Pharm Res (2016) 33:639–652
DOI 10.1007/s11095-015-1816-x

RESEARCH PAPER

In Vivo Toxicity and Immunological Characterization of Detoxified Recombinant Botulinum Neurotoxin Type A

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Received: 3 August 2015 / Accepted: 26 October 2015 / Published online: 3 November 2015

ABSTRACT

Purpose A double-mutant E224A/E262A full-length botulinum neurotoxin (BoNT) Type A with structural similarity to native BoNT/A but lacking the endopeptidase activity provides an ideal surrogate for testing pharmacokinetics and immunochemical characteristics of BoNT.

Methods We determined lethality (LD_{50}) of deactivated recombinant botulinum neurotoxin (drBoNT/A) to be 24.0 μg by intraperitoneal route (i.p). The polypeptide drBoNT/A labeled with near infra-red dye 800 (NIR 800) was used to examine its distribution to different organs using whole body imaging when administered to mice via intravenous (i.v) or i.p route. Also, drBoNT/A was used to evaluate its immunogenicity in Balb/C mice model.

Results drBoNT/A was found to be highly immunogenic when tested under various *in vivo* conditions in Balb/C mice model. For the first time we have demonstrated that a full length 150 kDa drBoNT/A, by administering via inhalation route in mice model, has evoked both circulating immunoglobulin levels of IgG and secretory IgA at the mucosal surface. The immunoglobulin levels were sufficient enough to protect against the challenge dose of native BoNT toxin in mice model. Tissue distribution of drBoNT/A seems to be similar to that of native toxin.

Conclusions Based on the characteristics described in this report this nontoxic holotoxin protein will assist us to explore the window of opportunity available for therapeutic treatment in case of unnatural poisoning, and also it can be an effective vaccine candidate.

KEY WORDS botulinum · Clostridium · deactivated neurotoxin · drug delivery · microsphere · mucosal vaccine · toxicity · vaccine

ABBREVIATIONS

ABSL-3	Animal Biosafety Level 3
BAL	Broncho-alveolar lavage
BCA	Bicinchoninic acid protein assay
Bis-Tris	[Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl]
BoNT/A	Full-length botulinum neurotoxin Type A
drBoNT/A	Deactivated recombinant botulinum neurotoxin type A
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-linked immunosorbent assay
FRET	Fluorescence energy resonance transfer
HC	Heavy Chain
i.p	Intraperitoneal route
i.v	Intravenous route
IgA	Immunoglobulin A
IgG	Immunoglobulin G
kDa	kilodalton
LC	Light Chain
LD_{50}	A dose that proves lethal to 50% of the given population
PLGA (50:50)	Poly Lactic-co-Glycolic Acid biodegradable polymer
PVDF	Polyvinyl difluoride membrane

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rHc/A-BoNT/A	Recombinant heavy chain of botulinum neurotoxin type A
rLC/A-BoNT/A	Recombinant light chain of botulinum neurotoxin type A
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNAP-25	Synaptosomal-associated protein 25
Vitamin-E TPGS	Vitamin – E ~ D -α-Tocopherol polyethylene glycol succinate

INTRODUCTION

Botulinum Neurotoxins (BoNTs) are a group of extremely potent toxins, which are produced by various strains of *Clostridium botulinum*, and in some cases by *C. butyricum* and *C. baratii*. There are seven serotypes (A–G) of BoNTs (1–3), each produced as a 150 kDa single polypeptide chain. The protein is post-translationally proteolyzed or 'nicked' by indigenous bacterial protease or *in vitro* with trypsin to form a dichain in which the heavy Chain (HC) and light Chain (LC) are linked through a disulfide bond (3). HC is composed of two 50 kDa domains, with the N-terminal half (HCN) involved in translocation across the endosomal membrane and the C-terminal half (HCC) which is involved in binding to neuronal receptors. LC plays a critical intracellular enzymatic role in the action of BoNT (3,4).

The active site of BoNT is composed of the HEXXH + E zinc-binding motif (5). 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 (6). General conformation and active site residues are conserved in all of the clostridial neurotoxins (7). Site-directed mutation studies with BoNT/A and TeNT (tetanus neurotoxin)-LC (5,8–10) have demonstrated that active site mutations result in either drastically reduced (E224D) or completely abolished (E224Q) endopeptidase activity. The loss of activity is due to interference with the hydrolysis step and not due to any change in the binding of the SNAP-25 or the Zn2+ ligand to the enzyme (5). Crystal structure and mutagenesis studies have shown that additional distal amino acid residues act as secondary coordination sites of zinc (8–10). The secondary coordination sites are very important for stabilizing the zinc binding and substrate specificity (11).

We have successfully constructed and expressed drBoNT/A with structural characteristics similar to native toxin, but lacking the endopeptidase activity that is critical to toxicity (12). Although, full-length drBoNT/A had been biochemically and biophysically characterized by comparing with the native holotoxin, still the *in vivo* toxicity levels and immunogenicity had not been examined. In this study for drBoNT/A the following studies were investigated, i) enzyme activity assay, ii) mouse lethality LD_{50} dose, iii) *in vivo* distribution in mice model, and iv) efficacy of drBoNT/A inducing circulating (IgG) and secretory antibody (IgA), engendering protection against the native toxin challenge dose. 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-BoNT/A, rendering the animal model fully protected against the BoNT/A challenge dose.

MATERIALS AND METHODS

Endopeptidase Assay Using SNAPtide

The endopeptidase activity was measured for reduced and non-reduced versions of drBoNT/A (100 nM) and rLc-BoNT/A (2 μM) samples. All the samples were incubated for 30 min at 37°C with 10 mM Hepes buffer, 0.1% tween-20 pH 7.4 along with 1 μM SNAPtide 521 (List Biological Laboratories, Inc, California). Native BoNT/A cleaves SNAP-25 at amino acid sequence Q-R which is incorporated in the sequence of SNAPtide. SNAPtide is a 12 amino acid long peptide containing FITC/DABCYL FRET pair, where the N-terminal is attached with a fluorophore, Fluoresceinisothiocyanate (FITC) is quenched by the C-terminally attached chromophore, DABCYL group. Cleavage of the substrate by the corresponding botulinum neurotoxin or light chain releases the fluorophore and restores the fluorescence fully. The increase in fluorescence intensity is directly proportional to the amount of cleavage, and in turn activity of the enzyme BoNT/A and/or rLc-BoNT/A. The relative fluorescence unit (RFU) signal was measured using a microplate reader at excitation 490 nm and emission is 523 nm (Molecular Devices, Sunnyvale, CA).

Full Length SNAP-25

SNAG (eGFP-SNAP-25-His) is a 54 kDa protein produced in our laboratory 2 nM light chain, 20 nM BoNT/A or 20 nM drBoNT/A were incubated with 2 μM SNAG at 37°C for 45 min. BoNT/A and light chain cleave SNAG into 28 and 24 kDa bands, as examined on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Cell Based – Western Blot Analysis

M17 neuroblastoma cells were grown in 12-well plate to 2× 105 cells per well in 5% CO2 and 95% humidity chamber at 37°C for 2 days until 80% confluence. In order to assay cleavage intracellular SNAP-25 by BoNT, M17 neuroblastoma cells were incubated for 24 h with a varied range of BoNT

concentrations, 1 nM, 100 nM and 1 μM of native BoNT/A or drBoNT/A. At the end of the incubation period cells were washed with serum free media followed by trypsinization with Trypsin EDTA for 2 min, followed by neutralization with media containing 10% serum. Then all the cells were harvested and centrifuged for 3 min at 6500 rpm (Epindorff Mini-Spin centrifuge). After removing all the media the cell pellet was resuspended in ice cold PBS. The cells were re-pelleted at 6500 rpm for 5 min. After removing the PBS 10 μl of mammalian protein extraction reagent (Thermo-Fisher Scientific, Pittsburgh, PA; cat#78503) was added to the tube and incubated for 10 min with occasional vortexing. The reaction was terminated by addition of Nupage-Lithinum Dodecyl Sulfate, 4X sample buffer from Invitrogen (Life Technologies, Grand Island, NY; cat # NP0008). Samples were run through 15% SDS-polyacrylamide gel (Bio-Rad) at 60v for 20 min and 130 v for 3 h. The proteins bands on the gel were then transferred on to a polyvinyl difluoride (PVDF) membrane using wet transfer western blot system, (mini trans-blot cell, Bio-Rad). The membrane was incubated in 5% dry milk overnight. Anti-SNAP-25 antibody produced in rabbit (1:2500), (Sigma-Aldrich, St. Louis, MO, USA; cat# S9684) was added in 1% dry milk. After washing 3 times with TBST, secondary antibody, anti-rabbit IgG alkaline phosphatase conjugate antibody (Sigma-Aldrich, St. Louis, MO, USA; cat # A3937), was incubated for 1 h. After washing three times with TBST the blot was developed with BCIP/NBT (Sigma-Aldrich, St. Louis, MO, USA; cat #B3804).

