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Dual-route targeted vaccine protects efficiently against botulinum neurotoxin A complex

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ABSTRACT

Clostridium botulinum readily persists in the soil and secretes life-threatening botulinum neurotoxins (BoNTs) that are categorized into serotypes A to H, of which, serotype A (BoNT/A) is the most commonly occurring in nature. An efficacious vaccine with high longevity against BoNT intoxication is urgent. Herein, we developed a dual-route vaccine administered over four consecutive weeks by mucosal and parenteral routes, consisting of the heavy chain (Hc) of BoNT/A targeting dendritic cell peptide (DCpep) expressed by *Lactobacillus acidophilus* as a secretory immunogenic protein. The administered dual-route vaccine elicited robust and long-lasting memory B cell responses comprising germinal center (GC) B cells and follicular T cells (Tfh) that fully protected mice from lethal oral BoNT/A fatal intoxication. Additionally, passively transferring neutralizing antibodies against BoNT/A into naïve mice induced robust protection against BoNT/A lethal intoxication. Together, a targeted vaccine employing local and systemic administrative routes may represent a novel formulation eliciting protective B cell responses with remarkable longevity against threatening biologic agents such as BoNTs.

1. Introduction

Botulinum neurotoxins (BoNTs) are among the most toxic substances known to man. These toxins are secreted as exotoxins from Gram-positive spore forming *Clostridium botulinum* (*C. botulinum*). *C. botulinum* can be cultivated from the soil, and its toxins can be mass produced as a bioweapon; one gram of aerosolized toxins can potentially eradicate one million individuals [1]. Due to its extremely toxic nature, *C. botulinum* is categorized as a select agent A. *C. botulinum* secretes neurotoxin serotypes (A-H) [2–4]; however, human botulism is commonly associated with toxin serotypes A, B, E, and F. These poisonous BoNTs can lead to death via airway obstruction secondary to muscle paralysis [2–4]. The BoNTs consist of two peptide chains bridged together with a disulfide bond [5]. The heavy chain (Hc) acts as the vehicle for the short chain to facilitate entry into nerve cells. BoNTs are susceptible to gastric enzymes and a low pH; however, they are protected from harsh conditions of the gastrointestinal tract by complexing with a hemagglutinin protein expressed by the bacteria. The short chain of BoNTs possesses zinc (Zn^{++})-dependent protease activity that cleaves soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) to inhibit acetylcholine vesicle-delivery at nerve synapses, leading to complete flaccid paralysis. Of the different serotypes of toxins produced by *C. botulinum*, serotype A is the most common. BoNTs or *C. botulinum* producing BoNTs can enter the host by various routes; however, the most common and natural mode of introduction is the orogastric route. Presently, due to lack of long-lasting and efficacious protective vaccines, the therapeutic and preventative approach(s) against BoNTs comprise the physical elimination of neurotoxins and provision of basic care to sustain life. However, anti-toxin antibody treatment may be considered a prime pathway for therapy [6–8]. More precisely, BoNTs are transcribed as a single polypeptide that is further cleaved into two chains bound to a disulfide link. The Hc of BoNTs binds to a neuronal receptor, SV2, for internalization of the toxins (e.g., BoNT/A), whereupon, neutralizing antibodies against BoNTs, particularly

Abbreviations: BoNT, clostridium botulinum neurotoxin; DCpep, dendritic cell binding peptide; Hc, heavy chain of BoNT/A; CL, colon; MLN, mesenteric lymph node; SP, spleen; PLN, peripheral lymph node; GC, germinal center; Tfh, T follicular helper cells.

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Hc-BoNT/A, prevent the devastating effects of the neurotoxin complex [6]. Thus, it is urgent to generate innovative vaccine strategies using BoNT subunits to significantly confer long-lasting protection against BoNT complex intoxication.

The commensal, *Lactobacillus acidophilus* (*L. acidophilus*), demonstrated the potential to be an excellent delivery vehicle for oral vaccine subunits, including protective antigen (PA) of *B. anthracis* [9,10]. Such an oral vaccine produced by this bacterium induced protective responses against pathogen challenge [9]. However, two major disadvantages of such employed plasmid-based delivery may account for the risk of losing the plasmid, being the lack of antibiotic selection and delivery of the antibiotic resistance gene to gut commensals. To over come these potential hurdles, *L. acidophilus* now expresses targeted Hc-BoNT/A to DCpep from a chromosomal location [11]. Oral vaccination using this new vaccine delivery system, when combined with intradermal injection of purified targeted Hc-BoNT/A-DCpep vaccine, elicited robust and long-lasting protective B cell responses against lethal BoNT/A complex intoxication, as well as remarkable longevity of humoral responses that protected mice against subsequent oral BoNT/A complex intoxication nine months later.

