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Subunit Vaccine Efficacy against Botulinum Neurotoxin Subtypes

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Abstract

Botulinum neurotoxins (BoNT) are classified into 7 serotypes (A-G) based upon neutralization by serotype-specific anti-sera. Several recombinant serotype-specific subunit BoNT vaccines have been developed, including a subunit vaccine comprising the receptor binding domain (HCR) of the BoNTs. Sequencing of the genes encoding BoNTs has identified variants (subtypes) that possess up to 32% primary amino acid variation among different BoNT serotypes. Studies were conducted to characterize the ability of the HCR of BoNT/A to protect against challenge by heterologous BoNT/A subtypes (A1–A4). High dose vaccination with HCR/A subtypes A1–A4 protected mice from challenge by heterologous BoNT/A subtype A1–A3, while low dose HCR vaccination yielded partial protection to heterologous BoNT/A subtype challenge. Absolute IgG titers to HCRs correlated to the dose of HCR used for vaccination, where HCR/A1 elicited an A1 subtype-specific IgG response, which was not observed with HCR/A2 vaccination. Survival of mice challenged to heterologous BoNT/A2 following low dose HCR/A1 vaccination correlated with elevated IgG titers directed to the denatured C-terminal sub-domain of HCR/A1, while survival of mice to heterologous BoNT/A1 following low dose HCR/A2 vaccination correlated to elevated IgG titers directed to native HCRc/A1. This implies that low dose vaccinations with HCR/A subtypes elicit unique IgG responses, and provides a basis to define how the host develops a neutralizing immune response to BoNT intoxication. These results may provide a reference for the development of pan-BoNT vaccines.

Keywords

Botulinum neurotoxin serotype A; subunit vaccine; vaccination; synaptic vesicles; botulism

Introduction

Botulinum neurotoxins (BoNTs) are the most potent protein toxins for humans [1]. BoNTs possess conserved overall structure and function properties with ~65% primary amino acid similarity and ~40% amino acid identity [2]. BoNTs are synthesized as single-chain proteins

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that are cleaved by endogenous or exogenous proteases into di-chain proteins, consisting of ~ 50 kDa light chain and of ~100 kDa heavy chain (HC) linked by a disulfide bond. The N-terminal catalytic light chain possesses zinc metalloprotease activity while the HC consists of a translocation domain and a receptor binding domain (HCR). BoNTs inhibit neuronal exocytosis at the peripheral neuromuscular junction eliciting flaccid paralysis [3].

BoNTs are classified into 7 serotypes (A-G) based upon serotype-specific antibody neutralization. Within each BoNT serotype, DNA sequencing has identified subtypes that differ by up to 32% at the primary amino acid level; for example, several BoNT/A subtypes have been identified that differ by up to 16% [4]. BoNT/A1 is the prototypic BoNT and has been licensed for numerous BoNT therapies that include neurological afflictions as well as cosmetic application [5]. BoNT/A2 was identified based upon DNA sequencing [6–8] and protein analysis [9]; the catalytic properties of LC/A2 are similar to LC/A1 [10]. Two recent studies have reported that BoNT/A2 elicits more rapid intoxication than BoNT/A1 with BoNT/A2 entering neurons more efficiently than BoNT/A1 [11, 12]. Less is known about the other BoNT/A subtypes. Clostridia producing BoNT/A3 were isolated from a botulism outbreak in Scotland in 1922, while clostridia producing BoNT/A4 were isolated from a case of infant botulism in 1988 [4, 13–15]. Recently, genome sequencing identified BoNT/A5 [16, 17], which has ~97 % identity with BoNT/A1. Initial studies showed that BoNT/A1 and BoNT/A5 had similar inactivation properties with neutralizing polyclonal anti-BoNT/A1 antibody [18].

BoNT/A binds to dual-host receptors, comprising a ganglioside and Synaptic Vesicle protein 2, SV2 [19] on α -motor neurons. The HCR domain of BoNT/A initially binds to gangliosides on the surface of unstimulated neurons and upon membrane depolarization and synaptic vesicle fusion with the plasma membrane, BoNT/A binds the luminal component of SV2. The structural interactions between the HCR domains of BoNT/A and SV2 have not been resolved [20]. The protein receptor for BoNT/B and BoNT/G is Synaptotagmin I and II (Syt) [21, 22], while BoNT/E, and BoNT/F use SV2 [23–25]. Receptor bound BoNT is internalized during synaptic vesicle cycling from the plasma membrane.