Mouse Lethality Assay (LD_{50})

The following recombinant proteins a) drBoNT type A, b) Heavy chain BoNT type A (rHc-BoNT/A) and c) Light chain BoNT type A (rLc-BoNT/A) were isolated and purified at the Botulinum Research Center at University of Massachusetts Dartmouth (UMassD). All the protein samples tested in the animal model were buffer exchanged against PBS, pH 7.4, prior to administration and the purity of those samples examined by electrophoresis method.

The total protein content was estimated by bicinchoninic acid protein assay (BCA; Sigma-Aldrich, St. Louis, MO, USA). Female Swiss Webster mice (20–25 g) were purchased from Charles River Laboratories International, Inc., (Wilmington, MA). Mice were housed at University of Massachusetts Dartmouth campus (UMassD) animal facility, and all procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committees, UMassD.

Briefly, varying doses of drBoNT/A, rHc-BoNT/A and rLc-BoNT/A (range, 2.5–125.0 μg/dose) was prepared in PBS, pH 7.4 solution. In each group, 10 mice per dose in total of 40 groups were injected intraperitoneally (i.p.) with 0.5 ml of a dose. A positive control group ($n=5$) of native BoNT/A toxin ($1LD_{50}$) was also injected via i.p route. All the intraperitoneal injections were performed in each mouse by carefully administering 0.5 ml of the test dilutions on the lower left quadrant of the mice abdomen, using 0.5 ml BD syringe fitted with 27 gauge, 0.5 in., stainless steel needles (BD Medical, Fisher Scientific, Pittsburgh, PA). The mice were then observed every 4 h, for a period of 4 days for symptoms of BoNT toxicity, including mortality.

In Vivo Imaging - Pearl® Impulse

In vivo imaging studies for drBoNT/A was carried out with NIR dye 800 CW labeled compound (LI-COR Biosciences, Lincoln, NE). The labeling was performed as described previously (Hale *et al.*, 2010). The labeled proteins were dialyzed against PBS buffer to remove excess dye and aliquots of the proteins were then analyzed with NuPAGE® Novex Bis-Tris [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl], a 4–12% pre-cast gels for separating small to mid-size molecular weight proteins using XCell SureLock™ Mini-Cell system. The gel electrophoresis was carried out initially at a constant voltage of 85 V for 90 min followed by 100 V for 15 min at room temperature (Life Technologies, Grand Island, NY 14072). After electrophoresis, protein band on the gel was scanned on the Odyssey Imaging System to ensure that the proteins had not degraded and the dye labels remained attached to the protein. Dye-to-protein ratios, calculated with equations provided in the vendor's protocol using OD readings at 280 and 800 nm, were approximately 3:1. Protein concentrations of labeled proteins were compared with unlabeled proteins in a micro-BCA assay (Sigma-Aldrich, St. Louis, MO, USA; cat # B9643) to ensure that the dose given represent the equivalent to the unlabeled protein..

Imaging studies were performed using the Pearl Imaging System. Prior to administration of the NIR labeled molecule to mice, hair was removed from the ventral region of mice to reduce interference by the fur's auto-fluorescence. Individual Swiss Webster mice were injected either via i.p. or intravenous (i.v.) route with 50 ng of drBoNT/A-800, or saline. After 5 or 24 h, each mouse was scanned individually using the same instrument settings (focal offset, 0; resolution, 169, quality, medium; intensity settings: 800 channel = 5 and 700 channel = 3). For visualization purposes, green color was used to indicate NIR fluorescence in the 800 nm channel and red was used to indicate fluorescence in the 700 nm channel. The mice were slightly anaesthetized with 2% isoflurane gas inhalation using a nebulizer box with 1 L/min oxygen flow and 2.5% isoflurane vaporizer. After the mice were immobilized, they were placed on the imager's heated stage. During the imaging process or scan, mice were continuously given isoflurane gas through a nose cone located within the imaging drawer. Immediately after collecting the needed images or upon completion of scan, mice were returned to their cages to recover from anesthesia in less than 2 min (13). The captured images were

analyzed by using the PEARL imager (*in vivo*) software. This method was adopted to evaluate the distribution and clearance pattern of their respective administered routes/molecule, without subjecting the mice to any stress or further isolating the organ or tissues for bioprocess of estimation.

Vaccination Protocol

A group of 5 female Balb/C mice, weighing 18–20 g were immunized via intranasal (i.n) route with the following antigens, a) drBoNT/A, b) rHc-BoNT/A and c) rLc-BoNT. The antigens dosed in this paradigm are either in soluble form or admixed with mucosal adjuvants, such as Vitamin-E TPGS (1.0%, *w/v*) or Forskolin (2.0%, *w/v*). An additional group of Balb/C mice ($n=10$) were dosed with the antigens mentioned above having adsorbed onto empty biodegradable PLGA (50:50, Lactic Acid: Glycolic Acid) microspheres via i.n route. All the groups of mice dosed via intranasal route were given by a single application of 5.0 μg / 20 μl (each antigen) in phosphate-buffered saline solution (pH 7.4) to the nares of mice (14–16).

In all vaccination paradigms, animals received a prime dose on day zero followed by booster doses on days 14 and 28. On days 21 and 42 specimen blood samples were collected for estimating antisera titers by ELISA method. The animal groups were moved to ABSL-3 and assessed for their ability to protect from the challenge dose with native toxin. Survivors were monitored for >1 week.

Intranasal toxin application was carried out by first lightly anesthetizing mice with inhaled gaseous mixture of Isoflurane (Abbott Laboratories, North Chicago, IL) and oxygen (300 cm^3 min^{-1}). The dose was administered by single application to the nares. The heads of animals were maintained in an upright position to minimize drainage into the posterior pharynx, bronchoalveolar lavage (BAL). When sufficient mucous was not collected with the syringe we used moistened fine cotton swab to collect nasal mucous. The collected sample was assayed directly or after dipping and rinsing with 0.25 ml PBS.

Statistical Analysis

Statistical significance using unpaired t-tests was determined using the Prism 4 statistics software (GraphPad Software Inc., San Diego, CA).

RESULTS

The series of experiments carried out with drBoNT/A, LCA and HCA were aimed at demonstrating the preparation of a non-toxic surrogate of BoNT/A by testing its endopeptidase activity, along with its *in vivo* toxicity in mice. The utility of drBoNT/A in trafficking studies was demonstrated using the mouse whole body imaging, after labeling it with fluorescent dye. Finally, the drBoNT/A's potential as a vaccine candidate was examined in comparison to its component chains (LCA and HCA) by determining its immunogenicity and protection against challenge with native toxin.

