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Enhancing toxin-based vaccines against botulism

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Abstract

Botulinum neurotoxins (BoNT) are the most toxic proteins for humans. BoNTs are single chain proteins with an N-terminal light chain (LC) and a C-terminal heavy chain (HC). HC comprises a translocation domain (HC_N) and a receptor binding domain (HC_C). Currently, there are no approved vaccines against botulism. This study tests a recombinant, full-length BoNT/A1 versus $LCHC_N$/A1 and HC_C/A1 as vaccine candidates against botulism. Recombinant, full-length BoNT/A1 was detoxified by engineering 3-amino acid mutations (E224A/R363A/Y366F) (M-BoNT/A1) into the LC to eliminate catalytic activity, which reduced toxicity in a mouse model of botulism by > 10^6-fold relative to native BoNT/A1. As a second step to improve vaccine safety, an additional mutation (W1266A) was engineered in the ganglioside binding pocket, resulting in reduced receptor binding, to produce M-BoNT/$A1^W$. M-BoNT/$A1^W$ vaccination protected against challenge by 10^6 LD_{50} Units of native BoNT/A1, while M-BoNT/A1 or M-BoNT/$A1^W$ vaccination equally protected against challenge by native BoNT/A2, a BoNT subtype. Mice vaccinated with M-BoNT/$A1^W$ surviving BoNT challenge had dominant antibody responses to the $LCHC_N$ domain, but varied antibody responses to HC_C. Sera from mice vaccinated with M-BoNT/$A1^W$ also neutralized BoNT/A1 action on cultured neuronal cells. The cell- and mouse-based assays measured different BoNT-neutralizing antibodies, where M-BoNT/$A1^W$ elicited a strong neutralizing response in both assays. Overall, M-BoNT/$A1^W$, with defects in multiple toxin functions, elicits a potent immune response to BoNT/A challenge as a vaccine strategy against botulism and other toxin-mediated diseases.

Keywords

Botulism; Botulinum neurotoxin; vaccine; Botulinum neurotoxin A1; Botulinum neurotoxin A2; ELISA

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Conflict of interest statement.

None.

1. Introduction

Botulinum neurotoxins (BoNT) are the most toxic proteins for humans [1]. There are seven BoNT serotypes, designated (A-G) with subsequent recognition of natural variants termed subtypes [2]. BoNT are produced as 150-kDa single chain proteins and processed into a 50-kDa light chain (LC) and a 100-kDa heavy chain (HC), which are linked by a disulfide bond. LC is a zinc metalloprotease, which cleaves plasma membrane or vesicle associated SNARE proteins, based upon serotype [3]. SNARE cleavage in peripheral motoneurons blocks neurotransmitter release, resulting in the flaccid paralysis typical of botulism. HC is organized into an N-terminal translocation domain (HC_N) and a C-terminal receptor binding domain (HC_C). While a chemically inactivated pentaserotype (ABCDE) toxoid has previously been used to vaccinate at risk populations, the use of this toxoid stock has been discontinued due to declining potency [4]. Currently there are no approved vaccines against botulism [4].

Several strategies address engineering the next generation BoNT vaccine, including DNA based-vaccine approaches such as viral-based delivery and plasmid-based delivery [5–9]. Protein based-BoNT vaccines include continued production of chemically detoxified BoNT [10, 11] and recombinant BoNT derivatives. HC_C of BoNT/A1 produced in *Escherichia coli* elicited a neutralizing immune response against BoNT/A1 challenge [12]. Subsequent studies developed HC_C as a vaccine, using heterologous expression systems [13–18] and an HC_C/A-HC_C/B vaccine is currently in clinical trials [19]. Other BoNT vaccine candidates include $LCHC_N$ expressed in *E. coli* [20] and full-length BoNT expressed in clostridia [21], *E. coli* [22], and the yeast *Pichia pastoris* [17, 18]. Molecular studies showed the structure of full-length BoNT/A1 with 3-amino acid mutations (E224A/R363A/Y366F) (M-BoNT/A1) was similar to native BoNT/A1 [23], while mutations within the ganglioside binding pocket reduced BoNT/A action [24]. In the current study, M-BoNT/A1 and M-BoNT/A1 with an additional mutation (W1266A) that prevents receptor binding (M-BoNT/$A1^W$) are tested as vaccines against botulism relative to two other BoNT vaccine candidates, M-$LCHC_N$/A1 (BoNT/A1(1-878)), and HC_C/$A1^W$ (BoNT/A1(879-1296)). These studies show M-BoNT/$A1^W$, engineered with defects in multiple functions, is a potent strategy for the development of vaccines against botulism and other toxin-mediated diseases.