The current penta-serotype (A, B, C, D, E) *C. botulinum*-derived vaccine against botulism [26, 27] is prepared in 0.6% formalin (BoNT toxoid) with aluminum phosphate as adjuvant and preserved with 0.01% thimerosal, but is no longer distributed by the CDC. This has prompted the development of several vaccine strategies that utilized recombinant derivatives of BoNTs to produce effective vaccines against the seven serotypes of BoNT. As proof of principle, Middlebrook and coworkers [28] produced an *Escherichia coli*-derived HCR serotype A (HCR/A) and showed that the HCR/A was an effective immunogen. Subsequent studies utilized HCRs expressed in the yeast *Pichia pastoris* as a heterologous host [26, 29] and *E. coli* [30, 31] to generate a subunit vaccine that protects against challenge by all seven serotypes of BoNTs [30] and a vaccine comprised of HCR/A and HCR/B that is in clinical trials [15, 32]. Domain mapping experiments showed that the HCR was the most potent immunogen for providing protection against BoNT intoxication in mice (reviewed in [33]). Vaccination with the HCR elicits neutralizing antibodies that are serotype specific [30, 34–41] and BoNT-neutralizing antisera derived from mice immunized with the HCR block HCR binding to gangliosides and neuronal plasma membranes, indicating the presence of an epitope close to the ganglioside binding pocket of the HC [34–40]. Other approaches have utilized non-catalytic holo-BoNT/A as an effective immunogen against challenge by BoNTs [42, 43] and DNA vaccination which elicits neutralizing antibody response to challenge by BoNT [28, 44, 45].

There has been limited consideration for the influence of BoNT subtypes in vaccine strategies [46]. We and others have shown that vaccination with HCR/A1 will protect

against challenge by heterologous BoNT/A subtypes [34, 42], but a characterization of the immune response to challenge related to protection has not been considered. In this report, the host response to HCR/A subtype vaccination and heterologous BoNT/A subtype challenge is evaluated.

Materials and Methods

Engineering recombinant HCRs of BoNT/A1–A4

pET-28a (Novagen) was modified to contain a 3x-FLAG epitope downstream of the resident (His6) tag. DNAs encoding HCR/A1–HCR/A4 were amplified and subcloned into KpnI and PstI sites of the modified pET- vector. DNA encoding the HCR domains of BoNT/A subtypes A1–A4 (residues 870–1296 for BoNT/A1 equivalents) was derived from: BoNT/A1, *C. botulinum* A str. ATCC 3502; BoNT/A2, *C. botulinum* A2 str. Kyoto F; BoNT/A3, *C. botulinum* A3 str. Loch Maree; and BoNT/A4, *C. botulinum* str. 657Ba and confirmed by DNA sequencing. *E. coli* BL-21-(DE3)RIL were transformed with plasmids encoding each pET28-HCR/A subtype and cultured in LB with 50 µg/ml of kanamycin and 100 µg/ml of chloramphenicol at 37 °C.

Production of HCR A1–A4

HCR/A1–HCR/A4 were purified as previously described [34]. Briefly, *E. coli* (pET28-HCR/A) were grown at 30°C for 2 h at 250 rpm to an OD of ~0.6, when 0.5 mM IPTG was added and cultured overnight at 16°C. Cells were harvested, broken with a French Press, and clarified by centrifugation (6,000 × g for 15 min) and filtered (0.45 µm cellulose acetate). The filtered lysate was subjected to 6-His affinity chromatography (Ni²⁺-NTA resin, Qiagen), size-exclusion chromatography (Sephacryl S-200HR, Sigma), and anion-exchange chromatography (DEAE Sephacryl, Sigma). Fractions, containing purified HCRs, were dialyzed overnight against 20 mM HEPES-KOH buffer (pH 7.6), 20 mM NaCl, and 1 mM EDTA. Purified proteins were then stored either at –20°C in the presence of 40% (v/v) glycerol or undiluted at –80 °C. HCR/A2 was stored in 200 mM NaCl to enhance solubility. Coomassie blue staining of purified HCR/A subtypes subjected to SDS-PAGE did not detect contaminating proteins (Figure 1, insert).

