# Development of recombinant protein based vaccine for BoNT

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## Abstract

Botulinum neurotoxins (BoNTs) are Category A biothreat agents which pose significant threat to public health and military personnel. There is currently no treatment available against botulism. Vaccination is an effective strategy for providing specific protection against exotoxins like botulinum toxin by eliciting neutralizing antibodies that prevent binding of the toxin to their specific receptors. A formulation of a pentavalent (ABCDE) botulinum toxoids by Parke-Davis in 1957, thereafter by the Michigan department of public health through CDC and DoD was distributed to immunize thousands of at-risk laboratory and military personnel as a preventive measure against botulinum toxin. However, it was still classified as an Investigational New Drug (IND), as a preventative measure against botulism. This toxoid vaccine contained formalin-inactivated BoNTs that was adsorbed onto aluminum phosphate, with thimerosal added as preservatives. The three injection routine of this toxoid was painful and caused reddening and swelling of area of administration. While this pentavalent toxoid was used over half a century to immunize thousands of individuals, its use has been discontinued since 2011 due to several shortcomings, including declining immunogenicity and potency, and occurrence of adverse reactions (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6042a3.htm). In the United States, there is currently only one FDA-approved treatment of infant botulism types A and B: BabyBIG. There is no other FDA-licensed prophylactic treatment against botulism available, and the antitoxin consisting equine antibodies have limited utility in terms of window for treatment. Development of recombinant protein based BoNT vaccine is a suitable alternative. The hypothesis of the proposed research can be achieved by pursuing the following components: 1) Formation of a complex between DrBoNT/A and NAPs. 2) NAPs mediated delivery of Dr. BoNT, and 3) evaluation of the vaccine potential of the construct. In the preliminary study, we tested the stability and immunogenicity of the proposed construct and drBoNT/A. Results suggest that BoNT and NAPs remain intact in the blood and different organs during trafficking and trafficking is not affected due to the presence of NAPs. DrBoNT/A has been found to be highly immunogenic when tested under various in vivo conditions in Balb/C mice model, could evoke circulating immunoglobulin (IgG) levels which are 25-fold higher than those evoked by its heavy chain. Based on preliminary data, we expect that immunoglobulin response of DrBoNT-NAPs complex would be more than rHC, proving DrBoNT/A-NAPs complex to be better vaccine candidate than rHC.

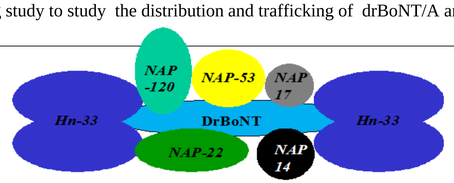
## Introduction

Botulinum neurotoxins (BoNTs) are produced by *Clostridium botulinum* as a complex with neurotoxin associated proteins (NAPs) which pose a significant threat to the public as potential bioterrorist weapons because of their extreme potency, relative ease of production and transport, and the burden that affected individuals would place on the public health care system. Thus, it is essential to develop an effective vaccine therapy for the prevention of these deadly diseases. A formulation of pentavalent (ABCDE) botulinum toxoid vaccine was developed by Parke-Davis in 1957 and thereafter by the Michigan Department of Public Health. CDC and DoD continued to distribute the pentavalent botulinum toxoid vaccine to immunize thousands of at risk laboratory and military personnel for over half a century. However, its use was discontinued in 2011 due to several shortcomings, including declining immunogenicity and potency and an increased occurrence of adverse reactions upon its continual use. In the USA, there is currently no FDA-licensed prophylactic treatment against botulism.