2. Materials and Methods

2.1 Biosafety and Biosecurity

Experiments conducted at the University of Wisconsin-Madison were approved by the Institutional Biosafety Committee. In addition, experiments were conducted in laboratories approved for this research by the Federal Select Agent Program by researchers who have undergone suitability assessments and adhere to institutional policies and practices. Animal experiments were approved and conducted according to the guidelines of the Animal Care and Use Committee at the University of Wisconsin-Madison. The genes and protein products of BoNT/A encoding three LC mutations ((E224A/R363A/Y366F), termed M) do not meet the regulatory definition of a select agent, allowing production of M-BoNT/A without select agent registration (§ 73.3 HHS select agents and toxins 42 CFR 73.3 (e)(1).

2.2 Botulinum neurotoxins

BoNT/A1,/A2,/A3 and/A5 were purified from *C. botulinum* strains Hall A-*hyper*, Kyoto-F, CDC A3 (provided by Susan Maslanka and Brian Raphael, Centers for Disease Control and Prevention) and A661222 by standard toxin purification protocols [25–28]. BoNT/A6 was purified from CDC41370 B2tox$^-$ (modified from strain CDC41370 to produce only BoNT/A6) toxin using previously described methods [29]. Toxin purity was confirmed by spectroscopy and SDS-PAGE analysis [30]. Purified toxins were stored in phosphate buffered saline with 40 % glycerol at −20°C until use. Activities of the five subtype preparations were determined using a standard intraperitoneal mouse bioassay (MBA) as previously described [31, 32]. The half-lethal dose of each toxin is 1 mouse LD_{50} Unit (U), defined as the amount of toxin injected IP into mice resulting in 50% deaths within 4 days. Specific activities of the BoNT/A subtypes were; 8 pg/U (A1), 7.9 pg/U (A2), 17 pg/U (A3), 7.3 pg/U (A5), and 5.9 pg/U (A6).

2.3 Recombinant BoNT derivatives

HC_C/A1(W1266A) ($HC_C/A1^W$), LC/A1(R363A/Y366F) ($LC/A1^{RY}$), $LCHC_N$/A1(E224A/R363A/Y366F) (M-$LCHC_N$/A1), BoNT/A1(E224A/R363A/Y366F) (M-BoNT/A1), BoNT/A1(E224A/R363A/Y366F/W1266A) (M-BoNT/$A1^W$) and non-catalytic-Tetanus toxin(R372A/Y375F) ($TeNT^{RY}$) were produced as previously described [16]. Briefly, *E. coli* expressing recombinant protein were broken with a French Press, centrifuged, and filtered through a 0.45 μm membrane (Thermo). Lysates were subjected to tandem gravity-flow chromatography using Ni^{2+}-NTA resin (Qiagen), p-aminobenzamidine-agarose (Sigma), and Strep-Tactin Superflow high-capacity resin (IBA). Purified proteins were dialyzed into 10mM Tris-HCl (pH 7.9), 200mM NaCl, and 40% glycerol and stored at −20°C. Recombinant proteins used in this study are shown (Figure 1).