Ganglioside binding assay

GT1b (stock 20 µg/µl in DMSO, Sigma) was added to 96-well non-binding plates (3474 Corning) at 1 µg/100 µl in MeOH/well, and allowed to dry at room temperature for 2 h. Plates were blocked with 200 µl 1% BSA (w/v) in 0.05 M Na₂CO₃ (pH 9.6) at 4°C for 1 h, then washed three times in PBS. HCR/A subtypes were added to wells in 3-fold dilutions (100 µl) and incubated at 4°C for 1 h. The plate was washed three times in PBS and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP, Pierce) at 1:40,000 dilution in 100 µl of PBS + 1% BSA for 1 h at 4°C. Wells were washed with PBS and incubated with 100 µl of 1-StepTM Ultra TMB-ELISA substrate (Thermo Scientific) for 30 min at room temperature (RT). The assay was stopped with an equal volume of 1 M H₂SO₄ and absorbance was read at 450 nm with a Victor3 V plate reader (Perkin Elmer). Background binding was determined in wells without added ganglioside and subtracted from test samples.

Synaptic vesicle purification

Synaptic vesicles were purified as previously described [47]. Briefly, cerebral cortex from 20 rats (PelFreez Bio) were thawed on ice and placed into ten volumes of buffered sucrose (320 mM sucrose, 5 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 1 mM PMSF, and mammalian protease inhibitor cocktail (Sigma)). Cortex was homogenized with a motorized

glass-Teflon homogenizer using 20 up-and-down strokes at 900 rpm. The homogenate was centrifuged for 10 min at 3000 rpm ($800 \times g$) to yield a pellet (P1) and post-nuclear supernatant (S1). The homogenization and centrifugation steps were repeated on the P1 fraction to increase the recovery of synaptic vesicles. S1 was decanted into fresh tubes and centrifuged for 45 min at $45,000 \times g$, the supernatant removed and discarded (S2), and the pellet (P2) was suspended in 0.64 M buffered sucrose and centrifuged ($45,000 \times g$) to yield a supernatant (S2') and pellet (P2'). The P2' pellet was suspended in hypotonic lysis buffer (5 mM HEPES-KOH (pH7.4) with 1 mM EDTA and 1 mM PMSF), stirred for 45 min, and centrifuged for 60 min at $45,000 \times g$. The pellet (P3) was discarded and the supernatant (S3) containing disrupted synaptic vesicles were centrifuged in a Beckman Ti50.2 rotor at 48,000 rpm ($\sim 150,000 \times g$). The pellet was suspended in either PBS buffer (1 mM EDTA, 1 mM PMSF) or PBS buffer containing 2% (w/v) CHAPS or Triton X-100 and stored at -80°C . Synaptic vesicle lysates were prepared by centrifugation of the detergent-extracted lysates for 25 min at $13,000 \times g$. The soluble synaptic vesicle lysate was stored at -80°C .

Synaptic vesicle binding assay

Synaptic vesicles (1 μg of protein in 100 μl /well) were incubated in a 96-well, high-bind plate (9018 Corning) for 2 h at 4°C in 0.05 M Na_2CO_3 (pH 9.6) and washed three times in PBS. Wells were blocked with 1% BSA (w/v) in 0.05 M Na_2CO_3 (pH 9.6) at 4°C for 1 h and washed three times in PBS. HCR/A subtypes, at 3-fold dilutions, were added to each well, 100 μl volumes, incubated at 4°C for 1 h and washed three times in PBS. Wells were then incubated with 100 μl of goat anti-mouse antibody conjugated-HRP at a 1:20,000 dilution in PBS for 1 h at 4°C . Wells were incubated with 100 μl of 1-StepTM Ultra TMB-ELISA substrate for 30 min at RT, stopped with an equal volume of 1 M H_2SO_4 , and read at 450 nm. Background absorbance, wells without vesicles or without HCR, was subtracted from test absorbance.

HCR/A subtype vaccination and BoNT/A subtype challenge

Two protocols were followed to determine the efficacy of different HCR/As to elicit a protective antibody response against challenge (96 hr) with homologous or heterologous BoNT/A subtypes. In the 3 dose vaccination, female ICR mice (18 to 22 g) were immunized intra-peritoneal (IP) with 1.0 μg of HCR/A1, HCR/A2, HCR/A3 or HCR/A4 on days 1 and 14 with Alhydrogel gel and day 28 alone. Serum was collected from mice on day 35 and mice were challenged on day 37 with BoNT/A1, BoNT/A2 or BoNT/A3. In the second protocol, a 2 dose vaccination, mice were vaccinated on days 1 and 14 with either 0.1 μg or 1.0 μg of the indicated HCR/A (HCR/A1 – HCR/A4) plus Alhydrogel, sera were collected on day 20 and mice vaccinated with HCR/A1 or HCR/A2 were challenged on day 22 with 1,000 LD₅₀ of the BoNT/A1, BoNT/A2, or BoNT/A3 [30]. These experiments were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison.