In Vitro Studies: Endopeptidase Assay

The endopeptidase activity is key to the toxic action of BoNT, as it catalyzes the cleavage of intracellular protein substrates, leading to the blockage of neurotransmitter release. The catalytic properties of drBoNT/A and LCA were determined with both a 13 amino acid short peptide and a full length SNAP-25 membrane protein. SNAPtide 521 is a fluorescence energy resonance transfer (FRET) synthetic 12 amino acid peptide, comprised of SNAP25 cleavage site residues (Q197-R198). The endopeptidase activity of control rLc/A (100 nM) under reduced and non-reduced conditions, had RFU values of 5817.05 ± 84.11 and 5459.35 ± 123.81, where the data represents average of three wells ± standard deviation, as shown in Fig. 1a. The endopeptidase activity of 100 nM drBoNT/A, under non-reduced and reduced conditions, yielded RFU values of 0.43 and 0.49, respectively. When ten times higher concentration of drBoNT/A was tested at 1 μM, the RFU value was 0.9 for non-reduced and 0.8 reduced. Thus upon mutation of the two of the active site residues (E224 and E 262) endopeptidase activity has drastically decreased to less than 1% of the activity of recombinant light chain A.

In the solution phase both the full length SNAP-25 and the small peptide may behave differently, so we confirmed the endopeptidase activity using full length SNAP-25. When rLc-BoNT/A cleaves the SNAP-25 near the C-terminus, it cleaves into two fragments of (a) 24 kDa and (b) 1 kDa, respectively. By using traditional method of SDS-PAGE it is very difficult to determine a 1 KDa difference of uncleaved recombinant from 25 kDa SNAP-25 proteins, with the cleavage product is of 24 – KDa protein. Therefore, the 25 KDa full length recombinant SNAP-25 is attached with N–terminal eGFP –SNAP25. The full length SNAP-25 assay is shown in Fig. 1b, where rLc-BoNT/A cleave 54 kDa SNAG proteins into 30 and 24 kDa fragments. Due to the vast difference in the molecular weight of cleaved products, it is easier to discern on a SDS-PAGE gel. Treatment of SNAG with 2 nM rLCA cleaved SNAG at the SNAP-25 cleavage site, whereas 20 nM drBoNT/A did not show any cleavage with SNAP-25, Fig. 1b.

In addition, a cellular assay was carried out with M17 human neuroblastoma cell model to confirm the non-toxic nature of drBoNT/A by comparing with native toxin at various concentrations. The extent of drBoNT/A endopeptidase activity was estimated by Western blot. The M17 cells were incubated with 1 nM, 100 nM and 1 μM of drBoNT/A or native toxin for 24 h. From the western blot (Fig. 1c) it is clear that native toxin cleaves the SNAP-25 at the concentration of

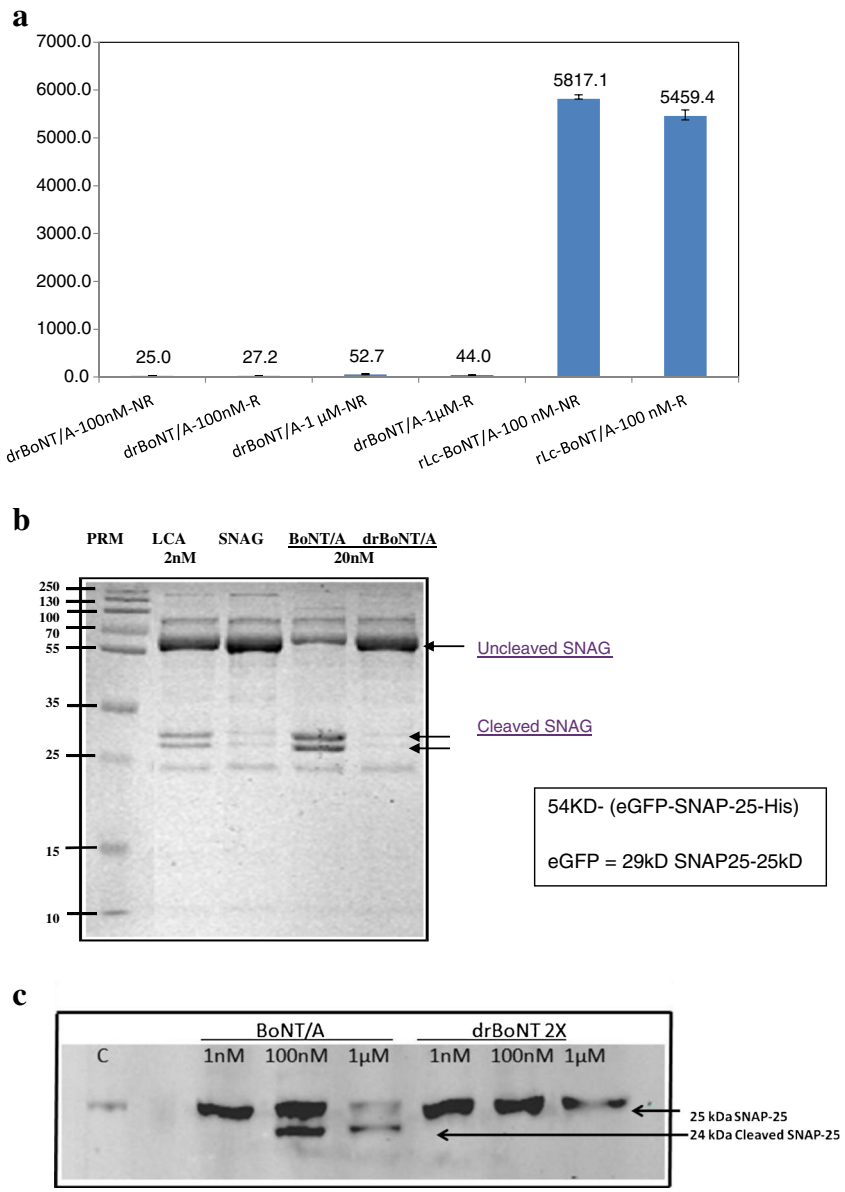


Fig. 1 (**a**) Endopeptidase activity of drBoNT/A reduced and non-reduced 100 nM and 1 μM at ten time higher concentration than 100 nM recombinant Light chain A with using SNAPtide521 as a substrate. In this assay positive control was 100 nM rLc-BoNT/A whereas the negative control SNAPtide itself. Negative control value was subtracted from the sample signals in all cases. *Error bars* represent the standard deviation from triplicate samples. (**b**) 54 kDa recombinant eGFP-SNAP25-his tag (SNAG) protein substrate was incubated with 2 nM rLCA and 20 nM BoNT/A and drBoNT for 45 min at 37 C. rLCA and BoNT/A cleaves full length SNAP-25 in to 24kd and 30 kDa protein. Where as Dr.BoNT does not cleave the SNAP-25 protein. Here, SNAG is negative control and LCA and BoNT/A are positive controls. Lane 1, Prestained Molecular Markers; Lane 2, 2 nM LCA incubated with 2 μM SNAG subatrate (Positive control); Lane 3, 2 μM SNAG Protein (Negative control), Lane 4, 20 nM BoNT/A incubated with 2 μM SNAG (Positive control); Lane 5, 20 nM dr.BoNT/A incubated with 2 μM SNAG. (**c**) Cell based Assay: M17 neuroblastoma cells were incubated for 24 h with toxin A and mutated toxin A at 1 nM, 100 nM and 1 μM. BoNT/A cleaved the SNAP-25. Detected by western blot using Rabit anti-SNAP-25 as a primary antibody and anti-rabit alkalin phosphatase as a secondary antibody. The signal detected using BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) as a substate. Lane 1, Untreated cells; Lane 2, empty lane; Lanes 3 to 5, BoNT/A concentration 1 nM, 100 nM and 1 μM, respectively; similarly Lanes 6 to 8, drBoNT/A concentrations 1 nM, 100 nM and 1 μM, respectively.

100 nM, whereas drBoNT did not show any cleavage of SNAP-25 at all irrespective of the concentration tested so far in this cellular model. This indicates that the active site mutation in the BoNT/A has completely inactivated the endopeptidase activity.

In Vivo Studies: Mouse Bioassay (LD_{50}) for drBoNT/A

Mouse bioassay is the gold standard for testing the toxicity of BoNT. Therefore, the detoxification of drBoNT/A was tested in mouse bioassay, and its toxicity was compared with LCA

and HCA, as the latter two are generally considered ineffective on their own.

