A recombinant Holo-toxoid Vaccine against Botulism

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Abstract

The botulinum neurotoxins (BoNT) are the most toxic proteins for humans and designated Category A Select Agents. The current vaccine against botulism is in limited supply and there is a need to develop new vaccine strategies. A recombinant BoNT/A toxoid was produced in *Clostridium botulinum* that contained a double amino acid substitution, R363A, Y365F (termed BoNT/A<sup>RYM</sup>). BoNT/A<sup>RYM</sup> was non-catalytic for SNAP25 and non-toxic for mice. Immunization with BoNT/A<sup>RYM</sup> protected mice from challenge at levels that were similar to chemically inactivated BoNT/A toxoid. BoNT/A<sup>RYM</sup> elicited an immune response against the light chain and heavy chain components of the toxin. Neutralizing anti-BoNT/A<sup>RYM</sup> sera blocked BoNT toxicity in primary cortical neurons and blocked ganglioside binding by the heavy chain. BoNT/A<sup>RYM</sup> represents a viable vaccine candidate for a holo-toxoid against botulism.
Introduction

The neurotoxins of *Clostridium botulinum* (BoNTs) are the most potent protein toxins for humans and are included in the list of Category A Select Agents and Toxins (16, 17, 24, 25). BoNTs comprise seven distinct neurotoxins – designated A to G (BoNT A-G); which are differentiated serologically by specific neutralization. Polyclonal antibodies derived for a specific neurotoxin can neutralize the toxic effects of that toxin but will not cross-neutralize another toxin serotype (31).

BoNTs are produced as 150 kDa nontoxic single-chain proteins that are activated by proteolytic cleavage to a di-chain molecule (16, 17, 24, 25). BoNTs comprise three functional domains, organized as an N-terminal catalytic domain (light chain, LC), an internal translocation domain (heavy chain translocation, HCT), and a C-terminal receptor binding domain (heavy chain receptor, HCR) (20). BoNTs enter neurons via receptor mediated endocytosis where neuronal tropism involves BoNT binding to unique host cell receptors. Fundamental studies by Chapman and colleagues demonstrated that luminal domain fragments of synaptic vesicle proteins synaptotagmin I and synaptotagmin II mediate the binding and entry of BoNT/B into cultured neuroendocrine cells (11). A similar approach demonstrated that Syt I and Syt II also mediate the binding and entry of BoNT/G, whereas isoforms of synaptic vesicle protein 2 (SV2) mediated the binding and entry of BoNT/A into cultured neurons (12, 27). Upon
delivery into the cytosol, LC cleaves one or more of the neuronal SNARE proteins SNAP25, Syntaxin and Synaptobrevin (16, 17, 24, 25).

The threat of bioterrorism has stimulated renewed efforts to generate vaccines and therapies against agents such as BoNTs. There are two available therapies against botulism, the human Botulinum Immune Globulin product (BabyBIG) used in infant botulism cases against type A and B toxins and an equine-based antitoxin treatment and concurrent intubation and ventilation assistance for respiratory failure (1, 22). Immunization with chemically detoxified proteins has led to successful vaccines against several bacterial pathogens. This approach was also used for generation of the current vaccine against botulism (1, 8). However, at present, there are no licensed vaccines for preventing botulism. The US Centers for Disease Control and Prevention (CDC) currently distributes a pentavalent (ABCDE) toxoid under Investigational New Drug status to individuals at high risk of exposure (1). The vaccine is composed of formalin inactivated crude isolates of BoNTs absorbed to aluminum phosphate and containing thimerosal as a preservative. This is a relatively impure preparation that is difficult to produce, which combined with the limited supply of this vaccine highlights the need to develop more efficient approaches for vaccine development against botulism.