Solid-phase determination of IgG titers following HCR immunization

One μg of HCR/A1, HCR/A2, or the corresponding C-terminal sub-domains HCRc/A1 and HCRc/A2 (residues 1096–1296 of BoNT/A1 equivalent) were directly spotted in duplicate or boiled (5 min in 0.1% SDS) and spotted onto nitrocellulose strips (Millipore). Spots were dried, blocked with 2% dried milk in 20 mM Tris-HCl (pH 7.4) and 140 mM NaCl with 0.1% Tween 20 (TBST) at RT for 20 min, washed TBS-0.5% Tween 20, and incubated with sera from mice vaccinated with either HCR/A1 or HCR/A2 at 1/2000 final dilution in 2% dried milk in TBST for 1 h at RT. Strips were washed in TBS-0.5% Tween 20, incubated with goat anti-mouse IgG-HRP (Pierce, 1:20,000 dilution) in TBST for 1 h at RT, washed with TBS-0.5% Tween 20, and incubated in Super Signal (Pierce) for 5 min at RT. Light emission was imaged with a digital camera (Cell Bioscience). For each serum, signal obtained from control protein 3x-FLAG-HCR/Tetanus toxin was subtracted. The control

serum signal varied with each serum and was < 10% of the signal detected for HCR/A derivatives. Signals were proportional to serum dilution and time of imaging, allowing a determination of the relative HCR-specific IgG signal as a function of survival to BoNT challenge. Values reported were uniformly divided by 15000 and reported as arbitrary units from two independent experiments performed in duplicate. Statistical analysis between sets of data was assessed by the Student T test, using GraphPad Prism 5.

Results

Binding of HCR/A subtypes A1–A4 to the ganglioside GT1b and synaptic vesicles

Initial experiments determined how recombinant HCR/A subtypes bound to host receptors; purified GT1b and SV2 in synaptic vesicles [20, 49, 50]. Each purified HCR/A1–HCR/A4 is visible as a single band (Figure 1, insert). A solid-phase binding assay showed that each HCR/A1–HCR/A4 bound GT1b with similar affinity where ganglioside binding was proportional to HCR concentration with a variation of ~2-fold with the order of binding: HCR/A4 > HCR/A1 > HCR/A2 = HCR/A3 (Figure 1). This is consistent with subtypes HCR/A1 – HCR/A4 possessing conserved amino acid residues within the ganglioside binding pocket of HCR/A1 equivalents: Phe1252, His1253, Ser1264, Trp1266, Tyr1267, Gln1270, and Gly1279 [4].

The protein receptor for BoNT/A is SV2, an integral membrane binding protein of synaptic vesicles [23, 34]. Disrupted synaptic vesicles were used in a solid phase binding assay to determine the SV2 binding capacity of the HCR/A subtypes. Under condition where HCR/A binding to synaptic vesicles was dose dependent, the HCR/A1 – HCR/A4 bound synaptic vesicles with similar efficiency with a variation of ~2-fold at a point in the curve where binding was proportional to HCR (Figure 2). Thus, HCR/A1 – HCR/A4 have similar affinities for GT1b and synaptic vesicles, indicating that the HCR/A subtypes possess similar biological activities.

HCR/A subtype vaccine protection to heterologous BoNT/A subtype challenge

Cross HCR/A1 – HCR/A4 protection from heterologous BoNT/A subtype challenge was determined with BoNT/A1, BoNT/A2 and BoNT/A3. BoNT/A4 was not available at the time of the experiments. Three immunization protocols were utilized. The first protocol utilized a high dose HCR/A vaccination. Mice vaccinated with 3 doses of 1.0 µg of HCR/A1, HCR/A2, HCR/A3 or HCR/A4 survived challenges with 1,000 LD50 of BoNT/A1, BoNT/A2, or BoNT/A3 (Table 2). Mice that survived the 1,000 LD50 challenge also survived a subsequent challenge with 10,000 LD50 of the respective BoNT/A subtypes four days after the initial challenge (data not shown). A second protocol vaccinated with 2 doses of either 1.0 µg or 0.1 µg HCR/A. Mice vaccination with 2 doses of 1.0 µg of HCR/A1 or HCR/A2 showed complete or partial challenge from homologous or heterologous BoNT/A subtype. Mice vaccinated with a 2 doses of 0.1 µg of HCR/A1 or HCR/A2 showed 90% or complete protection against homologous BoNT/A subtype challenge, but lower protection to challenge by the heterologous BoNT/A subtype (Table 2). These experiments showed that a protective antibody response in mice to challenge with heterologous BoNT subtypes is a function of the amount of immunogen and number of vaccinations. Low dose vaccination yielding partial protection to heterologous BoNT/A subtype challenge indicates the stimulation of neutralizing antibodies to unique epitopes within each HCR/A subtype, or the presence of common epitopes that have varied affinity for neutralizing antibodies within each HCR/A subtype.