2.4 Vaccine challenge

$HC_C/A1^W$, M-$LCHC_N$/A1, M-BoNT/A1, or M-BoNT/$A1^W$, at the indicated concentration, were mixed with an equal volume of alhydrogel as an adjuvant and used to intraperitoneally vaccinate groups of female ICR mice (18 to 22 g). Non-trypsinized M-BoNT/A1 and M-BoNT/$A1^W$ were used as vaccines. Vaccines were administered on day 1 and 14, blood was collected by maxillary bleed on day 21, and mice were challenged with BoNT/A1, BoNT/A2, or a BoNT-/A2,/A3,/A5, A6 cocktail as indicated on day 26. At least eight mice per group were used in each experiment as indicated. Results were evaluated for statistical relevance by two-tailed, paired student t-test with a p=0.05.

2.5 ELISA

ELISAs were performed as previously described [16]. Briefly, BoNT derivatives or $TeNT^{RY}$ (250 ng/well) were bound to high protein binding 96-well plates (Corning) overnight at 4°C. Plates were washed and blocked at room temperature (RT) for 30 min with 0.2 ml of PBS with 1% (wt/vol) bovine serum albumin (1% BSA). Plates were incubated at RT for 1 h with the indicated serum dilution from individually vaccinated mice, either 1: 20,000 or 1: 30,000 in PBS with 1% BSA (0.1 ml). After washing, plates were incubated at RT for 1 h with goat α-mouse IgG-horseradish peroxidase (IgG-HRP) (Thermo) diluted to 1: 20,000 in PBS with

1% BSA. Plates were washed and incubated with 0.1 ml per well tetramethylbenzidine (TMB; Thermo Ultra TMB) as substrate. Reactions were terminated, and absorbance was read at 450 nm. Measuring α-HA and α-FLAG epitopes showed similar amounts of antigens, within 15%, were bound to the plates (data not shown). For the ELISA, statistical analyses were performed on groups of individually analyzed sera (n=10) based upon immunization and/or challenge conditions by two tailed, unpaired Student t test with $P < 0.05$= *, 0.01=**, 0.001=***, and 0.0001=**** (GraphPad Prism 7). Individual sera were analyzed by at least two-independent ELISAs performed in duplicate. ELISA on serum dilutions from mice vaccinated with M-BoNT/A1, surviving challenge with BoNT/A1, established a serum dose-response range for subsequent experiments (Supplemental Figure 1).

2.6 Cell based assay for detection of neutralizing antibodies

Cell based neutralization assays were performed as previously described [33]. Briefly, human induced pluripotent stem cell (hiPSC) derived neurons (Cellular Dynamics International, WI) were seeded into poly-L-ornithine and matrigel coated 96-well TPP plates (Midwest Scientific, MO) at a density of ~35,000–40,000 cells per well and maintained in iCell Neurons culture media (Cellular Dynamics International, WI) for 7 days prior to the neutralization assay. To detect neutralizing antibodies in mouse sera, 2 pM BoNT/A1 was combined with serial dilutions of sterile filtered sera in culture media and incubated for 1 h at 37°C. BoNT/A1 without sera was used as a 'no antibody' reference. Serum from naïve mice was used as a positive control and serum without BoNT/A1 was used as a negative control. Fifty μl of each antibody-toxin mixture was added per well of hiPSC derived neurons in at least duplicates, and cells were incubated for 24 h at 37°C, 5 % CO2. Cell lysates were prepared in 50 μl of lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) and analyzed by Western blot for SNAP-25 cleavage [34, 35]. Images were obtained using PhosphaGlo reagent (KPL, Gaithersburg, MD) and a Fotodyne/FOTO/Analyst FX imaging system (Hartland, WI). Cleaved (24 kDa) versus uncleaved (25 kDa) SNAP-25 signal was analyzed by densitometry using TotalLab Quant software (Fotodyne, Hartland, WI). Protection was determined by comparison to the 'no-antibody' control, and IC_{50} values, using GraphPad Prism 6 software and a nonlinear regression, variable slope, four parameters.