The purified drBoNT/A was tested for its lethality level in an *in vivo* model, by determining the LD_{50} dose, the dose which kills 50% of the mice population. Totally 14 groups of Swiss Webster mice ($n=10$), female were dosed with drBoNT/A, via intraperitoneal route, in the range of 2.5–100 μg per mice. The post administered mice group was given plenty of food and water. Those animals were monitored, visually for every hour initially for 8 h, there after every 2 h to record any sign or symptoms of stress it may go through. If any stress was evident, those animals were isolated and immediately euthanized humanely as per the UMassD IACUC approved protocol.

As shown in Fig. 2a, the molecular bands in lane 1 &2 correspond to un-nicked drBoNT/A, reduced and non-reduced sample. Whereas the bands in lane 3 & 4 refers to nicked drBoNT/A, reduced and non-reduced samples, in lane 5 was protein bovine serum albumin as positive control. In Fig. 2b, the lane 6 represents the reduced BoNT/A holotoxin having two bands, where the first band at 100 KDa was Heavy chain and second band at 50 KDa was light chain. Whereas the lane 7 & 8 represent reduced rHc-BoNT/A and rLc-BoNT/A respectively.

As shown in the Fig. 3, the group of mice which received drBoNT/A, dose 2.5–21.0 μg did not show any stress sign during the window of observation time period, i.e., 0–96 h. When the dose level of drBoNT/A was increased to 22 μg per animal, mice did not show any sign of sickness or stress, until 36 h, but there after from 40 h, 20% of animal population started showing the following symptoms, a) hair straightening, b) heavy breathing, c) no movement but crouching in the corner, but not showing any other signs of typical botulism such as droopy eyes, muscle weakening, etc. For the next 36 h, 80% of the administered population remained healthy. Finally death occurred to only to 20% of mice population, and 80% of mice population which received drBoNT/A survived the observation period of 96 h. In the next group of mice which received 23 μg of drBoNT/A, the physiological symptoms were similar to the dose mentioned as above, and 70% of population survived. Since we wanted to identify the LD_{50} value for drBoNT/A, we continued to increase the dose level to 24 μg, for which 60% of mice group survived till 96 h. The group which received 25 μg started showing symptoms of hair straightening followed by crouching but no symptoms of botulism, yet the animals started dying at very early stage of observation period (96 h). At 24 h only 20% of the dosed animal groups were alive. The trend continued to be similar for other groups of animals dosed each with 50, 75 and 100 μg of drBoNT/A. So the acute toxicity, the dose at which drBoNT/A killed the whole population of mice with acute toxicity was found to be 75.0 μg among the tested dose groups of Swiss Webster model.

Mouse Bioassay (LD_{50}) for rHc-BoNT/A

The receptor binding domain (Hc) of BoNT/A was tested for its acute toxicity and the lethal dose of 50%, for i.p. route and the results are shown in Fig. 4. Initially the doses selected to test in Swiss Webster mice model were 5.0, 10.0, 15.0, 20.0, and 25.0 μg ($n=10$) per mouse. The post administered animals were observed for up to 96 h as described in methods section. The animal groups which received the rHc-BoNT/A, even at low doses like 10.0 μg per animal, started showing rapid onset of illness and symptoms as described above for drBoNT/A assay. Within 36 h the toxic severity increased as

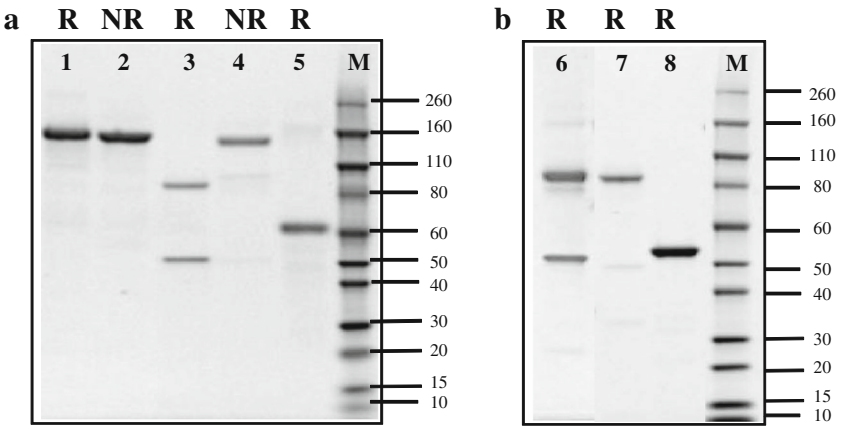


Fig. 2 (**a**) Novex Bis-Tris (XCell SureLock™ Mini-Cell system) separation of purified soluble proteins in Lane 1 & 2, dr BoNT/A, un-nicked; Lane 3 & 4 , drBoNT/A, nicked; Lane 5, Bovine serum albumin as control. (**b**) Lane 6, native BoNT/A toxin as positive control; Lane 7, rHc-BoNT/A; Lane 8, rLC-BoNT/A, and M, Molecular weight marker, Novex® Sharp Standard. *R* Reduced, *NR* Non reduced. Proteins were separated on 4–12 Novex Bis-Tris [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl] gel. The gel electrophoresis was carried out initially at a constant voltage of 85 V for 90 min followed by 100 V for 15 min at room temperature (Life Technologies, Grand Island, NY 14072). All the samples were boiled and either under reducing and non-reducing conditions; β-mercaptoethanol used as a reducing agent. The expression, purification and characterization for detoxified recombinant botulinum neurotoxin (drBoNT/A) methods was adopted as described by Yang *et al.*, (12).

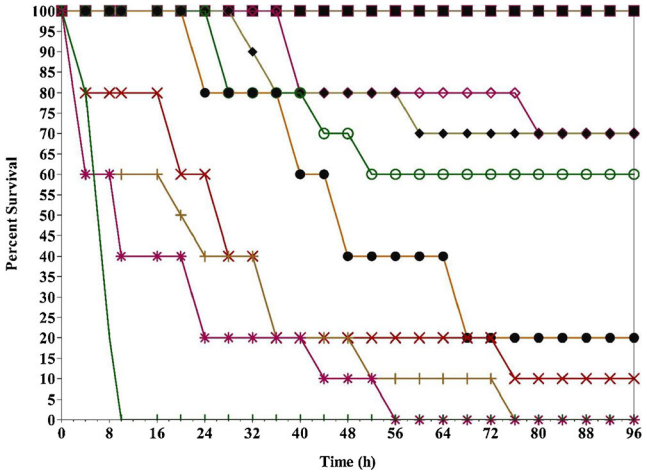


Fig. 3 Survival curves of female Swiss Webster mice treated with 150-kDa drBoNT/A via i.p route. Percent survival was plotted over time. In total of 14 doses were tested in mice. In this study the total amount of drBoNT/A was administered in 500 μl of PBS buffer, pH 7.4, per mouse. They are as follows: 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 movement-crouching, labored breathing and survival time till 96 h.

the dose increased, and the animals which received 15.0, 20, 25 or 50 μg of rHc-BoNT/A succumbed to death within 24 h.