IgG titers to HCRs following vaccination with HCR/A subtypes

Previous studies showed that, while less efficient than HCR/A, HCRc/A1 (residues 1096–1296 of BoNT/A1) elicits a neutralizing immune response to BoNT challenge [31], supporting the presence of epitopes that stimulated neutralizing antibodies within the HCRc sub-domain. Pooled sera from mice immunized with 2 low doses of 0.1 µg of HCR/A1 – HCR/A4 were analyzed for reactivity to the four HCR/A subtypes. Each of the four HCR/A subtypes stimulated an IgG response to homologous and heterologous HCR/A subtypes with similar titers (Figure 3); 50% serum titers occurred between 1/2000 and 1/6000 serum dilution. Thus, analysis of pooled sera did not resolve the basis for partial protection to heterologous BoNT/A subtype challenge observed following low dose HCR/A vaccination.

IgG titers to HCR/A and HCRc/A following high- and low- dose HCR/A vaccination

To enhance resolution of the host IgG response to HCR/A subtype vaccination, a solid phase binding assay measured the IgG titers to homologous and heterologous HCR/A and HCRc/A relative to the survival status of individual mice. This analysis focused on BoNT/A1 and BoNT/A2 and their derivatives, since the BoNT/A subtypes cluster into two groups; BoNT/A1 (BoNT/A1, BoNT/A4, and BoNT/A5) and BoNT/A2 (BoNT/A2 and BoNT/A3) and to date recombinant HCRc/A3 and HCRc/A4 subdomains have not been engineered. We therefore felt that characterizing the two best characterized subtypes (A1 and A2) would allow generation of meaningful data. Figure 4 shows the purity of the HCR/A1 and HCR/A2 and HCRc/A1 and HCRc/A2 used in this analysis. IgG titers to HCR/A and HCRc/A (native) and following boiling in 0.1 % SDS (denatured) were assayed. Serum from 5 mice that survived BoNT/A challenge after 2 high doses of HCR/A had IgG titers to HCR/As and HCRc/As that were ~200-fold greater than IgG titers from mice that survived BoNT/A challenge following 2 low doses of HCR/A vaccination (Figure 5). Sera from mice vaccinated with high dose HCR/A1 or HCR/A2 had similar IgG titers to the homologous HCR/A and HCRc/A, which indicated that much of the IgG response was directed to HCRc/A. Sera from mice vaccinated with high dose HCR/A1 had statistically higher IgG titers to HCR/A1 than HCR/A2, which indicated the presence of a HCR/A1 subtype-specific epitope or that HCR/A1 had a higher affinity for an epitope that was shared with HCR/A2. An HCR/A2 subtype-specific epitope was not detected.

Protection by low dose HCR/A vaccination correlates with high IgG titers to HCRc

Vaccination with low dose HCR/A elicited a partial protection to challenge by heterologous BoNT/A challenge, which allowed a statistical characterization of the host IgG response to heterologous BoNT/A subtype challenge (Figure 6). Sera from mice vaccinated with HCR/A1 or HCR/A2 that survived heterologous BoNT/A challenge had similar IgG titers to native HCR/A and native HCRc/A, indicating that the C-terminal sub-domain of HCR elicited a dominant linear immune response. The differences in titers to HCR/A2 (boiled) were not significant in the HCR/A2 sera from mice dead and alive due to the variance in the IgG titers among the alive mice. One unique property of mice protected from heterologous challenge was observed. Sera from survivor mice vaccinated with HCR/A1 had a similar IgG titers for native HCRc/A and denatured HCRc/A, indicating that HCR/A1 stimulated the production of antibodies to linear epitopes within HCRc/A, while sera from survivor mice vaccinated with HCR/A2 had higher IgG titers for native HCRc/A than denatured HCRc/A, indicating that HCR/A2 stimulated the production of antibodies to native epitopes within HCRc/A (Figure 6).