3. Results

3.1 M-BoNT/A1 is not toxic to outbred mice or neurons in culture

Ten μg of either trypsinized- or non-trypsinized- M-BoNT/A1 per mouse (ICR) injected intraperitoneally did not result in observable signs of botulism, indicating M-BoNT/A1 was at least 10^6-fold less toxic than native BoNT/A1. In addition, incubation of human iPSC derived neurons with 80 nM M-BoNT/A1 did not yield detectable SNAP-25 cleavage, while incubation with 50 fM native BoNT/A1 cleaved SNAP-25, also indicating that M-BoNT/A1 was at least 10^6-fold less toxic than native BoNT/A1 (data not shown).

3.2 M-BoNT/A1 and M-BoNT/A1^W, and M-LCHC$_N$/A1 are more protective vaccines than HC$_C$/A1^W

Vaccine challenges, using a primary immunization followed by one boost, were conducted on outbred ICR mice (n=8–10) to reflect natural immune variance of the host [36] (Table 1). Since previous studies showed HC$_C$/A1(W1266A) (HC$_C$/A1^W) had similar vaccine potency in the mouse model of botulism as HC$_C$/A1 [16], M-BoNT/A1^W was also tested as a vaccine.

Mice vaccinated with 0.3 μg/mouse of single chain M-BoNT/A1 or M-BoNT/A1^W were fully protected against challenge by 10^3 LD$_{50}$ of native BoNT/A1 and native BoNT/A2. Partial protection was observed against challenge by 10^6 LD$_{50}$ of native BoNT/A2, while mice vaccinated with 0.1 μg/mouse of HC$_C$/A1^W were partially protected against challenge by 10^3 LD$_{50}$ of native BoNT/A1 and native BoNT/A2 (Table 1, **Experiments 1 and 2**). Thus, at equimolar doses, M-BoNT/A1 and M-BoNT/A1 ^{W} vaccines showed similar protection and were ~1,000-fold more protective than the HC$_C$/A1^W vaccine.

Mice vaccinated with 0.3 μg/mouse of single chain M-BoNT/A1^W were protected against challenge by 10^6 LD$_{50}$ of native BoNT/A1 or 10^5 LD$_{50}$ of a native BoNT/A subtype cocktail (2.5×10^4 LD$_{50}$ each A2, A3, A5, A6), while mice vaccinated with 0.3 μg/mouse of HC$_C$/A1^W were partially protected against challenge by 10^5 LD$_{50}$ of native BoNT/A1 and 10^5 LD$_{50}$ of the native BoNT/A subtype cocktail (Table 1, **Experiment 3**). Thus, at equal concentrations (a 3-fold molar excess of HC$_C$), M-BoNT/A1^W vaccine was more protective than the HC$_C$/A1^W vaccine.

Mice vaccinated with 0.2 μg/mouse of M-LCHC$_N$/A1 or 0.2 μg of M-LCHC$_N$/A1/+ 0.1 μg/mouse of HC$_C$/A1^W were protected against challenge by 10^6 LD$_{50}$ of native BoNT/A1 and 10^5 LD$_{50}$ of the BoNT/A subtype cocktail (Table 1, **Experiment 3**). Thus, M-LCHC$_N$/A1 had a similar vaccine potency as M-BoNT/A1^W, and addition of HC$_C$/A1^W in *trans* had a limited influence on the vaccine potency of M-LCHC$_N$/A1.

Overall, the vaccine studies show the protective vaccine potencies of M-BoNT/A1 and M-BoNT/A1^W are similar, as well as M-BoNT/A1, M-BoNT/A1^W, and M-LCHC$_N$/A1 being more potent vaccines than HC$_C$/A1^W.

