNT/B [51,52]. In addition to developing vaccines, current research has developed novel anti-neurotoxin therapies that utilize antibodies and/or small molecules to promote neutralization and clearance of BoNT post-intoxication, respectively. In contrast to vaccination, these therapies are intended for rapid elimination of circulating BoNT via immune complex clearance, without long lived protection. The role of serum antibodies in BoNT neutralization was demonstrated in a guinea pig model who were passively immunized with immunoglobulin from human volunteers vaccinated with the pentavalent BoNT toxoid [53]. Subsequent studies using immunoglobulin from human volunteers vaccinated with a recombinant BoNT-HCR vaccine also demonstrated efficacy to BoNT challenge

[54]. Extending the development of passive immunity as a BoNT therapy showed that while a monoclonal antibody (mAb) directed against BoNT was not sufficient for neutralization, simultaneous administration of two or three mAbs that recognized non-overlapping epitopes was efficient in neutralizing a BoNT challenge [55,56]. A novel approach utilizes recombinant peptide termed 'tagged-binding agents' decorated BoNT to facilitate immune clearance of neurotoxins. The tagged-binding agents are derived from the B-cell repertoire of a hyperimmune sheep and are not potent neutralizing agents individually, but when multiple, unique binding agents are combined, and a mAb directed against an epitope tag is co-administered, protection from a lethal BoNT challenge is observed via immune complex clearance [57•]. This approach was applied to a clinically relevant post-intoxication model and was efficacious when administered up to three hours post intoxication [58].

Figure 2



Structure based alignment of BoNT/A subtypes. The receptor binding domains (HCR, residues 875–1295) from BoNT/A1 (Hall Strain, Accession number: A5HZZ9), BoNT/A2 (Q58GH1), BoNT/A3 (Lock Maree, Q3LRX9), BoNT/A4 (C3KS13) are aligned based on structural similarity. The β strands and α helices are defined and labeled above the sequence alignment. Residues highlighted yellow are conserved in identity between subtypes. Residues on while background are not conserved among the BoNT/A subtypes. Residues highlight green represent a conserved motif that defines the ganglioside binding pocket.

Natural BoNT variants complicate development of a pan-vaccine against botulism

While an effective, subunit vaccine against the seven prototypical BoNT serotypes has been achieved [43••], the presence of BoNT variants as subtypes or chimeras challenges the development of pan-BoNT vaccines and therapies [59–61]. BoNT subtypes are natural variants of the prototype BoNT serotype that vary in primary amino acid sequence by between 3 and 26% depending on the serotype, while BoNT chimeras are natural variants that appear to have derived by recombinant events between two BoNT serotypes. A structure based alignment created for the BoNT/A subtypes (A1, A2, A3 and A4) reveals that overall, the subtypes are very similar. Notably, the signature residues of the Ganglioside Binding Pocket are conserved among subtypes (Figure 2). Despite high amino acid identity, subtype activity can deviate from the prototypical BoNT/A. For example, BoNT/A subtype A2 intoxicates neurons with more rapid kinetics than the prototype BoNT/A1, where HCR/A2 binds neurons under resting and stimulated conditions more efficiently than HCR/A1, indicating that BoNT/A1 is more restricted in neurons than BoNT/A2 [62]. In addition, the catalytic properties of the LCs subtypes possess unique properties that may also contribute to functionality that diverges from the prototypical BoNT/A1 [63,64]. Thus BoNT subtypes have unique activities relative to the prototypical BoNT serotype. In addition to variable toxicity between BoNT/A subtypes, the subtle divergence in amino acid sequence also influences the host immune response to vaccination [50,65•]. Vaccination with HCR/A1 and HCR/A2 subtypes elicit unique protection profiles and low-dose HCR vaccination (Table 2) showed a subtype specificity where mice immunized with an HCR/A subtype survived challenge by the homologous subtype BoNT challenge, but only partial protection a heterologous BoNT subtype challenge. Immune analysis showed that differential HCR/A subtype vaccination yielded unique immune epitope responses [65•].

BoNT chimeras are a property of the BoNT/C and /D family. The genes encoding BoNT/C and/D are located on bacteriophage that may contribute to the propensity of these neurotoxin genes to undergo recombination that results in unique neurotoxin variants [66,67]. BoNT serotype D-South Africa (BoNT/D-SA) is a chimera of BoNT/D and BoNT/C. Antiserum to the LC of BoNT/D, but not LC BoNT/C, cross reacted with BoNT/D-SA, antiserum to HC of BoNT/C, but not HC BoNT/D, cross reacted with BoNT/D-SA [68]. Amino acid sequence alignments revealed a high degree of identity between the LC of BoNT/D-SA and the LC of BoNT/D (96%), a conserved HCT between the three toxins (~80–90%), and high homology between the HCR of BoNT/C and BoNT/D-SA (74%) [69]. Studies attempting to create a bivalent vaccine effective against BoNT/C and BoNT/D observed that following vaccination with HCR/C or HCR/D, challenge by BoNT/D-SA was not completely neutralized [70•]. Analysis of the HCR/C and HCR/D-SA crystal structures revealed while the overall structures were well aligned, subtle differences within loop segments at extreme C terminus of the HCRs were identified that may represent unique neutralizing epitopes (Figure 1C) [71••].

Conclusions

The antigenic variability observed among the seven BoNT serotypes has hindered efforts to produce pan-serotype vaccines and therapies. A novel approach to vaccine design that utilizes structure-based knowledge produced a single molecule containing the immunodominant epitopes of multiple, antigenic distinct variants towards the development of a meningococcal pan-vaccine [72•]; a similar approach may allow the development of a pan-vaccine strategy. Continued research into the structure–function properties of BoNT will expand our understanding of the three-dimensional organization and may lead to insight into the rational design of pan-vaccines. Conversely, these natural BoNT serotype

Table 2

Cross protection of BoNT/A subtypes challenge by subunit HCR/A vaccination

HCR/A immunogen (# of immunizations)	Survival of BoNT/A subtype challenge (1000 LD_{50})		
	BoNT/A1	BoNT/A2	BoNT/A3
1.0 μg (3)[a]			
A1	4/4	4/4	4/4
A2	4/4	4/4	4/4
A3	4/4	4/4	4/4
A4	4/4	4/4	4/4
0.1 μg (2)[b]			
A1	9/10	7/10	5/10
A2	4/10	10/10	7/10

[a] Mice were immunized the 3 doses of 1.0 μg of the indicated HCR/A subtype and then challenged with the indicated BoNT serotype (1000 LD_{50}). After 3 days, survivors were scored.
[b] Mice were immunized the 2 doses of 0.1 μg of the indicated HCR/A subtype and then challenged with the indicated BoNT serotype (1000 LD50). After 3 days, survivors were scored. Adapted from Henkel, J, *et al.* (2011).

variants may be developed to extend the clinical applications of BoNT-based therapies.

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- • of special interest
- •• of outstanding interest

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