There is a need to develop novel strategies for vaccine and therapies against botulism. Several studies have reported the immunogenic properties of
bacterial- or yeast-derived HCRs directed against various BoNT serotypes (5, 9, 13, 21, 30, 32). The HCR component of BoNTs has several potential advantages over currently available *C. botulinum* derived antigens. Production of HCR in a heterologous system facilitates large scale production and removes the possibility of contamination with other neurotoxins and clostridial components. We recently reported the neutralizing capacities and immunogenic properties of *E. coli*-derived HCRs directed against BoNT serotypes A and E. The immunogenic potency of the *E. coli*-derived HCRs demonstrated the suitability of this strategy for the development of the next generation of vaccines against botulism (5). However, recent studies demonstrated that the Light chain (LC) of BoNT/A alone can stimulate a protective immune response. These observations indicate the presence of neutralizing epitopes on the LC and suggest that a holotoxin-derived immunogen may constitute an optimal BoNT vaccine. Here we report the generation of a genetically engineered holo-toxoid that elicits a protective immune response similar to chemically inactivated BoNT.
Materials and Methods

Materials. Unless otherwise stated, molecular biology grade chemicals and reagents were obtained from Sigma-Aldrich Co. Restriction enzymes were from New England Biolabs. Bacterial culture media and components were purchased from Becton Dickinson and Thermo Fisher Scientific. Anti-SNAP25 monoclonal antibody clone MC-6053 was purchased from R&D Systems. Sprague-Dawley rat embryonic day 18 cortices were from Brainbits LLC and handled as described by the supplier. BoNT/A was purified as described previously (23) and was below the maximum amounts excluded from regulation as defined by the CDC Select Agent Program.

Construction and Expression of recombinant HCR/A in Escherichia coli. The construction and expression of HCR/A in E. coli has been described in detail previously (5).

Construction and Expression of recombinant BoNT/A<sup>RYM</sup> in C. botulinum. In order to comply with the Guidelines for Research involving recombinant DNA molecules, specifically, cloning of toxin genes with LD<sub>50</sub> of less than 100 ng/kg of body weight (NIH Guidelines), the gene for expression of non-toxigenic BoNT/A holotoxin was constructed in several steps. First, the LC and HC regions of the neurotoxin gene were amplified by PCR in separate reactions using C. botulinum chromosomal DNA from strain ATCC 3502 as a template. Unique restriction sites for SacI (5’) and BgII (3’) were added to the LC/A gene fragment (coding for
amino acid residues 1-450). The PCR fragment was inserted into a TA cloning vector pGEM-T (Promega) and the nucleotide sequence of the cloned fragment verified. Next, site-directed mutagenesis was performed to introduce two mutations in the catalytic region of the LC/A, Arg362Ala and Tyr365Phe, using a QuickChange kit (Stratagene) following the manufacturers instructions. The HC/A gene region (coding for amino acid residues 451-1296) with added restriction sites for BamHI (5’) and SphI (3’) was inserted into a modified pCITE4a(+) vector (Novagen), and the HC/A nucleotide sequence was verified. The modifications of the pCITE4a(+) vector included insertion of additional restriction sites into the multicloning site and the histidine tag (C-terminal His x 6), followed by the stop codon. The next step was insertion of the altered LC/A_RYM in front of the HC/A, to form a recombinant BoNT/A_RYM gene with a C-terminal His-tag. During the last cloning step, six additional nucleotides were introduced in the junction site between the LC and HC regions (BglII/BamHI), thus two additional amino acid residues, Arg and Ser, were added to the recombinant holotoxin. To remove these extra nucleotides from the junction site, the neurotoxin gene region between restriction sites Bsu361 and BstBI (nucleotides1140-2317) was amplified by PCR using chromosomal DNA of C. botulinum strain ATCC 3502 as a template. Then the Bsu361 and BstBI fragment in the recombinant gene was replaced with the same DNA fragment from the wild type gene, and the nucleotide sequence of final gene construct was verified. Lastly, the recombinant BoNT/A_RYM gene was inserted into a clostridial expression vector pMTL9361 (N. P. Minton, University of Nottingham, UK) between restriction sites SacI and NotI.
The resulting plasmid, pMVP410 contained a recombinant nontoxigenic neurotoxin gene, which differs from the wild type gene by two mutations (Arg362Ala and Tyr365Phe) in the catalytic region and a C-terminal His-tag.

Introduction of the pMVP410 expression vector into nontoxigenic C. botulinum strain LNT01. The expression vector pMVP410 was first transformed into E. coli donor strain CA434, and then transferred by conjugation to a nontoxigenic C. botulinum strain LNT01 as described previously (7).