3.3 Antibody responses to BoNT vaccination varied qualitatively and quantitatively in outbred mice

Vaccination with M-BoNT/A1 or M-BoNT/A1 ^{W} provided partial protection to challenge by 10^6 LD$_{50}$ of native BoNT/A2 (Table 1, **Experiment 2**), allowing an assessment of the basis for protection. M-BoNT/A1 or M-BoNT/A1^W vaccination elicited dominant antibody titers to BoNT and LCHC$_N$ that were not statistically different for mice surviving or not surviving native BoNT/A2 challenge (Supplemental Figure 2). Thus, partial protection against native BoNT/A2 challenge appears to be due to specific differences in the composition of neutralizing epitopes to the BoNT/A subtypes, not the ability of the vaccinated mice to mount an immune response to the delivered vaccine.

For mice surviving BoNT/A1 challenge, M-BoNT/A1^{W}, M-LCHC$_{N}$/A1 or HC$_{C}$/A1^{W} vaccination elicited unique antibody responses. M-BoNT/A1^{W} vaccination (Supplemental Figure 3, **lower left**) elicited dominant antibody titers to BoNT (mean titer 2.2 (range 1.3 – 2.6)) and LCHC$_{N}$ (mean titer 1.7 (range 0.8 – 2.4)), while titers to HC$_{C}$ varied among vaccinated mice (mean titer 0.41 (range 0.07 – 1.83)). Titers to LC/A1 were not statistically above controls, which indicated the majority of antibody response was directed towards the HC. M-BoNT/A1 vaccination elicited similar antibody titers as M-BoNT/A1^{W} vaccination (data not shown). M-LCHC$_{N}$/A1 vaccination (Supplemental Figure 3, **upper left**) also elicited dominant antibody titers to BoNT and LCHC$_{N}$, which on average were lower titers than M-BoNT/A1^{W} vaccination (Supplemental Figure 4). M-LCHC$_{N}$/A1 + HC$_{C}$/A1^{W} vaccination (Supplemental Figure 3, **upper right**) elicited antibody titers to BoNT and LCHC$_{N}$ that were qualitatively similar to M-BoNT/A1^{W} vaccination and quantitatively similar with mice vaccinated with M-LCHC$_{N}$/A1 alone (Supplemental Figure 4). HC$_{C}$/A1^{W} vaccination (Supplemental Figure 3, **lower right**) elicited antibody titers to HC$_{C}$ that correlated with survival to BoNT/A1 challenge. Note, HC$_{C}$/A1 vaccination elicited varied HC$_{C}$ antibody titers, from non-detectable to approaching 1.0 A450, when administered alone, within M-BoNT/A1^{W}, or in *trans* with M-LCHC$_{N}$/A1 (Supplemental Figure 3), indicating the varied host response was due to the varied immunogenicity of HCc, independent of other components within the vaccine. BoNT-vaccinated mice possessed limited antibody titers to TeNT^{RY} (Supplemental Figure 3), indicating that the observed antibody responses were BoNT-specific. Variance in the range of titers was due to the varied antibody titers among individual mice, not to variance in the ELISA replicates.

3.4 Properties of sera from individually vaccinated mice surviving BoNT challenge

Within each vaccination group of mice surviving native BoNT/A1 challenge (Supplemental Figure 3), M-BoNT/A1^{W}, M-LCHC$_{N}$/A1, or HC$_{C}$/A1^{W} vaccination elicited different immune responses (Supplemental Figure 5). M-BoNT/A1^{W} vaccination elicited dominant antibody titers to BoNT and LCHC$_{N}$ with **(#3)** or without (**#7**) a detectable antibody response to HC$_{C}$. Since the antibody response to M-LCHC$_{N}$/A1 vaccination has not been previously characterized, serum from three LCHC$_{N}$/A1 vaccinated mice are shown. M-LCHC$_{N}$/A1 vaccination elicited dominant antibody titers to BoNT and LCHC$_{N}$ (**#21, #24, #25**). For mice surviving native BoNT/A1 challenge, HC$_{C}$/A1^{W} vaccination elicited a dominant antibody titer to HC$_{C}$ (**#78**). These individual sera were next tested for the capacity to neutralize native BoNT/A1 in cell culture.