Two control groups were included to test the animal death to examine whether it was due to the total protein injected or

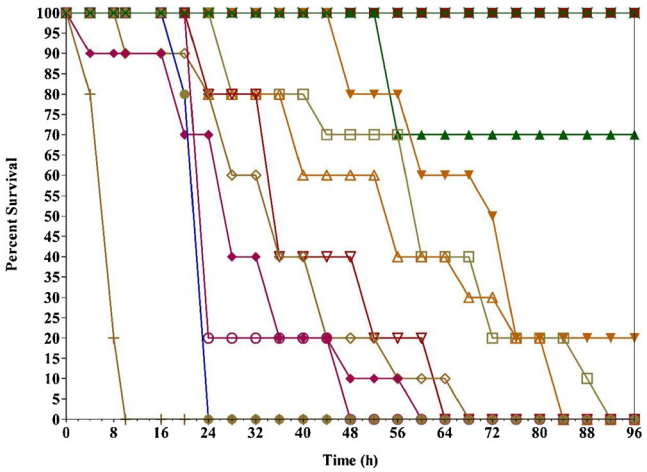


Fig. 4 Survival curves of female Swiss Webster mice treated with rHc-BoNT/A via i.p route. Percent survival was plotted over time. In total of 12 doses were tested in mice. In this study the total amount of rHc-BoNT/A was administered in 500 μl of PBS buffer, pH 7.4, per mouse. They are as follows: 2.5 μg/mouse (), 5.0 μg/mouse (), 6.0 μg/mouse (), 10.0 μg/mouse (), 15.0 μg/mouse (), 20.0 μg/mouse (), 22.0 μg/mouse (), 24.0 μg/mouse (), 25.0 μg/mouse (), 50.0 μg/mouse (), 6.0 μg/mouse + α rHc-BoNT/A (), and () positive control. 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.

due to the nature of rHc-BoNT/A administered that killed the animals: a) Anti-Hc (IgG) obtained from mice serum, incubated with rHc-BoNT/A antigen at room temperature for 60 min (1:1 ratio; Ab *vs* Ag) and followed by i.p injection (in total of 500 μl), b) Bovine serum albumin (BSA) 6.0 μg alone injected via i.p route. No death occurred to those control groups studied and outcome of this study shows that antibody did protect animals from death and even when they received equivalent amount of protein BSA, no symptoms of stress or sickness was shown by the model till 96 h. Even a low dose of 2.5 μg was tested to check the lethality of rHc-BoNT/A. For rHc-BoNT/A LD_{50}, dose which considered killing 50% of mice population, female Swiss Webster, was 5.0 μg and an acute toxicity was estimated to be 15.0 μg when administered via i.p. route.

Mouse Bioassay (LD_{50}) for rLc BoNT/A

In Fig. 5, totally 14 doses of rLc-BoNT/A were tested for its ability to kill 50% of the animal population via i.p. route, as mentioned in methods section. The light chain (rLc-BoNT/A) did not show any significant clinical symptoms of stress or other visual clinical parameters as observed for the rHc-BoNT/A, at any of the following doses, 3.0, 5.0, 7.0, 9.0, 10.0, 20.0, 25.0 and 50.0 μg. Further testing at the dose level of 100.0 μg per mice, the animals did not show any sign of stress until 72 h and only 20% of the population was dead at the end of observation period 96 h. When tested with a dose at 110.0 μg per mice ($n=10$), onset of sickness started at 44 h, having more than 70% of survival rate amongst the administered group of animals. Further, a higher dose levels were tested in Swiss Webster animal model, with doses of 115.0, 120.0 and 125.0 μg, wherein the LD_{50} was estimated to be 115.0 μg for rLc-BoNT/A and its acute toxicity was estimated to be 120.0 μg. The clear manifestation of the symptoms for native toxin or BoNT's neurotoxin was not observed in either of the proteins, rHc BoNT/A, rLc-BoNT/A, when tested in the mice model.

Bio-Imaging Studies for Near Infra-Red Dye Cross-Linked with drBoNT/A

Trafficking of BoNT is important to devise intervention protocols. It has been difficult to monitor BoNT trafficking due to its extreme toxicity. The surrogate drBoNT/A provides an opportunity to address BoNT trafficking under *in vivo* conditions.

The image screening performed with drBoNT/A ~ NIR 800 (Fig. 6) was in parallel to those studies performed by Hale *et al.* (2010), but the dose administered were 10 times lower than the previous investigation. The study provided insight about the movement and localization of NIR ~ drBoNT/A in the *in vivo* system. The most of administered dose appeared

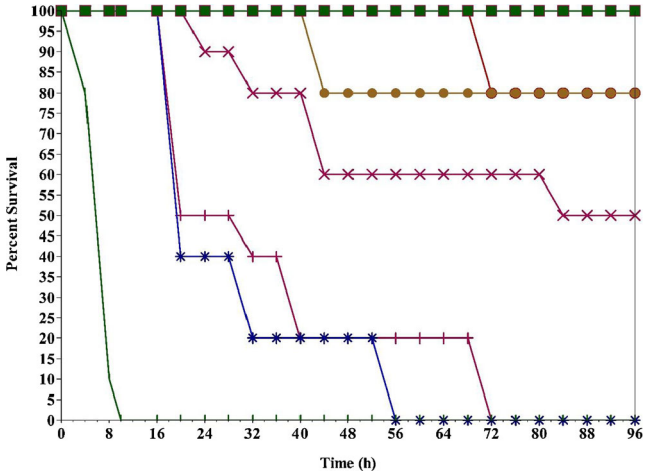


Fig. 5 Survival curves of female Swiss Webster mice treated with rLc-BoNT/A via i.p route. Percent survival was plotted over time. In total of 14 doses were tested in mice. In this study the total amount of rHc-BoNT/A was administered in 500 μl of PBS buffer, pH 7.4, per mouse. They are as follows: 3.0 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.

in the in the intestine and bladder area irrespective of the dosage route chosen either i.p. or i.v. routes.

In this preliminary data, when examined the post administered NIR ~ drBoNT/A, (i.p. route, 5 h), in the upper and lower regions of intestine, including bladder, where most of the protein appeared to be retained (Fig. 6a). The mouse which received NIR ~ drBoNT/A via i.v. route, it retained the protein in the upper intestine region and within hind-limbs (Fig. 6b). In order to facilitate for examination of the distribution results, a grid was superimposed over the mouse model which was adopted with permission from Vasqueszet *et al.*(17) (Fig. 6c).

Immunogenicity of Recombinant Light Chain, Heavy Chain and Deactivated Botulinum Neurotoxin and Its Survival Studies Against Native Toxin Challenge

Currently there is no vaccine available against BoNT, and the vaccine under development involves only the c-terminus half of the HCA. A full length vaccine candidate is obviously better alternative due to its potential high immunogenicity. Comparative immunogenicity of drBoNT/A and its constituent chains was therefore examined with intransal administration, as such administration would be more convenient in case mass immunization is warranted.

Immunogenicity was tested for the following antigens, a) rLc-BoNT/A, b) rHc-BoNT/A and c) drBoNT/A either in the presence or absence of adjuvants, such as Vitamin-E TPGS or Forskolin, Also an empty microsphere of PLGA (50:50) was used as an adjuvant, where the antigen was adsorbed on to the surface of microspheres.

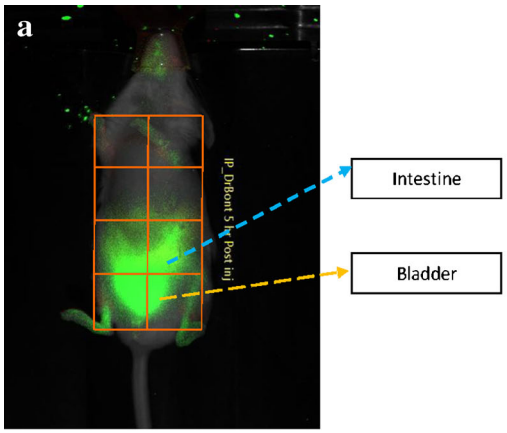
The immunoglobulins (IgG) evoked by the soluble antigens of rLc-BoNT/A *vs.* rHc-BoNT/A *vs* drBoNT/A are shown in Fig. 7a. In the IgG titers, there was a 2-fold increase for rHc/A when compared with rLc/A, statistically very significant ($P<0.0001$), and 52-fold higher for soluble drBoNT/A in comparison to rLc/A, statistically significant ($P<0.0001$). The rHc/A rendered 25 fold lower IgG titers when compared with drBoNT/A, which was statistically very significant ($P<0.0001$).