Purification of pMVP410BoNT/A<sup>RYM</sup>. The recombinant holotoxin (BoNT/A<sup>RYM</sup>) was purified from C. botulinum strain LNT01 carrying expression vector pMVP410. Cultures were grown in an anaerobic chamber (ThermoForma), using initial gas mixture comprised of 80% N<sub>2</sub>/10% CO<sub>2</sub>/10% H<sub>2</sub>. Cultures were incubated at 37° C in 1 liter of TPM media (2% casein hydrolysate [NZ Case TT]. 1% yeast extract, and 0.5% glucose, pH 7.2) supplemented with 10 mM EGTA and 50 µg/ml erythromycin. When the culture reached 0.3 OD<sub>600</sub>, toxin expression was induced by addition of 1mM IPTG and then the culture was further incubated at 30° C for an additional 18 hours. Bacterial cells were precipitated by centrifugation at 6,000 x g for 15 min at 4° C. The cell pellet was suspended in 50 ml of buffer (0.2 M KH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, pH6.0), stirred gently for 2 hours at room temperature, followed by centrifugation at 10,000 xg for 20 min at 20° C to clear the cell extract. BoNT/A<sup>RYM</sup> was purified from the clarified lysate using chromatography on Ni<sup>2+</sup>-NTA resin (5 ml bed volume, Qiagen). Protein samples
from the chromatography fractions were separated by SDS-PAGE using a 12% NuPAGE gel (Invitrogen) followed by a semi-dry transfer to an Immobilon-P membrane (Millipore). A Western blot was preformed using a 6-His monoclonal antibody (Covance) as a primary antibody and the His-tag AP Western blot kit (Novagen). Protein concentrations were determined using a BCA protein assay kit (Pierce).

**Determination of BoNT/A\textsuperscript{RYM} toxicity.** Recombinant BoNT/A\textsuperscript{RYM} was tested for toxicity in both the single and di-chain forms. The native single chain recombinant BoNT/A\textsuperscript{RYM} was converted to a di-chain form by mild trypsinization in 0.1 M Na-Acetate buffer pH 6.0 (40:1, wt:wt, BoNT/A\textsuperscript{RYM}: trypsin, 37°C, 30 minutes). SDS-PAGE analysis of the trypsinized BoNT/A\textsuperscript{RYM} showed a single band of ~150 kDa in the unreduced sample and two bands of ~100 kDa and ~50 kDa in the reduced sample corresponding to the heavy chain and light chain domains respectively. Female ICR mice (18-22 g) were injected intraperitoneally with up to 1 μg/mouse of either the single chain or di-chain forms of the recombinant BoNT/A\textsuperscript{RYM} and monitored for 96 hours at which point survival was scored. All mice survived challenge with up to 1 μg of the single or di-chain form of the recombinant BoNT/A\textsuperscript{RYM} and showed no symptoms of botulinal intoxication.

**Immunization of mice with recombinant HCR fragments and BoNT\textsuperscript{RYM}**. Female ICR mice (18 to 22 g) were immunized intraperitoneally with either 1 μg or 0.1 μg total protein (recombinant HCRs or BoNT/A\textsuperscript{RYM}) in PBS mixed with an equal
volume of Alhydrogel as adjuvant. Mice were vaccinated at 0, 14 and 28 days, the adjuvant was omitted for the final boost. Seven days after the final boost mice were challenged with the indicated amount of the native BoNT holotoxins and monitored for 96 h, at which point survival was scored.