Discussion

Recognition of BoNT subtypes and/or mosaic toxins [48] prompted this analysis on the ability of HCR vaccines to protect against heterologous subtype challenge. BoNT/A was

chosen based upon current knowledge of the protein's molecular and cellular properties and the availability of biochemical and biological assays to assess subunit vaccine efficacy; specifically BoNT/A subtypes (A1–A4), which showed overall primary amino acid identity between 89 and 84% (Table 1). Recently, BoNT/A5 was identified in a genome analysis of a subset of *C. botulinum* with 97% homology to BoNT/A1 [16]. The immune reactivity of BoNT/A5 was not tested in this study based upon the limited known properties of this subtype and since preliminary studies indicated that BoNT/A1 and BoNT/A5 possessed similar neutralization responses to polyclonal anti-BoNT/A1 sera [18].

High dose vaccination with HCR/A was observed to protect against challenge by heterologous BoNT/A subtypes. This supports earlier studies that reported the ability of HCR/A to stimulate a protective immune response to heterologous BoNT/A subtype challenge. The partial protection to heterologous BoNT/A subtype challenge by low dose vaccination indicates that HCR/A stimulated unique host immune response during the development of a protective response. High dose vaccination and low dose vaccination with HCR/A1 and HCR/A2 stimulated unique IgG host responses. High dose HCR/A1 vaccination stimulated a similar antibody response to HCR/A1 and HCRc/A1 which was greater than the antibody response to HCR/A2, while high dose HCR/A2 vaccination elicited a similar antibody response to HCR/A2 and HCR/A1. Thus, HCR/A1 vaccination stimulated an A1 antibody subtype-specific response that is not observed with HCR/A2 vaccination. Since high dose vaccination with either HCR/A1 or HCR/A2 stimulated a cross subtype BoNT/A protection, this HCR/A1-specific response does not correlate with the protective state of the host. Antibody response to low dose vaccination by HCR/A1 and HCR/A2 also showed subtype specificity. Low dose HCR/A1 vaccination showed similar IgG titers for native HCRc/A2 and linear HCRc/A2, while low dose HCR/A2 vaccination showed higher IgG titers for native HCR/A1 than linear HCR/A1. Thus, while the immune response to the HCRs matures, HCR/A1 stimulates the production of antibodies to linear epitopes within HCRc that correlates to protection to heterologous BoNT/A subtype challenge, while HCR/A2 stimulates production of antibodies to conformational epitopes within HCRc that correlates to protection to heterologous BoNT/A subtype challenge. Understanding the molecular basis for the unique maturation of the host response to HCR vaccination may provide a basis to formulate strategies for pan-subtype-specific vaccines against botulism.

Atassi and coworkers have provided a detailed characterization of the human response to “natural” BoNT immunization following long term therapy with BoNT/A1 (BoTox) and researchers who had been vaccinated with the chemically inactivated BoNT vaccine [51]. In these patients and researchers, IgG titers to nine peptides within HCR/A1 were detected. Alignment of HCR/A1 and HCR/A2 identified seven immunoreactive peptides that contained amino acid subtype variance, representing candidate subtype-specific linear epitopes. The ganglioside binding pockets among the BoNT/A1 and BoNT/A2 is conserved with 7 of 8 contacts between GT1b [20]. One immune reactive peptide in the C terminus of the BoNT/A (1275–1296) is included within HCR/A that is present in neutralizing immune response to BoNT/A challenge [52, 53]. This C-terminal region of BoNT/A is also the site of two variations of primary amino acid sequence for BoNT/A1 and BoNT/A2: residues 1271–1275 and 1292–1295 and are candidate regions for subtype specific epitope(s). The non-overlapping, immune-reactive yet mechanistically similar, epitopes between BoNT/A and BoNT/B may explain the inability of serotype-specific immunization to produce a neutralizing response against cross serotype challenge.

Low dose immunization with HCR/A2 protected against challenge by BoNT/A2, but partial protection against challenge with BoNT/A1 or BoNT/A3 (Table 2). The partial protection against BoNT/A3 was unexpected since HCR/A2 and HCR/A3 are 98% identical (422/427

residue identity). The variation between HCR/A2 and HCR/A3 include: I1012M, S1090P, V1140M, E1152M, and I1246V. Each of these variants lie outside the immune reactive toxin peptides identified in serum from patients with cervical dystonia unresponsive to BoNT/A1 therapy and serum from hyper immunized individuals [51]. The inability to correlate a linear epitope to the subtype specificity between HCR/A2 and HCR/A3 implicates a role for conformational neutralizing epitope(s) as contributing to subtype-specific neutralization, or the presence of additional linear neutralizing epitopes within the HCR that lie outside the identified immune reactive peptides. The basis for the partial subtype protection elicited by HCR/A2 to BoNT/A3 challenge is currently under investigation.