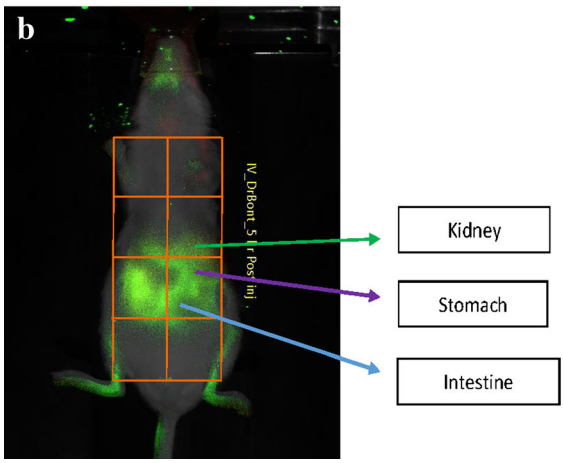
The efficacy of engendering IgG antibody levels in mice model was in the following order, rLc/A < rHc/A < drBoNT/A.

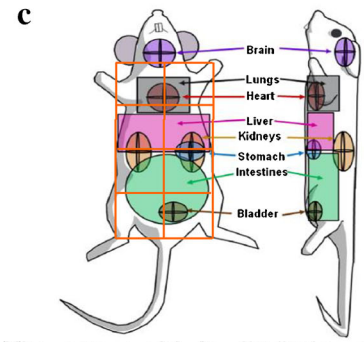
The effects of immunoadjuvants were examined on the efficacy of antigens in evoking immunoglobulins, IgG, (Fig. 7b). The water soluble adjuvant Vitamin-E TPGS, seems to evoke higher amount of IgG titers for rLc/A, a 3-fold increase in their IgG titers when compared with antigen administered alone (no adjuvant). There was a 4-fold higher IgG titers for rHc/A compared to rLc/A, and a 8-fold higher response in drBoNT/A compared to rHc/A. Furthermore, when the antigens tested with PLGA microsphere as an adjuvant, there was 2 fold less amount of anti-rLc/A (IgG) compared with anti-rHc/A (IgG), which was very significant ($P<0.0001$). With PGLA microsphere as an adjuvant, drBoNT/A showed only 4-fold higher IgG titers than rLcA, which was again statistically very significant ($P<0.0001$). The trend in evoking IgG levels for antigens remained the same, even in the presence of adjuvant (Vitamin-E TPGS), i.e., rLc/A < rHc/A < drBoNT/A.

The drBoNT/A was tested for its ability to evoke immunoglobulin (IgG) in four different conditions, a) as soluble antigen, b) admixed with adjuvant Vitamin-E, c) adsorbed onto PLGA microsphere, and d) adjuvant Forskolin. The results are shown in Fig. 7a and b. When the anti-drBoNT/A (IgG) levels are compared among the groups administered as soluble "drBoNT/A" and drBoNT/A plus Vitamin-E, a significant increase of 2-fold IgG levels was observed ($P<0.0022$). Also, a 2-fold difference was noticed when serum from anti-drBoNT/A plus PLGA, compared with serum anti-drBoNT/A (IgG) levels ($P<0.0001$) and Forskolin, serum anti-drBoNT/A (IgG) ($P<0.0001$), respectively. However, among the group of adjuvants, there was no significant difference observed in the levels of anti-drBoNT/A (IgG) ($P=0.98$).

The levels of secretory immunoglobulin IgA (sIgA) in response to different antigens in Balb/C is shown in Fig. 8. From each antigen paradigm a group of Balb/C animals ($n=5$) was separated at the completion of third dose and nasal wash samples were collected 1 weak after the third immunization. The PLGA adsorbed dosage system for rLc/A showed







Mice organ model for distribution studies adopted with permission from Vasquezet et al. (17)

Fig. 6 PEARL® Imaging system (LiCor Biosciences, Lincoln, NE) was used to scan Swiss Webster mice injected with drBoNT/A~800 (NIR dye), by intraperitoneal (**a**) and by intravenous route (**b**). The images scan above represents 5 h post administrated drBoNT/A ~800 localized at various regions of intestine, stomach, bladder etc. The Fig. 6(**c**), a mice model representation was adopted from Vasquezet *et al.* (17) to demonstrate the organ/tissue regions in a typical mouse *in vivo* system, in order to facilitate understanding the pathways of labeled compound. The image scanned at white light 700 and 800 nm and fluorescent signals examined later with PEARL® Cam Software.

significant increase (8-fold) in sIgA when compared with soluble antigen of rLc/A (P=0.0009), whereas rHcA antigen showed no significant (p=0.4032) difference in their estimated levels of IgA, although 2-fold difference was observed with PLGA delivery system. sIgA evoked by drBoNT/A administered with Forskolin was 4-fold higher than the soluble version of drBoNT/A (p=-0.0035), and 2-fold difference was noticed in sIgA response between the adjuvant Forskolin compared with PLGA (p<0.0153) delivery system.

All the rLc/A, rHc/A and drBoNT/A, vaccinated animal groups, with or without adjuvants along with control animals were challenged i.p. with 1000 LD_{50} native toxin BoNT/A doses delivered in 50 µl are shown in Table. I. The control animals became very seriously ill within 100 min or less. Of the animals vaccinated with no adjuvant, only 30% of them survived the challenge, and 70% of animals which received Vitamin-E adjuvant survived. Also, 100% survived for PLGA adjuvant group showing no signs of illness. In the animal group which received soluble protein rHc-BoNT/A, survival rate was 40% only, and the group of animals which received adjuvants Vitamin-E and PLGA there was complete protection from the challenge dose. All the groups of animals which were immunized with drBoNT/A showed no sign of illness, when challenged with native toxin irrespective of the dosage delivery system, i.e., presence or absence of mucosal adjuvants. All of them survived during the observation period of 96 h and even further beyond the stipulated time for observation.

DISCUSSION

BoNT's are deadly substance that requires extraordinary biosafety precautions. It has multidomain structural features which has specific biological roles such a) receptor binding domain (Hc), b) translocation domain (NH) and c) enzymatic active domain (Lc) (18). It would be easier and safe to work with a non-toxic mutant analogue of BoNT/A is the drBoNT/A. drBoNT/A has mutation at the LC residues E224 to glutamine and E262 to alanine. The E224 is located in zinc-binding domain and active site of enzymatic activity of BoNT/A, and E262 interacts directly with zinc (12).

Both amino acids play critical roles in peptide hydrolysis of the BoNT/A substrate, SNAP-25. The endopeptidase activity of the drBoNT/A, along with mutant LC and wild-type HC was tested against BoNT/A's natural neuronal substrate SNAP-25, to ensure complete loss of the enzymatic activity (1,19,20).

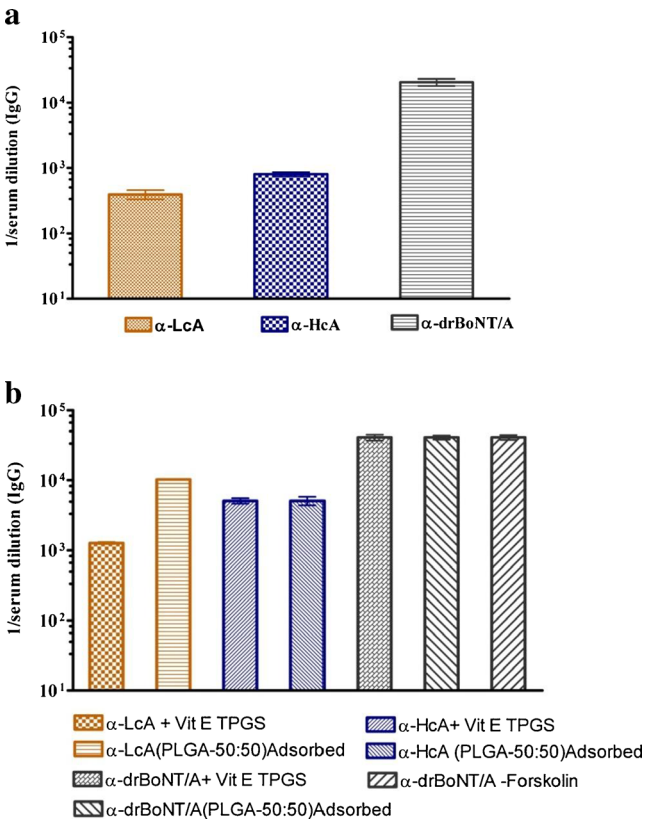


Fig. 7 Immunoglobulin (IgG) response to the following antigens i) rLc-BoNT/A (*brown*), (ii) rHc-BoNT/A (*blue*) and iii) drBoNT type A (*black*), in female Balb/C mice via intranasal route ($n = 10$ per /antigen/adjuvant). (**a**) Initially screening was undertaken for the soluble antigen alone and then the (**b**) antigen plus vitamin-E TPGS, antigen adsorbed onto PLGA (50:50) microsphere as mucosal adjuvant and finally antigen plus Forskolin. The mice were vaccinated at day 0, 14, 28. At 6 week, aliquots of blood were obtained for IgG. 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.