ELISA. Native BoNT/A was diluted to 0.5 µg/ml in coating buffer (50 mM Na₂CO₃, pH 9.6) and 100 µl was added to each well of an enhanced binding enzyme-linked immunosorbent assay (ELISA) plate (enzyme immunoassay/RIA high binding plate; Corning) and allowed to adhere overnight at 4°C. Column 1 was incubated with coating buffer alone (no-antigen control). Plates were then washed four times with 400 µl PBS and blocked for 1 h at 37°C with 200 µl per well of 2% (wt/vol) bovine serum albumin in coating buffer. Following a washing step as outlined above, plates were incubated for 1 h at 37°C with serial dilutions of the sera in binding buffer (1% wt/vol bovine serum albumin in PBS, 100 µl per well). As controls, no antigen was incubated with the lowest dilution of the serum, while no primary antibody was incubated with binding buffer alone. Following a washing step, plates were incubated for 1 h at 37°C with either donkey anti-mouse or donkey anti-mouse immunoglobulin G- horseradish peroxidase conjugate (1:12,000) in binding buffer. Plates were washed six times with 400 µl PBS and then incubated with 100 µl per well of Ultra-TMB (tetramethyl benzidine, Pierce) as substrate. The reaction was terminated by addition of 100 µl per well 0.2 M sulfuric acid and absorbance read at 450 nm using an ELISA plate reader (Victor 3V, Perkin Elmer).
Ganglioside binding assay. Porcine brain gangliosides (Avanti Polar Lipids) were dissolved in methanol and applied to high-affinity 96-well plates (0.2 µg mixed gangliosides in 100 µl/well). The solvent was evaporated at RT and wells were washed with PBS. Non-specific binding sites were blocked by incubating for 1 hr in sodium carbonate buffer, pH 9.6 supplemented with 1% w/v BSA. Binding assays were performed in binding buffer (10 mM Tris-HCl, 150 mM NaCl, 1% w/v BSA, pH 7.6, 100 µl/well) for 1 h at RT containing wild-type or mutated HCR/A domains (150 nM final concentration) which had been pre-incubated for 30 mins at 37°C with either control mouse serum or sera from mice immunized with antigens. Unbound protein was removed by three washes each with 400 µl of PBS. Bound HCR domains were detected by incubation with α-FLAG M2 monoclonal antibody-HRP for 15 min at 4°C. TMB-Ultra served as substrate for HRP. The reaction was terminated by addition of 0.2 M H₂SO₄ and the absorbance at 450 nm was determined using an ELISA plate reader (Victor 3V, Perkin Elmer).

Neutralization of BoNT activity in rat cortical neurons. Rat cortical neurons were cultured on laminin coated glass coverslips in Neurobasal medium supplemented with 2 mM glutamine, 55 µM β-mercaptoethanol and B27 supplement for 10-14 days prior to use. Cells were treated with Low K⁺ solution (15 mM Hepes, 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 0.5 mM ascorbic acid, and 0.1% BSA, pH 7.4) or high K⁺ solution (same as control
solution but adjusted to 95 mM NaCl and 56 mM KCl) for 15 min at 37°C, in the
presence of 10 nM BoNT/A which had been pre-incubated for 30 mins at 37°C
with either control mouse serum or sera from mice immunized with antigens.

Cells were washed with PBS and incubated for a further 48 hr at 37°C in fresh
Neurobasal media : conditioned Neurobasal media (1:1). Following treatment,
cells were washed three times with PBS, fixed with 4% w/v paraformaldehyde in
PBS (15 min at RT), permeabilized with 0.1% Triton X-100 / 4% formaldehyde in
PBS (for 10 min at RT), and stained with mouse α-SNAP25-C (clone MC-6053).

Staining was visualized using goat anti-mouse IgG conjugated to Alexa Fluor 568
dye (Molecular Probes). Images were captured at room temperature using a
Nikon TE 2000 microscope equipped with a CFI Plan Apo VC 60X Oil, N.A. 1.4
type lens and a Photometrics CoolSnap EZ camera. Image acquisition and
subsequent analysis was performed using Metamorph version 7. Figures were
compiled using Photoshop CS2 (Adobe).

Cleavage of SNAP25bHA. Endopeptidase activity of the recombinant LC proteins
was assayed in 40-µl reaction mixture containing: 2 µg GST-SNAP25b and
indicated concentrations of LC/A or LC/A_RYM. After incubation for 5 mins in
reaction buffer A (20 mM K⁺-HEPES, pH 7.4, 150 mM potassium glutamate +/-
20 µM ZnCl₂) at 37°C, reactions were stopped by adding an equal volume of 2×
SDS–PAGE sample buffer. The products were resolved by electrophoresis on a
10% SDS-PAGE gel and stained with Coomassie Blue.
Results

The crystal structure of LC/A containing a double mutation at Arg362 and Tyr365 was recently solved and observed to retain a three dimensional structure that could be superimposed upon wild type LC/A, indicating that these mutations did not perturb LC/A structure (14). Previous reports demonstrated single mutations at Arg and Tyr did not influence substrate binding affinity, but reduced the catalytic rate (6). rLC/A (residues 1-425) containing the double mutation R362A, Y365F (hereafter referred to as LC/A$^{RYM}$ or BoNT/A$^{RYM}$) failed to cleave human SNAP25b at all tested concentrations. Addition of exogenous Zn$^{2+}$ to the reactions did not stimulate catalysis, which is consistent with the proposed role of Arg362 and Tyr365 in facilitating the proper alignment of His222 and Glu261, respectively, for the Zn$^{2+}$ coordination sphere. Thus, the lack of catalytic activity and conservation of overall LC/A$^{RYM}$ structure validates BoNT/A$^{RYM}$ holotoxoid as a vaccine candidate.