The active site of BoNT is composed of zinc-binding motif, HEXXH+E. The crystal structure of type A BoNT has revealed that H223, H227, and E262 of the HEXXH+E motif directly coordinate the zinc, and E224 coordinates a water molecule as the fourth ligand. Site-directed mutagenesis studies with BoNT/A have demonstrated that active site mutation result in either drastically (E224D) or completely abolished (E224Q) endopeptidase activity. Based on the above information, we have successfully constructed and expressed drBoNT/A (Detoxified Recombinant BoNT/A). In this study for rBoNT/A we demonstrated the catalytic activity, determined mouse lethality (LD50) dose, efficacy of drBoNT/A inducing circulating (IgG) and secretory (IgA), protection against the native toxin challenge dose, and examine the pharmacokinetics of the distribution. Results demonstrated in both in vitro enzymatic assay and in vivo mouse bioassay that the drBoNT/A was drastically less toxic compared with native BoNT/A toxin and also evoked abundant immunoglobulin's when compared with rLC/A-BoNT/A or rHc/A-BoNTA, rendering the animal model fully protected against the BoNT/A challenge dose. We also found that that BoNT and NAPs remain intact in the blood and different organs during trafficking, and trafficking is not affected due the presence of NAPs. In conclusion, Botulinum neurotoxin and its associated protein appear to remain together during trafficking of BoNT/A complex in mouse model. Based on the above observations, we hypothesize that DrBoNT/A can be used as an immunogen for the production of vaccines and antitoxins as well as research and drug development applications. This botulinum vaccination can address biothreat, and can also assist research workers from the risk of exposure during research with larger quantities of the toxin. Furthermore, the full length BoNT vaccine will be reconstructed with neurotoxin associated proteins as an oral/nasal delivery vehicle for needle-free administration.

## Experiment s

1. Endopeptidase assay with peptide substrate, full length substrate, and cleavage of SNAP-25 in M17 cells.
2. Determination of mouse $LD_{50}$ of drBoNT/A.
3. Detemination of circulatory and secretory response of drBoNT/A alone, and effect of adjuvant on these responses.
4. Comparison of immunoglobuline responses of drBoNT/A withrHC/A and rLC/A.
5. Vaccination potential of drBoNT/A with and without adjuvant.
6. Protection against the native toxin challenge dose (1000 $LD_{50}$).
7. Recomplexation of drBoNT/A with NAPs.
8. Mouse imaging study to study the distribution and trafficking of drBoNT/A and drBoNT/A + NAPs construct.




**Recomplexed drBoNT/A**

## Endopeptidase Activity

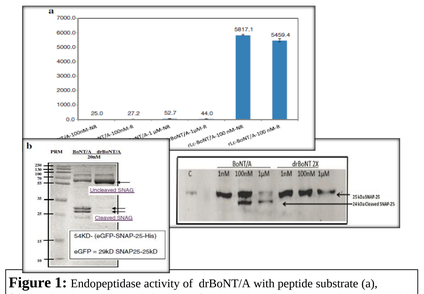



**Figure 1:** Endopeptidase activity of drBoNT/A with peptide substrate (a), full length substrate (b), and cleavage of SNAP-25 inside M17 neuroblastoma cells (

## Distribution and trafficking of drBoNT with and without NAPs

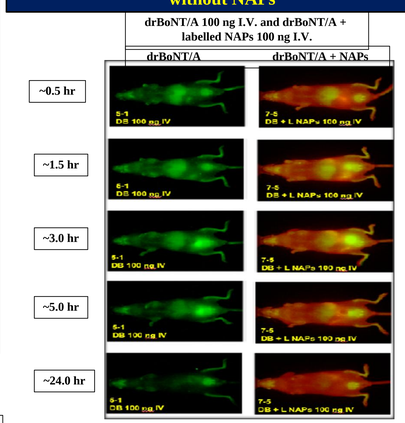



**Figure 5:** SKH1-E nude mice scanned on the Pearl Imaging system. Mice were treated with 100 ng DrBoNT/A-800 (upper panel) or 100 ng DrBoNT/A-800 mixed with 400 ng NAPs-700 by injection in the tail vein. Images for each scan captured at white light, 700 nm, and 800 nm and fluorescent signals were analyzed using the Pearl Cam software.