Other bacterial toxin variants have been described that potentially correlate with vaccine protection, including two antigens that are components of the acellular pertussis vaccine, pertussis toxin and pertactin [54]. The appearance of S1 subunit variants correlate with vaccine production and is possibly based upon vaccine selection [55]. In this system, the variations are minor changes to the primary amino acid sequence of the S1 subunit. For example, several sequence variants encode up to three amino acid substitutions within the S1 subunit [56]. The implication for the development of a pan-BoNT vaccine is that only a fraction of the amino acid variance observed within a BoNT subtype or serotype may be involved eliciting BoNT neutralization.

The evolution of the BoNTs and their host clostridia are complex [17]. BoNTs are produced by several strains of clostridia [57], including *C. butyricum*, and *C. baratii*, in addition to *C. botulinum*, where the physical location of gene encoding the BoNT appears to be associated with specific species [48]. In addition, BoNTs may be encoded on plasmids [58], extending the possibility for genetic exchange among clostridia. The evolutionary pressures that select each BoNT serotype and subtype are not clear, but may involve environmental pressures to enhance the survivability of clostridia, genetic transfer mechanisms, the biology and biochemistry of BoNT intoxication, and the sporulation process of clostridia. Understanding the evolution of the BoNTs may provide insight for developing vaccines that provide pan-serotype coverage to BoNT intoxication.

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Highlights

- High dose vaccination with HCR/A protected mice from challenge by heterologous BoNT/A subtypes.
- Low dose HCR/A vaccination yielded partial protection to heterologous BoNT/A subtype challenge.
- Vaccination with HCR/A1 elicited an A1 subtype-specific IgG response.
- Survival to heterologous BoNT/A2 challenge with HCR/A1 vaccination correlated with IgG to the denatured HCRc/A1
- Survival to heterologous BoNT/A1 challenge with HCR/A2 vaccination correlated with IgG titers to native HCRc/A1.

Figure 1. HCR/A subtype ganglioside binding assay

GT1b was fixed to a 96-well plate, blocked with 1% BSA, incubated with HCR/A1–A4 in a dilution series, and probed with mouse α -3x-FLAG antibody (Sigma). Assay was developed using 1-Step™ Ultra TMB-ELISA for 30 min, stopped with equal volume of 1 M H_2SO_4 , and absorbance read at 450nm. Data shown are duplicate determinations and are representative of 3 repeat experiments. Background is subtracted for each subtype through subtraction of absorbance of wells incubated without HCR/A. Inset panel shows purified HCR/A1–A4; 1 μg of each HCR was run on 12% SDS-PAGE and visualized with Coomassie Brilliant Blue staining. HCR/A subtypes migrated as ~50 kDa bands.

Figure 2. HCR/A subtype binding to synaptic vesicles

Purified synaptic vesicles were fixed to 96-well, high-bind plates, blocked with 1% BSA, and incubated with purified HCR/A1–A4 in a dilution series. HCR/A bound to the plate was detected by probing with mouse α -3x-FLAG antibody, then developed with 1-StepTM Ultra TMB-ELISA for 30 min, stopped with equal volumes of 1M H₂SO₄, and absorbance read at 450 nm. Data shown are n=2 and representative of at least 3 repeat experiments. Background for each subtype through subtraction of absorbance of wells incubated without HCR/A.

Figure 3. Antibodies from HCRA/1-A4 vaccinated mice bind HCR/A subtypes

HCR/A subtypes A1–A4 (100 ng/well) were fixed to high-bind, 96 well plates, blocked with 1% BSA, incubated with a dilution series of sera from mice vaccinated with 0.1 μ g of either (A) HCR/A1, (B) HCR/A2, (C) HCR/A3, and (D) HCR/A4 in 1% BSA for 1 hr then probed with goat anti-mouse HRP. The assay was then developed with 1-StepTM Ultra TMB-ELISA for 30 min, stopped with H₂SO₄, and absorbance read at 450nm. Data shown are n=2 and representative of at least 3 repeat experiments. Background is subtracted for each subtype through subtraction of absorbance of wells incubated with non-vaccinated mouse sera.

Figure 4. Purification of HCR/A1, HCR/A2, HCRc/A1, and HCRc/A2

(Upper panel) HCR/A1 (A1), HCR/A2 (A2), HCRc/A1 (cA1), and HCRc/A2 (cA2) (2.0 μ g) were subjected to SDS-PAGE and Coomassie blue staining. HCR/T was included in the protein maker lane. (Lower panel) Schematic of BoNT/A1 (1296 aa), HCR/A1 (428 aa), and HCRc/A1 (208 aa) that were used in this analysis.