BoNT/A$^{RYM}$ expression system. Cloning and expression of BoNT/A$^{RYM}$ was facilitated with the use of a clostridial expression vector pMTL9361. In addition to the pCD6 replicon and erythromycin selection marker, the plasmid contained a $\text{P}_{\text{fac}}$ promoter / operator and an oriT region to allow mobilization from $\text{E. coli}$ donors. The recombinant BoNT/A$^{RYM}$ gene containing a C-terminal His-tag was inserted into the vector yielding the expression vector pMVP410. The BoNT/A$^{RYM}$ expression vector was introduced into the nontoxigenic $\text{C. botulinum}$ strain
LNT01 by conjugal transfer from *E. coli* donor strain CA434 as described previously (7). Plasmid pMVP410 was stably maintained in *C. botulinum* in the presence of erythromycin (50 µg/ml) and produced approximately 1.0 mg of BoNT/A<sup>RYM</sup> from 1 liter batch culture that was purified by Ni<sup>2+</sup>-NTA chromatography.

**Determination of BoNT/A<sup>RYM</sup> toxicity.** While mutation of Arg362 and Tyr365 resulted in a catalytically inactive LC *in vitro*, experiments were performed to determine residual LC/A<sup>RYM</sup> catalytic activity that could cause toxicity when introduced into the holo-BoNT. To address this concern, mice were challenged with 1 µg of purified BoNT/A<sup>RYM</sup> (~3.3 × 10<sup>4</sup> mouse LD<sub>50</sub>) and monitored for 96 h. All mice survived challenge with 1 µg of single-chain or trypsin nicked di-chain BoNT/A<sup>RYM</sup>.

**Immune protection of HCR/A, BoNT/A toxoid or BoNT/A<sup>RYM</sup> against BoNT/A.** Previously we demonstrated the efficacy of HCR/A and HCR/E as vaccine candidates against challenge with the homologous BoNT serotype (5). These observations prompted the subsequent studies that addressed the suitability of BoNT/A<sup>RYM</sup> as an alternate vaccine candidate.

Mice were immunized with either 0.1, 1, or 10 µg HCR/A, BoNT/A toxoid or BoNT/A<sup>RYM</sup> in aluminum hydroxide adjuvant (Alhydrogel) and challenged with either 1000 or 10,000 mouse LD<sub>50</sub> of BoNT/A. As reported previously, mice
immunized with HCR/A were protected against subsequent challenge with BoNT/A (Table 1). Mice immunized with 1.0 or 10.0 µg of each of the three vaccines were protected against challenge by either 1000 or 10,000 LD$_{50}$ of BoNT/A, while vaccination with 0.1 µg of vaccine provided at least a partial protection against challenge with 1000 LD$_{50}$ of BoNT/A when immunized with BoNT/A toxoid or BoNT/A$^{RYM}$ where in one experiment mice were partially protected against challenge with 1000 mouse LD$_{50}$ of BoNT/A, and completely protected in the second experiment. This indicated that 0.1 µg of these vaccines approximates a minimal protective dose at a 1000 LD$_{50}$ challenge of BoNT/A in this mouse model of intoxication.

Immunoreactivity of mouse anti-HCR/A, BoNT/A toxoid and BoNT/A$^{RYM}$ antibodies. Pooled sera isolated from mice immunized with either 0.1 or 1 µg of antigens were analyzed by ELISA and Western blotting. ELISA analysis using native BoNT/A as antigen confirmed that sera of mice from 3 independent immunizations were immunoreactive and displayed similar titers (Figure 1A). Sera from mice immunized with either HCR/A, BoNT/A toxoid or BoNT/A$^{RYM}$ reacted against both the heavy chain of native BoNT/A and recombinant HCR/A as determined by Western blotting (Figure 1B). Sera from mice immunized with BoNT/A toxoid or BoNT/A$^{RYM}$, but not HCR/A, also reacted with the LC of BoNT/A. This demonstrates the immunogenicity of the LC within BoNT/A toxoid or BoNT/A$^{RYM}$ and the specificity of the immune response to each immunogen.
Inhibition of BoNT/A activity with anti-sera in cultured rat neurons. At present the mechanisms of serum neutralization remain largely unknown. To address this question, the ability of sera from mice resistant to BoNT intoxication, was tested for the ability to neutralize BoNT/A toxicity of cortical neurons. Treatment of cortical neurons with BoNT/A alone or with BoNT/A incubated with control mouse serum resulted in cleavage of SNAP-25, as visualized by staining with a monoclonal antibody which specifically recognizes only the cleaved form of SNAP-25 (Figure 2) (12). In contrast, incubation of sera from mice immunized with chemically inactivated BoNT toxoid, BoNT/A$_{RYM}$, or HCR/A blocked the action of BoNT/A on the cultured neurons. The neutralizing capacity and unique serum reactivity of the mouse sera promoted subsequent studies to determine the mechanisms of antibody neutralization.

