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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.

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

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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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 10 mM Tris-HCl (pH 7.9), 200 mM NaCl, and 40% glycerol and stored at −20 °C. Recombinant proteins used in this study are shown (Fig. 1).

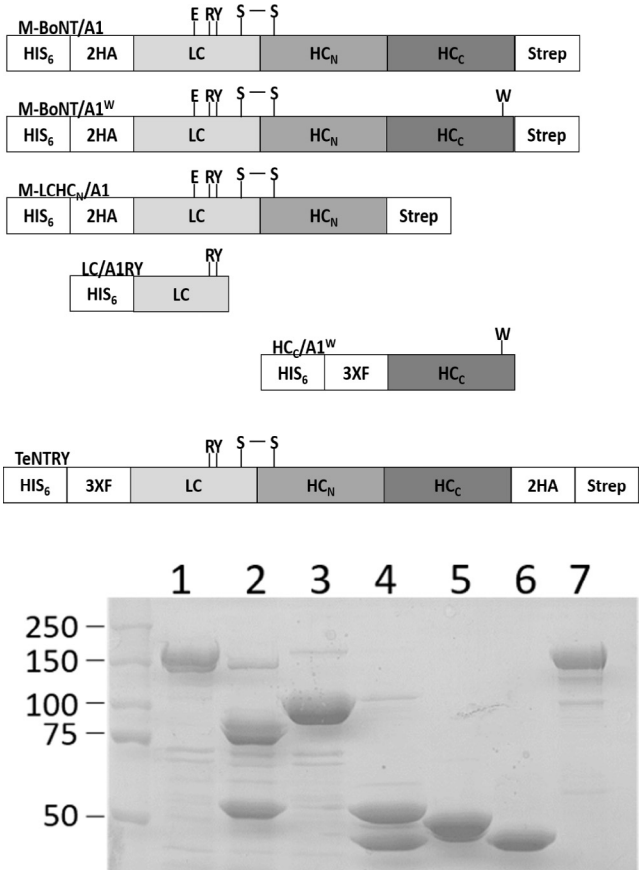

Fig. 1. Schematic of the recombinant proteins used as vaccines and/or antigens to assess the host immune response to vaccination. (**Upper panel**) BoNT-derivatives used in this study are shown. His$_6$ and Strep epitopes were used for protein purification, while 3X-FLAG and two sequential hemagglutinin, 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.

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 (18–22 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 = .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