Figure 5. Solid-phase determination of IgG titers following HCR immunization
HCR/A1 (A1), HCR/A2 (A2), HCRc/A1 (cA1) and HCRc/A2 (cA2) (0.5 μ g) were spotted, in duplicate, onto nitrocellulose strips. Strips were probed with sera from mice vaccinated with 1 μ g (1:20000) or mice vaccinated with 0.1 μ g (1:2000) of HCR/A1 (A1 vaccinate) or HCR/A2 (A2 vaccinate) and challenged with the heterologous BoNT/A (A2 challenge or A1 challenge), bound IgG was detected with goat anti- mouse IgG-HRP. Strips were incubated in Super Signal and light emission was imaged. Backgrounds were subtracted using 3X-FLAG HCR/Tetanus toxin as a control protein. P values: ns= not significant, *=0.05, **=0.01, and *** =0.001. In A1 Vaccinate: A2 Challenge (0.1 μ g), 7 of 10 mice survived. In A2 Vaccinate: A1 Challenge (0.1 μ g), 4 of the 10 mice survived. In the A1 Vaccinate: A2 Challenge (1 μ g), 5 of 5 mice survived. In the A2 Vaccinate: A1 Challenge (1 μ g), 5 of the 5 mice survived.

Figure 6. IgG titers to HCR and HCRc following low dose HCR/A subtype vaccination
HCR/A1 (A1), HCR/A2 (A2), HCRc/A1 (cA1) and HCRc/A2 (cA2) (0.5 μ g) were spotted, in duplicate, or boiled for 5 min in the presence of 0.1% SDS and spotted onto nitrocellulose strips. Strips were probed with sera (1:2000) from mice vaccinated with 0.1 μ g of HCR/A1 (A1 vaccinated) or HCR/A2 (A2 vaccinated) and challenged with the heterologous BoNT/A (A2 challenge or A1 challenge). Strips were incubated with goat anti-mouse IgG-HRP, incubated in Super Signal, and light emission was imaged. Backgrounds were subtracted using 3X-FLAG HCR/TeNT as a control protein. P values: ns= not significant, *=0.05. In A1 Vaccinate: A2 Challenge, 7 of 10 mice survived. In A2 Vaccinate: A1 Challenge, 4 of the 10 mice survived.

Table 1

Primary amino acid identity among the BoNT/A subtypes A1–A5^a

Subtype	% identity to BoNT/A1 ^b				
	Total (1296 aa)	LC (1–448 aa)	HC (449–1296 aa)	HCR (870–1296 aa)	HCR _C (1096–1296 aa)
A2	89	95	87	87	90
A3	84 (1292 aa)	81	86	86	90
A4	89	88	89	91	97

^a A1, C. botulinum A str. ATCC 3502 accession number YP_001253342; A2, C. botulinum A2 str. Kyoto F accession number YP_002803127; A3, C. botulinum A3 str. Loch Maree accession number YP_001715703; and A4, C. botulinum Ba4 str. 657Ba accession number YP_002860313.

^b Identity determined by BLASTP (PubMed) analysis

Table 2

Cross protection of BoNT/A subtype challenge by subunit HCR/A vaccination.

HCR/A immunogen (number of immunizations) ^a	Survival of BoNT/A subtype challenge (1000 LD50)		
1.0 µg (3) ^a	BoNT/A1	BoNT/A2	BoNT/A3
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
1.0 µg (2) ^b			
A1	4/5	5/5	4/6
A2	5/5	4/5	6/6
0.1 µg (2) ^b			
A1	9/10	7/10	5/10
A2	4/10	10/10	7/10

^a Mice were immunized with 1.0 µg of the indicated HCR/A subtype (A1–A4) with alum for the primary (day 1) and secondary (day 14) and alone on day 28. On day 35 sera was collected and on day 37 mice were challenged with 1,000 LD50 of the indicated BoNT/A subtype. Survival was scored at 96 hr. Controls showed that immunization with adjuvant alone did not protect mice (0/4 survived) from challenge with 10 LD50 BoNT/A1, BoNT/A2, or BoNT/A3 and that unvaccinated mice did not survive a challenge by 2 LD50 of BoNT/A1, BoNT/A2, or BoNT/A3 (0/2 survived).

^b Mice were immunized with 0.1 µg or 1.0 µg of the indicated HCR/A subtype (A1/A2) with alum for the primary (day 1) and secondary (day 14). On day 20 sera was collected and on day 22 mice were challenged with 1,000 LD50 of the indicated BoNT/A subtype. Survival was scored at 96 hr.