Inhibition of BoNT/A binding to mixed gangliosides by anti-sera. The interaction of BoNT/A with gangliosides is well documented and is proposed as an initial step in BoNT/A intoxication of the neuromuscular junction (4). To further elucidate the mechanisms of BoNT immune sera inhibition, a solid phase ganglioside binding assay was developed. Porcine brain gangliosides were immobilized on microtiter plates and incubated with either wild-type HCR/A or a mutated HCR/A (W1266L) which was previously shown to lack ganglioside binding (28). The amount of bound protein was quantified by employing a monoclonal antibody against the N-terminal FLAG epitope of recombinant HCR/A. Wild-type HCR/A efficiently bound to porcine brain gangliosides in a
dose dependent manner while HCR/A\textsuperscript{W1266L}, showed a reduction in ganglioside binding relative to wild-type HCR/A (Figure 3). Control experiments showed that in the absence of ganglioside coating, HCR/A binding was not detected above blank wells (data not shown). Pre-incubation of HCR/A with anti-sera against chemically inactivated BoNT toxoid, BoNT/A\textsuperscript{RYM}, or HCR/A, but not control mouse sera, inhibited ganglioside binding by HCR/A (Figure 3). Thus, serum neutralization of BoNT is mediated, at least in part, by inhibition of ganglioside binding. To our knowledge this is the first demonstration that neutralizing sera block the interaction of BoNTs with their cognate ganglioside receptors.
Discussion

Effective vaccines and therapies against botulinum neurotoxins are limited. A pentavalent vaccine that protects against BoNT(A-E) and a separate monovalent vaccine that protects against BoNT serotype F are available, but have several limitations including cost, efficacy and accessibility. The use of recombinant-derived HCRs and the recombinant holotoxin-derived vaccine described in the current study constitute BoNT vaccines which can be produced in large quantities and under low bio-containment. In the present study, development of a recombinant catalytically inactive holotoxin based upon a previously described mutated LC/A is described. In agreement with previous observations, mutation of Arg362 and Tyr365 to alanine and phenylalanine respectively, reduced the catalytic rate of LC/A to undetectable levels (6). The recently solved crystal structure of LC/A R362A / Y365F (hereafter referred to as LC/A\(^{RYM}\)) revealed conservation of overall structure relative to the wild type LC/A further validating BoNT/A\(^{RYM}\) as a potential vaccine candidate (5).

Recombinant BoNT/A\(^{RYM}\) holotoxin gene was inserted into a clostridial expression vector pMTL9361, making pMVP410 and the recombinant holotoxin was expressed in \(C.\ botulinum\) strain LNT01 (7, 26). The non-toxigenic \(C.\ botulinum\) type A strain (LNT01) was chosen as the expression strain because it is a native host for botulinum neurotoxins and it does not produce any neurotoxin to interfere with purification of modified BoNT derivatives. The purified holotoxin was non-toxic \textit{in vivo}, and did not cleave SNAP-25 \textit{in vitro}. Aside from the loss of
toxicity the other major difference between the recombinant and native BoNT/A toxins was that the former was not nicked. *C. botulinum* strain LNT01 is a transposon Tn916 mutant of 62A (18). Upon transposon insertion this strain has lost a 32.5 kb region containing the entire neurotoxin gene cluster and regions flanking the cluster (unpublished data). A gene encoding a protease responsible for the cleavage of the neurotoxin may be located within the region deleted from this strain. Our previous studies have shown that BoNT gene clusters and their flanking regions are highly homologous between 62A and the genome strain ATCC 3502. Analyzing descriptions of putative ORFs in the region deleted from LNT01, the most likely candidate for nicking the neurotoxin gene is an ORF (CBO0812), a putative amidohydrolase (29). This ORF is located 4 kb downstream of the toxin gene therefore its close proximity to the cluster suggests a possible role in toxin formation and activation. However, more studies are necessary to determine the role and functions of the CBO0812. Since the BoNT/A<sup>RYM</sup> could be readily nicked by trypsin treatment, this was not expected to have a major influence on neurotoxin properties, because all serotypes of BoNTs are initially produced as single-chain polypeptides that are subsequently cleaved by proteases to yield fully active double-chain molecules. The single chain form of serotype E is associated with human disease where gut proteases nick and activate the toxin (19).