3.5 BoNT/A and HC$_{C}$ vaccines elicit greater BoNT/A neutralizing antibody response than the LCHC$_{N}$ vaccine

The six representative individual sera shown in Supplemental Figure 5 were analyzed for the ability to neutralize BoNT/A1 cleavage of SNAP25 in a cell based assay, using hiPSC derived neurons (Supplemental Figure 6). These sera showed a ~ 10-fold range of potency for the neutralization of native BoNT/A1 in the cell based assay. Serum from HC$_{C}$/A1^{W} vaccination (**#78)** and M-BoNT/A1^{W} vaccination (**#3**), which contained antibody titers to HC$_{C}$ (Figure 4), were more potent inhibitors of BoNT/A1 cleavage of SNAP-25 than sera without HC$_{C}$ antibody titers (**#7, #21, #24, #25**). Thus, in this cell-based assay, sera

containing antibodies to HC_C correlated with a greater BoNT neutralizing activity than sera not containing antibodies to HC_C.

These data indicate that the HC_C domain of BoNT/A1 elicits a stronger neutralizing antibody response than $LCHC_N$ in the cell based BoNT-neutralization assays, which contrasts M-$LCHC_N$/A1 vaccine potency in eliciting a neutralizing response to BoNT-challenge (Table 1). Overall, the cell-based assays and mouse-based assays measure different antibody properties that neutralize BoNT and support the potency of the M-$BoNT/A1^W$ vaccine, which elicited a strong neutralizing response in both assays.

4. Discussion

In an outbred mouse model of botulism, M-BoNT/A1, M-$BoNT/A1^W$ and M-$LCHC_N$/A1 were more potent vaccines than $HC_C/A1^W$. M-$BoNT/A1^W$ elicited a similar protective immune response relative to M-BoNT/A1, showing host cell binding epitopes were not necessary to elicit high vaccine efficacy. The ability of $LCHC_N$ to elicit a strong BoNT-neutralizing response in the mouse model of botulism (Table 1), along with HC_C-eliciting a strong BoNT-neutralizing response in the cell based assays (Supplemental Figure 6), shows the advantage of a full-length BoNT-based vaccine, which elicited potent neutralizing responses in both assays. Thus, full-length BoNT engineered with defects in both catalysis and receptor binding domains represents a novel strategy for development of vaccines against botulism. The importance of vaccines possessing multiple, independent mechanisms is supported by the observation that second-site mutations partially reverted a genetically inactivated diphtheria toxin vaccine candidate [37]. In addition, recent studies by Smith and coworkers show the need for greater attenuation of BoNT-based vaccines than only reduction of catalysis for several BoNT serotypes [18].

Smith and coworkers [18] reported that catalytically inactive BoNT showed greater potency to challenge by a 1000 LD_{50} toxin challenge after single vaccination than the corresponding HC_C. These studies measured protection to threshold toxin challenges, which differed from the current study which measured protection to endpoint toxin challenge. Thus, by measurement of protection to a threshold challenge or to an endpoint challenge, full-length BoNT vaccines are more potent than their respective HC_C subunits. The utility of M-$BoNT/A1^W$ as a vaccine candidate addresses a concern that genetic inactivation of catalytic function alone may not provide a sufficient margin of safety for vaccine development of full-length BoNT [18].

In an earlier study, $LCHC_N$ was described as a BoNT vaccine candidate [20]. We observed that $LCHC_N$ was a potent vaccine by direct comparison to full-length BoNT and HC_C vaccine candidates [20]. Consistent with the presence of neutralizing epitopes within $LCHC_N$, neutralizing LC-specific and HC_N-specific monoclonal antibodies that neutralized BoNT/A action have been reported [38–40]. In addition, HC_N was reported to possess neuron-binding properties, which supports the potential for BoNT-neutralizing epitopes within this domain [41]. HC_C is a popular vaccine against botulism, using DNA- and viral-vectors, as well as protein-based vaccines[42], due, in part, to the ease of production [15]. Smith and colleagues expressed HC_C in the yeast, *Pichia pastoris*, and reported HC_C to elicit

protective immunity [13] and a bivalent vaccine composed of recombinant HC_C/A and HC_C/B (rBV A/B) is now in clinical trial [14]. The presence of immune epitopes within LC and HC_N has been reported [43, 44]. Since M-BoNT/$A1^W$ vaccination elicited a greater antibody response than M-$LCHC_N$/A1 (Supplemental Figure 2), and HC_C vaccination elicited antibodies with the strong neutralizing potency in cultured cells, M-BoNT/$A1^W$ should be more protective in a 'high-dose' BoNT challenge than $LCHC_N$ or HC_C vaccine derivatives.