## Comparison of survival curves treated with drBoNT/A rHC BoNT/A rLC-BoNT/A

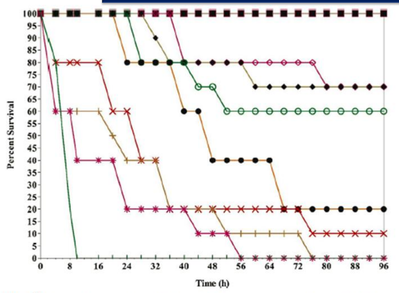



**Figure 2:** Survival curves of female Swiss Webster mice treated with 150-kDa drBoNT/A via i.p route. Percent survival was plotted over time. In total of 14 doses were tested in mice. In this study the total amount of drBoNT/A was administered in 500 µl of PBS buffer, pH 7.4, per mouse. They are as follows: 2.5 0 µg/mouse (), 5.0 µg/mouse (), 10.0 µg/mouse (), 15.0 µg/mouse (), 20.0 µg/mouse (), 21.0 µg/mouse (), 22.0 µg/mouse (), 23.0 µg/mouse (), 24.0 µg/mouse (), 25.0 µg/mouse (), 50.0 µg/mouse (), 75.0 µg/mouse (), 100.0 µg/mouse () and () positive control. Post administered mice group was monitored for these classic symptoms for botulism: ruffled fur, wasp-waist, difficult in move-ment-crouching, labored breathing and survival time till 96 h.

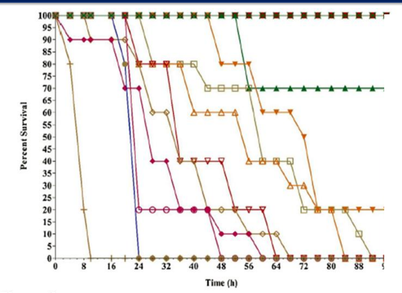



**Figure 3:** Survival curves of female Swiss Webster mice treated with rHc BoNT/A via i.p route. Percent survival was plotted over time. In total of 1 doses were tested in mice. In this study the total amount of rHc-BoNT/A wa administered in 500 µl of PBS buffer, pH 7.4, per mouse. They are as follow 2.5 µg/mouse (), 5.0 µg/mouse (), 6.0 µg/mouse (), 10.0 µg/mouse (), 15.0 µg/mouse (), 20.0 µg/mous (), 22.0µg/mouse (), 24.0 µg/mouse (), 25.0 µg/mous (), 50.0 µg/mouse (), 6.0 µg/mouse + α rHc-BoNT/ (), and () positive control. Post administered mice group wa monitored for these classic symptoms for botulism: ruffled fur, wasp-wais difficult in movement-crouching, labored breathing and survival time till 96 l

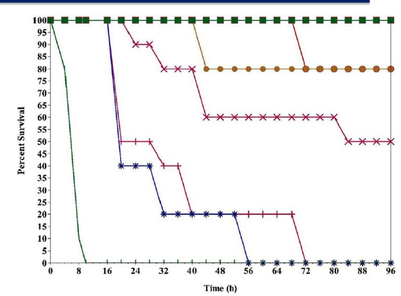



**Figure 4:** Survival curves of female Swiss Webster mice treated with rLc-BoNT/A via i.p route. Percent survival was plotted over time. In total of 14 doses were tested in mice. In this study the total amount of rHc-BoNT/A was administered in 500 µl of PBS buffer, pH 7.4, per mouse. They are as follows: 3.0 µg/mouse (), 5.0 µg/mouse (), 7.0 µg/mouse (), 9.0 µg/mouse (), 10.0 µg/mouse (), 20.0 µg/mouse (), 25.0 µg/mouse (), 50.0 µg/mouse (), 100.0 µg/mouse (), 110.0 µg/mouse (), 115.0 µg/mouse (), 120.0 µg/mouse (), 125.0 µg/mouse (), and () positive control BoNT/A. Post administered mice group was monitored for these classic symptoms for botulism: ruffled fur, wasp-waist, difficult in movement-crouching, labored breathing and survival time till 96 h.