The ability of BoNT/A<sup>RYM</sup> to stimulate protective immunity was tested following i.p. injection of antigen and compared to recombinant HCR/A and
formalin inactivated BoNT/A toxoid. Mice immunized with ≥ 1 µg HCR/A,
BoNT/A<sup>RYM</sup> or BoNT/A toxoid were resistant to challenge with at least 10,000
mouse LD<sub>50</sub>. The loss of protection occasionally observed when the BoNT/A<sup>RYM</sup>
dose was lowered to 0.1 µg could be overcome by pre-treatment of the protein
with formalin (data not shown), suggesting the native antigen may be less stable
than the recombinant HCR protein. Alternatively, formalin treatment may
increase the immunogenicity of the antigen through cross-linking protein
molecules. ELISA and Western blot analysis demonstrated the production of
antibodies against native BoNT/A toxin. Sera from mice immunized with
BoNT/A<sup>RYM</sup> or chemically inactivated BoNT/A toxoid – but not HCR/A, was
reactive against LC/A demonstrating both the antigenicity of LC/A and the
specificity of the observed antibody response. The three immunogens stimulated
a strong antibody response against HC/A with similar titers supporting earlier
observations that the HCR domain contains immuno-dominant epitopes (2).
Moreover, antibodies against HCR/A, BoNT/A<sup>RYM</sup> or BoNT/A toxoid were able to
neutralize the activity of the toxin in a cultured neuron model (Figure 2).

Despite the widely reported neutralizing epitopes within the HCR subunit,
the role of anti-HCR antibodies in toxin neutralization remains largely unknown.
Based on the longstanding double receptor model proposed by Montecucco (3,
25), BoNT is proposed to interact initially with lipid- and/or protein-linked
oligosaccharides such as ganglioside G<sub>T1b</sub>, concentrating the toxin on the
presynaptic membrane. Subsequent to initial capture, calcium influx resulting
from an action potential stimulates synaptic vesicle membrane fusion. This
exposes synaptic protein complexes to the extra-cellular milieu allowing
neurotoxin binding and subsequent uptake through clathrin mediated
endocytosis. Two independent studies on the interaction of BoNT/B with its
cognate protein receptor, synaptotagmin II, have provided the first physical
evidence for this model (4, 10, 15).

The observation that the synaptotagmin-binding domain is adjacent to the
ganglioside-binding site raises the possibility that neutralizing antibodies against
the HCR domain may block one or both of the binding sites. In support of this
model, antibodies against HCR/A, BoNT/A_RYM or BoNT/A toxoid were
demonstrated to inhibit the interaction of HCR/A with immobilized porcine
gangliosides (Figure 3). Neutralizing BoNT/A anti-sera also blocked the
intoxication of primary cortical neurons by BoNT/A, implicating the ability of the
anti-sera to block the interaction of BoNT/A with receptors. However, this
interpretation is complicated by the need to perform the binding component of the
experiment at 37 °C, since the exposure of the BoNT receptor requires synaptic
vesicle fusion to the plasma membrane (12). While the interaction of SV2 with
BoNT/A can occur independent of gangliosides (12), the possibility remains that
inhibition of BoNT/A intoxication of primary neurons is due to steric hindrance
due to the presence of antibody bound to the ganglioside binding pocket.
This study describes the development of an expression and purification strategy for a catalytically inactive BoNT/A holotoxin. The immunogenic potency of the BoNT\textsuperscript{RYM} protein observed in the current study represents a tool that allows genetic manipulation to develop the next generation of vaccines and therapies against botulism, as well as reagents to elucidate the mechanisms of serum neutralization and the cell biology of BoNT intoxication of neurons.