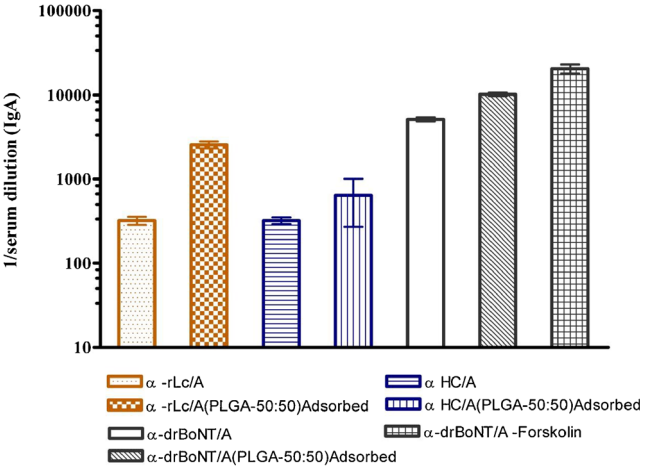


Fig. 8 Immunoglobulin (IgA) response to the following antigens i) rLc-BoNT/A (*brown*), (ii) rHc-BoNT/A (*blue*) and iii) drBoNT type A (*black*), in female Balb/C mice via intranasal route ($n = 10$ per /antigen/adjuvant). The initial screening was undertaken for the soluble antigen alone followed by the group which received antigen adsorbed onto PLGA (50:50) microsphere as mucosal adjuvant and finally antigen plus Forskolin. At day 42, aliquots of blood were obtained for IgA assay. The data show that the adjuvant significantly assisted in evoking higher level of secretory IgA antibody for drBoNT/A and rLc/A. However, for rLc/A statistically there was no significant increase in their response to secretory IgA.

The endopeptidase activity of double mutant rLc-BoNT/A, drBoNT/A, and rHc-BoNT/A against SNAP-25 was found to be negligible, compared to native BoNT/A or wild type LCA, which were considered as 100% activity (19). The current bioassay confirms the absence of cleavage for drBoNT/A at 20 nM or 1.0 μM range with full length SNAP-25 and FRET assays. The results thus demonstrate that we can completely remove the endopeptidase activity of BoNT/A through mutations within the LC region, while the HC has no endopeptidase activity of its own.

Furthermore, drBoNT/A toxicity assay was performed with M17 human neuroblastoma cell model. Among the other neuronal cell line models, the M17 cell line test has been selected in this study, since it has proved to have a higher sensitivity for BoNT sero types A, C & E detection (21). Testing of antidotes for BoNT intoxication has been compared with M17 cell lines (22–24), where study shows the advantage of M17 neuroblastoma cell line in screening potential inhibitors at high throughput level without even using a holotoxin. This M17 cell would simplify the regulatory requirements and thus greatly reduce the cost involved in the project. In this study with the M17 cell line, the ability of binding and internalization of drBoNT/A seems to be similar to native toxin, except that the latter cleaved the SNARE proteins at 100nM, whereas the drBoNT/A does not show any cleavage. When tested even at the higher concentration (1.0 μM), drBoNT/A showed no cleavage of SNARE proteins confirming the elimination of toxicity.

The drBoNT/A was labeled with near infra-red dye 800 and the purified conjugate of drBoNT/A~800 was subjected for imaging studies using the Pearl Imaging System developed by Licor Inc. The labeled compound was stable *in vivo* and fluorescence data correlated well with presence of drBoNT/A (13, 25) in the mice. The majority of the administered dose in the tissue or organ distribution of radio labeled native BoNT/A was reported to be localized within organ/tissues such as liver, spleen, kidney, heart, and lung (25).

Current *in vivo* imaging studies with drBoNT/A~800 was done in parallel to the previous studies carried out by Hale *et al.* (13), where an injected (400 ng; i.v,) dose appeared in the upper abdomen and thorax region within 1.5 h. When screened at 5th h, the fluorescence began to dissipate rapidly from the kidney and bladder region (13). In this study, the distribution pattern observed with lesser amount of labeled drBoNT/A~800 (40 ng) injected via i.p. route. The fluorescence localization was observed in the region of upper intestine and bladder even after 5 h. However, when labeled drBoNT/A was given via i.v. route, fluorescence rapidly

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)

cleared from the region of bladder indicating the rate of elimination from whole body was faster than the i.p route.

Earlier studies showed that when BoNT/A, native toxin, was given by i.v. route to mice, it took approximately 230 min to eliminate the 50% of dose from blood stream, the rest of administered dose being localized at various organs such as liver, kidney, spleen, etc. (25). Our data gathered with lower dose levels was similar to earlier studies. However, further extensive examination is essential at various time intervals and different routes to conclude the bio-distribution of NIR ~ drBoNT/A. Based on pharmacodynamics results, one could develop remedial treatment for a clinical patient intoxicated with BoNT's. So far, it has never been shown clearly that even after exceeding the $t_{1/2}$ time period for native toxin, the following issues have never been investigated; a) duration of toxin localization within tissues or organ, b) the region i.e., type of tissues or organ where it has been localized.

Sera from the current mice study were not analyzed for the presence of drBoNT/A, but changes in the intensity of fluorescence detected in the abdomen region suggested dispersion of the administered toxin dose. *In vitro* studies of drBoNT/A with mouse neuromuscular junctions, hind limb reflex experiment, clearly demonstrated the void of toxicity at two different doses, 150 ng or 3.2 pg, respectively and even after internalization and localization at the motor nerve the drBoNT/A structure remain unaltered (26). Further extensive *in vivo* studies using various concentrations administered by different routes will shed more details regarding the movement of toxin from one biological compartment to another before unleashing its toxicity at the peripheral neuromuscular junction, including their localization and clearance mechanism. Also these studies will provide sufficient knowledge about the window of opportunity to develop an effective choice of treatment, during a real time crisis.

Mouse bioassays were then performed to test whether the abolishment of endopeptidase activity corresponded to a similar reduction in toxicity in an *in vivo* model (20,27). Pier *et al.* (28) tailored BoNT/ARYM a recombinant BoNT/A with two single-point mutations (R365A and Y365F) on the light chain domain rendered toxins inactive. An *in vivo* challenge test for both single chain and trypsin-nicked dichain of recombinant BoNT/A in animal model showed the tolerance level of 1 μg (28). When the BoNT serotype C was mutated at the light chain region (H229—G, E230—T, and H233—N), it resulted in complete detoxification of the modified recombinant holotoxin, and the acute toxicity dose was 10 μg (29) in animals.