In summary, this study shows BoNT and $LCHC_N$ are more potent vaccinec than HC_C. M-BoNT/$A1^W$ elicited a common dominant antibody response to $LCHC_N$, but a varied HC_C antibody response in outbred mice. The lower potency of the HC_C vaccine correlated with the varied ability of the vaccinated mice to mount an immune response to HC_C vaccination. The reduction of both catalysis and receptor binding support the use of single chain M-BoNT/$A1^W$ as a safe vaccine against botulism. Further studies will determine if this vaccination strategy is viable against other BoNT serotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BoNT	Botulinum neurotoxins
TeNT	Tetanus toxin
LC	Light Chain of Botulinum neurotoxins
HC	Heavy Chain of Botulinum neurotoxins
HC_N	Translocation domain of Botulinum neurotoxins
HC_C	Receptor binding domains of Botulinum neurotoxins
LD_{50}	Half-lethal dose
IC_{50}	Half maximal inhibitory concentration
SNARE	Soluble NSF attachment protein receptor

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Highlights

- In a mouse model of botulism, M-BoNT/A1 was not toxic at > 10^6-fold greater amounts than native BoNT/A.
- M-BoNT/A1(W1266A) (M-BoNT/A1^W) was created to prevent neuronal cell binding.
- M-BoNT/A1 vaccination protected against challenge by 10^6 LD_{50} of native BoNT/A1
- $LCHC_N$ elicited a higher neutralizing antibody titer than HC_C, showing neutralizing epitopes within $LCHC_N$.
- Engineered BoNT with defects in catalysis and receptor binding is a novel vaccine strategy against botulism.

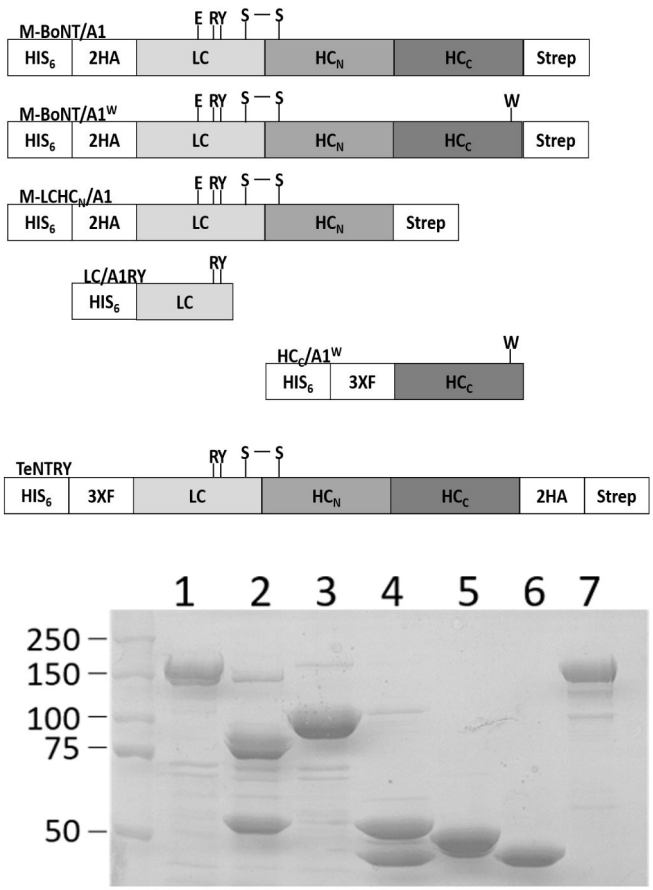

Figure 1. Schematic of the recombinant proteins used as vaccines and/or antigens to assess the host immune response to vaccination