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Sequence variation within botulinum neurotoxin serotypes impacts antibody

2000. Modeling Pichia pastoris growth on methanol and optimizing the
production of a recombinant protein, the heavy-chain fragment C of botulinum
Table 1 – Protection from BoNT/A intoxication by BoNT/A antigens

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<th>Antigen</th>
<th>Dose (µg antigen)</th>
<th>Mouse Survival&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>Exp. 1 1000 LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Exp. 2 1000 LD&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>rHCR/A</td>
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<td>3/3</td>
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<tr>
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<tr>
<td>BoNT/A Toxoid</td>
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<sup>a</sup> Mice were immunized with the indicated antigen and then challenged by the indicated amount of BoNT/A. Mice were observed for 96 hr and scored for survival or death.

<sup>b</sup> Each experiment is an independent replicate.

* One mouse died of symptoms not consistent with botulinal intoxication.
Figure 1 – Immunological characterization of mouse anti-sera to BoNT derivatives. (A) ELISA of mouse anti-sera to native BoNT/A (50 ng per well toxin as capture antigen) was performed as described in the methods section, using antisera generated against, HCR/A (HCR serum, ■), BoNT/A<sup>RYM</sup> (RYM serum, ▲) chemically inactivated BoNT/A (Toxoid serum, ●) or control serum (♦). (B) rHCR/A (0.35 µg, left lanes) and BoNT/A (1 µg, right lanes), were separated by SDS-PAGE and visualized by Western blotting with mouse anti-sera raised against HCR/A (α-HCR), BoNT/A<sup>RYM</sup> (α-RYM) and chemically inactivated BoNT/A (α-Toxoid). * indicates the position of rHCR/A, arrows indicated positions of full length BoNT/A (Non-reduced BoNT/A), BoNT/A HC, and BoNT/A LC. The blot shown is a composite of three independent experiments with the indicated primary anti-sera.
Figure 2 – Neutralizing sera inhibit uptake of BoNT/A into primary cortical neurons. BoNT/A (10 nM) was incubated for 30 min at 37°C in the buffer alone or in buffer with mouse anti-sera raised against HCR/A (α-HCR), BoNT/A<sup>R</sup>-RYM (α-RYM), and chemically inactivated BoNT/A (α-Toxoid) at the indicated dilutions. Following incubation, cortical neurons were exposed to each of the reaction mixtures for 15 min at 37°C. Cells were then rinsed and incubated for an additional 48 h at 37°C. Cleavage of SNAP25 by BoNT/A was detected using anti-SNAP25-C that recognizes the cleaved form of SNAP-25 (panel A). SNAP25 cleavage by BoNT/A was reduced ~80% by sera against HCR/A, BoNT/A<sup>R</sup>-RYM and BoNT/A toxoid (P<0.001, T-Test), but not by control serum (1/100 dilution, P>0.05, T-Test) (panel B).
Figure 3 – BoNT/A neutralizing sera block BoNT/A binding to gangliosides.

HCR/A (150 nM) was incubated for 30 min at RT in buffer alone or in buffer with mouse anti-sera raised against HCR/A (HCR), BoNT/A<sup>RYM</sup> (RYM), and chemically inactivated BoNT/A (Toxoid) as indicated. Following incubation, reaction mixtures were incubated with mixed gangliosides for 2 h at 4°C and binding was determined with a FLAG specific antibody-HRP conjugate, using TMB-Ultra as substrate. Data represent mean values ± S.D. of three to four independent experiments performed in triplicate.
A

![Graph showing absorbance (450 nm) against serum titer for different sera. The x-axis represents serum titer (10^2 to 10^6), and the y-axis represents absorbance. The graph includes data points for control serum, HCR serum, RYM serum, and toxoid serum, each represented by different markers.]

B

![Image of a gel electrophoresis with markers for α-HCR, α-RYM, and α-Toxoid. The gel shows bands for non-reduced BoNT/A, BoNT/A HC, BoNT/A LC, and rHCR/A.]

- Control serum
- HCR serum
- RYM serum
- Toxoid serum

- Non-reduced BoNT/A
- BoNT/A HC
- BoNT/A LC
- rHCR/A
The graph shows the absorbance (450 nm) for different treatments of HCR/A and HCR/A (W1266L) with α-sera. The treatments include no HCR, premixed with α-HCR/A, α-RYM, and α-Toxoid. The absorbance values are normalized and indicated by the bars.