In the present study, drBoNT/A was administered to groups of mice with a sequential increase in concentrations and monitored for their survival. Other than the loss of the catalytic activity and neurotoxicity compared with native toxin, the drBoNT/A protein purified as an unnicked protein, it exists in di-chain form when isolated. So while testing in animal models the drBoNT/A was nicked by trypsin digestion to ensure it exist di-chain form. The mice group which received a higher dose of drBoNT/A, 24.0 μg per mice (1,200,000 MLD); exhibited no toxicity. It also suggested drBoNT/A is about 1.2 million fold less toxic than the native toxin. Currently there are many studies in progress to deliver drugs to potentially treat various neuronal disorders, such as Alzheimer disease, Parkinson's disease, epilepsy and multiple sclerosis (20). These neurological disorders are affected due to their biochemical malfunctioning at cellular levels of central or peripheral nervous system. The literature shows that there is sufficient information on the steps involved with the BoNT's holotoxin is known to undergo, a) binding, b) internalization, c) endocytosis and finally cleavage of SNARE complex, thus blocking acetylcholine release. Using these steps our laboratory is trying to exploit the possibilities of drBoNT/A as a neuronal cargo delivery system. Preliminary studies in cell based model were attempted to examine the feasibility of drBoNT/A as a drug delivery system (20). The cell lines used to test their selective entry in nature was human SH-SH5Y neuroblastoma and rhabdomyosarcoma cells respectively, where drBoNT/A mostly localized to the cell surface without internalization in rhabdomyosarcoma cells and internalized inside the cell membrane with the other neuronal cells are SH-SH5Y (20).

Recently, a new generation of recombinant botulinum vaccine (rBV A/B) is being developed to prevent fatal botulism following exposure to botulinum neurotoxin complex serotype A, subtype A1 (BoNT/A1) and serotype B, subtype B1 (BoNT/B1). The antigenic components of rBV A/B vaccine by Dynport Vaccine Company for the US Department of Defense (DoD), tested the antigens in the ratio of 1:1 mixture, 20 μg antigen A and 20 μg antigen B, which was adsorbed on to Alhydrogel. The efficacy of the rBV A/B vaccine is based on the capacity of neutralizing antibodies induced by vaccination, which binds to BoNT and prevent its action at the cholinergic neurons. Both studies showed that mice immunized with recombinant proteins were protected against challenge with active BoNT. Sera from vaccinated mice also contained high titers of neutralizing antibodies (30–32). BoNT/ARYM admixed with adjuvant Alhydrogel, has been shown to be highly immunogenic as a vaccine candidate (28). All the above mentioned studies with recombinant antigens were tested admixed with FDA approved mucosal adjuvant Alhydrogel or the number of booster dose was more than 2 doses. Another report demonstrated with heavy chain (HC_{50}) fragments of serotypes A, B & E for the receptor binding domain was tested in mice, either as soluble antigens or mixed with adjuvant, via inhalation route. But the total dose administered was four times higher compared to our study. Although the immunoglobulin levels was sufficient enough to protect the challenge dose of 1000 LD_{50}, the mice model was never tested histopathologically for any damage it may have caused to organs such as liver, kidney etc., or achieving the same protection level with fewer dose of antigens (16).

In addition to study immunization with drBoNT/A, other recombinant proteins such as rHc-BoNT/A and rLc-BoNT/A were also tested in mice. The efficacy of those antigens in evoking their corresponding immunoglobulin G or A (IgG or IgA) were compared as the following groups, i) soluble antigen alone, ii) antigen mixed adjuvant Vitamin-E TPGS, iii) antigen adsorbed on to PLGA (50:50) microspheres as mucosal adjuvant and finally, iv) antigen admixed with Forskolin as adjuvant.

The data presented in this study suggest that antibodies evoked by soluble antigens alone were weak. The challenge study with native toxin rendered only partial protection (30–40%) of the mice group received rLc and rHc BoNT/A, respectively. Whereas in the soluble antigen drBoNT/A paradigm, their antibody response was much higher than other antigens (>65%) and all the animals from this group survived the challenge dose. In order to seek a safe, needle free and better immunization paradigm, we admixed the above mentioned antigens, initially with a mucosal adjuvant Vitamin-E TPGS. The mucosal adjuvant vitamin-E TPGS was extensively tested for its ability to enhance the antibody response with antigens like tetanus toxoid and diphtheria toxoid (33). The addition of Vitamin-E significantly induced better anti-rHc-BoNT/A (IgG) production than anti-rLc-BoNT/A. However, only a 2 fold difference was measured in the levels of anti-drBoNT/A (IgG). When the immunized group was challenged with native BoNT/A toxin, the mucosal adjuvant Vitamin-E gave only 50% protection for rLc-BoNT/A, but it rendered complete protection (100%) to the challenge dose for the mice group which received rHc-BoNT/A and drBoNT/A. Further, when all those antigens delivered by adsorbing onto PLGA (50:50) microsphere, it engendered higher level of IgG and all the groups of animals survived the challenge dose to native toxin, thus achieving our goal. In addition, Forskolin was tested for the first time as an adjuvant for drBoNT/A. The engendered immunoglobulin-G levels were similar to that of other adjuvants, whereas for immunoglobulin A levels, there was a significant increase compared to the rest of delivery system, especially for drBoNT/A and gave complete protection to the challenge dose with native BoNT/A toxin.

Several investigators have contributed in the field of botulinum to study the efficacy of HC_{50} domain for all BoNT serotypes, which were mostly injected multiple times to evoke sufficient levels of immunoglobulin IgG response. It has also been shown that the inactive holotoxin in the form of toxoid and the full length of HC (100 kDa) for several (BoNT's) serotypes, when tested in animal models, administered either via oral or inhalation routes, evoked enough IgG or IgA antibody levels and survived to the native toxin challenges. Immunization with BoNT chains individually could offer advantage of lower toxicity without a need to mutate amino acid residues. Larger proteins such as full length drBoNT/A requires higher protein load which can be avoided with truncated antigens like HCA or LCA. However, we found that HCA is more toxic than drBoNT/A (Figs. 3 and 4), and drBoNT/A was more effective as a vaccine candidate (Table I).

In this study for the first time, the data presented above shows that full length inactive holotoxin of serotype A, given via inhalation route, can evoke sufficient enough antibody to protect the challenge dose level of up to 1000 LD_{50} doses of native toxin. Further optimization needs to be attained to achieve this level of protection or even higher level, in the following categories, a) fewer number of doses and most importantly b) delivering the antigen dose via oral route with a suitable formulation delivery system such as biodegradable pH sensitive polymers, to protect those antigens from any degradation.

There are several potential advantages to the use of an intranasal vaccine, but two are paramount. Firstly, if the portal of entry and/or site of toxin action are at the mucosal surface, then a mucosal antibody response would be desirable. Secondly, administration of a mucosal vaccine, such as an intranasal or oral vaccine, eliminates the undesirable byproducts of needles and syringes, ranging from accidental needle sticks and blood borne diseases to medical waste and

more importantly the reduction of environmental contamination. Inhalation is a likely route for BoNT entry in the case of a potential bioterrorist incident. Therefore, the existence of a multiple mucosal layer of neutralizing antibodies is likely to be beneficial. The challenges involved in developing a vaccine delivery system will be delivering the antigen dose needed to evoke sufficient level of antibodies. By designing an effective mucosally deliverable mono and multicomponent particulate vaccine against botulism that incorporates catalytically deactivated full length protein, it is possible to induce a rapid, strong, long lasting, and protective immune response. In recent years, despite the difficulties involved in oral vaccine delivery system, considerable efforts has been made to deliver such vaccines with edible foods, such as potatoes, tomatoes, bananas as a delivery vehicles. Researchers involved with oral vaccine systems need to be focused on the properties of peptides or protein to survive the harsh condition of gastrointestinal system, involving the effects of pH and enzyme proteolysis. Even if there is no damage to proteins/peptides, it must not cause any toxicity to the digestive system and post absorbed proteins or peptides must possibly retain their conformational properties to effectively evoke sufficient neutralizing antibodies. Finally, a single dose vaccine could be administered safely as needle free, preferably via oral delivery system.

ACKNOWLEDGMENTS AND DISCLOSURES

The study was supported in part by a NIAID (NIH) grants [1U01A1078070-01and R03AI103868] and a contract [HSHQDC-12-C-00071] from the Department of Homeland Security through Omni Array Biotechnology. I would like to sincerely thank Mr. Paul Lindo, Ms. Koyel Ghosal and Ms. Harkiran Dhaliwal for being part of animal husbandry team.

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