(Upper panel) BoNT-derivatives used in this study are shown. His_6 and Strep epitopes were used for protein purification, while 3X-FLAG and two sequential hemagglutinin, 2HA, epitopes were included for cellular studies. Domain junctions were defined, using the crystal structure of BoNT/A1 (PDB:3BTA). Single amino acid designations indicate amino acid substitutions used to reduce catalysis (LC) or receptor binding (HC_C). Note, single chain BoNT and $LCHC_N$ were used for vaccination. **(Lower panel)** Four μg of the indicated proteins were subjected to SDS-PAGE and Coomassie blue staining. Lanes: 1, M-BoNT/A1; 2, M-BoNT/A1 trypsin nicked and reduced; 3, M-$LCHC_N$/A1; 4. M-$LCHC_N$/A1 trypsin nicked and reduced; 5, LC/$A1^{RY}$; 6, $HC_C/A1^W$; and 7, $TeNT^{RY}$. Migration of molecular weight marker proteins (kDa) are shown in left lane. Note, in lane 2 nicked HC runs at ~ 80 kDa, which other experiments showed was due to cleavage of the belt region of HC by trypsin.

Table 1

Vaccine potency of recombinant BoNT and BoNT-derivatives in the mouse model of botulism

Vaccine Primary & Boost (μg)[a]	**Challenge BoNT serotype**	**Survivors/Challenged Units of BoNT/A LD_{50} challenge (U)**[b]			
		10^3 U	**10^4 U**	**10^5 U**	**10^6 U**
Experiment 1					
M-BoNT/A1 (0.3)	A1	10/10	_[c]	-	-
	A2	10/10	-	-	-
M-BoNT/$A1^W$ [d] (0.3)	A1	10/10	-	-	-
	A2	10/10	-	-	-
$HC_C/A1^W$ (0.1)	A1	7/10	-	-	-
	A2	6/10	-	-	-
Alum	A1	0/5	-	-	-
Experiment 2					
M-BoNT/A1 (0.3)	A2	-	8/8	8/8	5/9
M-BoNT/$A1^W$ (0.3)	A2	-	8/8	8/8	3/9
Alum	A2	-	-	-	0/5
Experiment 3					
M-BoNT/$A1^W$ (0.3)	A1	-	-	-	10/10
M-$LCHC_N$/A1 (0.2)	A1	-	-	-	10/10
M-$LCHC_N$ (0.2) + $HC_C/A1^W$ (0.1)	A1	-	-	-	10/10
$HC_C/A1^W$ (0.3)	A1	-	-	7/10	-
M-BoNT/$A1^W$ (0.3)	A(subtype cocktail)[e]	-	-	10/10	-
M-$LCHC_N$/A1 (0.2)	A(subtype Cocktail)	-	-	10/10	-
M-$LCHC_N$/A1 (0.2) + $HC_C/A1^W$ (0.1)	A(subtype Cocktail)	-	-	9/10	-
$HC_C/A1^W$ (0.3)	A(subtype Cocktail)	-	-	7/10	-
Alum	A(subtype Cocktail)	-	0/5	-	-

[a] Mice were immunized IP with the indicated vaccine with alhydrogel as adjuvant. Vaccines were administered on day 1 and 14, blood was collected on day 21, and mice were challenged as indicated on day 26

[b] U = One half-lethal dose of a botulinum neurotoxin at 72 h post challenge is defined as 1 mouse LD_{50}

[c] – = not determined

[d] W = W1266A mutation within the ganglioside binding domain of HC/A1

[e] A(subtype cocktail) = 25,000 LD_{50} U of BoNT/A2/A3/A5 and/A6 (total 100,000 LD_{50} U)