## Circulatory and secretory antibody response

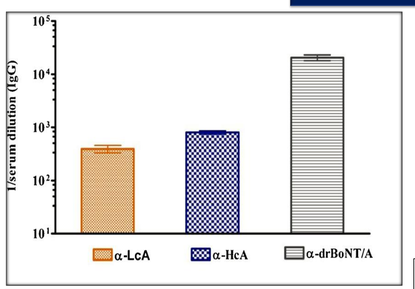



**Figure 6:** IgG response to r-LC/A, rHC/A, and drBoNT/A.

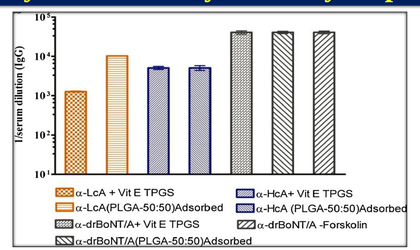



**Figure 7:** Adjuvant effect on IgG response. The data show that the adjuvant slightly enhanced the antibody level for drBoNT/A, whereas rHC/A and rLC/A there was significant increase in the response of IgG.

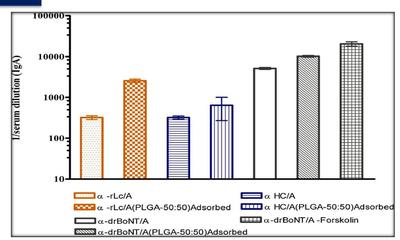



**Figure 8:** IgA response and effect of adjuvant for drBoNT/A, rHC/A, and rLC/A. The data show that the adjuvant significantly enhanced the antibody level for drBoNT/A and rLC/A. However, for rHC/A there was no significant increase in their response to secretory IgA.

**Detoxified Recombinant Botulinum Neurotoxin Type A Immunotoxicity**

**Table I** Summary of Protective Efficacy for the Following Antigens drBoNT/A, rLC-BoNT/A & rHc-BoNT/A in Balb/C Mice

| Vaccine paradigm | Number of mice surviving challenges | | | |
|---|---|---|---|---|
| | Soluble antigen | Adjuvant –Vit-E TPGS | PLGA (50:50), adsorbed | Adjuvant – Forskolin |
| α Lc-BoNT/A | 3/10 | 7/10 | 10/10 | NA |
| α Hc-BoNT/A | 4/10 | 10/10 | 10/10 | NA |
| α drBoNT/A | 10/10 | 10/10 | 10/10 | 10/10 |

BoNT/A challenge dose were administered by the intraperitoneal route

All the animals that survived showed none of the sign pertaining towards botulism

Control animals challenged with native BoNT/A toxin were dead within 8 h, ($n = 5$ per group)

## Conclusions

1. drBONT/A construct is enzymatically inactive.
2. drBoNT/A was not toxic to the mice in the range of 2.5 – 21.0 µg and acute toxicity was 75.0 µg. However, for rHC/A LD50 was 5.0 µg and acute toxicity was 15.0 µg. In case of r LC/A $LD_{50}$ and acute toxicity was estimated to be 115.0 µg and 120.0 µg, respectively. Which means drBoNT/A is 1.2 million fold less toxic than native toxin.
3. rHC/A is more toxic than drBoNT/A.
4. The majority of administered drBoNT/A complex is localized in liver, kidney, intestines, spleen and bladder.
5. Antibody response of soluble antigens rHC/A and rLC/A were weak compared to drBoNT/A.
6. Imaging study suggest that drBoNT/A and NAP do not dissociate opening a possibility for oral vaccination. In future, we will examine the vaccination potential of this construct.
7. This study demonstrated that by having a catalytically deactivated full length protein, it is possible to induce strong, long lasting and protective immune response.

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