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from NCI and Charles River and reared at the animal facility at the University of Florida. Mice (6–8 week) were used in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida under protocol number, 2014107129.

2.2. Vaccination

L. acidophilus strain, NCK2310, was generated, previously [11], which expresses Hc-BoNT/A-DCpep as fusion protein. NCK2310 was grown in static MRS media anaerobically at 37 °C. NCK2310 was used for gavaging BALB/c mice (10^9 CFU/100 μL sterile PBS/mouse).

2.3. Preparation of the vaccine

Hc-BoNT/A-DCpep was isolated from NCK2310 culture supernatants that were first freeze-dried and resuspended in 1/10th of the initial volume. The concentrated material was dialyzed against water for 48 h to remove excessive salt and other media components. The remaining contents were concentrated by a centrifuge concentrator (20 kD cut-off), and confirmed by Western blot analyses using anti-Hc-BoNT/A antibodies (Metabiologics Inc., Madison, WI).

2.4. Botulinum neurotoxin

Clostridium botulinum neurotoxin serotype A (BoNT/A) complex for mouse oral intoxication, and *Clostridium botulinum* serotype A neurotoxin (BoNT/A) were purchased from Metabiologics Inc. (Madison, WI) and kept under strict vigilance with appropriate documentation for their use, in accordance with Environmental Health and Safety (EHS) guidelines at the University of Florida (University of Florida Acute toxin registration number AT-4164). Mice were orally intoxicated with BoNT/A complex (1 or 3 μg/-mouse), as indicated in the respective figure legends. BoNT/A was used for ELISA or its neutralization by sera derived from surviving mice.

2.5. In vivo neutralization assay

To test the quality of serum antibodies developed by the vaccination, 100 μL sera isolated from the vaccinated or naïve mice were incubated with 50 picograms (pg) of BoNT/A for one hr at 4 °C before injecting them into naïve BALB/c mice . Mice survival was evaluated every hour for the next six hr and then every 12 h for ten days.

2.6. Flow cytometry

Single cells were isolated from the colon, spleen, mesenteric lymph nodes (MLNs), and the peripheral lymph nodes (PLNs) [12]. After incubation with Fixable Blue Dead Cell Stain (Life Technologies, Carlsbad, CA) and Fc blocking (Miltenyi Biotec, San Diego, CA), cells were stained with antibodies, or reagents and fixed with PFA 4% for 20 min. Flow cytometric analyses were performed using a BD LSR II Fortessa (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, Version 10.1r7). A list of the antibodies used can be found in the Supplemental Table 1. Gating strategies are depicted in the Supplemental Figs. 1–3.

2.7. Enzyme linked immunosorbent assay (ELISA)

ELISAs were used to determine the anti-Hc-BoNT/A antibodies in the sera (for IgG titer) and fecal samples (for IgA titer) of vaccinated and surviving mice. Standard ELISA protocols were followed to quantitate antibody responses to Hc-BoNT/A. Commercial BoNT/A (5 μg/mL) was used to coat MaxiSorp microtiter plates (Nunc, Thermo Fisher Scientific, Waktham, MA) overnight at 4 °C. Sera or fecal extracts from individual mouse groups were evaluated for anti-BoNT/A antibodies. Mouse fecal suspensions (10% w/v) were generated in PBS with 50 μg/mL soybean trypsin inhibitor (Sigma-Aldrich, Saint Louis, MO). HRP-goat anti-mouse IgA, and IgG (Southern Biotechnology Associates, Birmingham, AL) were used for detection. Endpoint titers were defined as the highest reciprocal of dilution of samples giving an absorbance at OD_{415} above 0.100 OD units above negative controls after 1 h of incubation at 25 °C.

2.8. Memory B cells transfer

BALB/c mice were orally vaccinated four times with NCK2310 (10^9 CFU/mouse), once a week, followed by 2 boosts (Fig. 1A). Seven days later, splenic memory B cells were magnetically sorted using the mouse memory B cell isolation Kit (Miltenyi Biotec, San Diego, CA). Briefly, 50 μL each of Anti-IgG1-APC, Anti-IgG2ab-APC and 100 μL Biotin Antibody Cocktail were incubated with 108 isolated splenic cells from the vaccinated mice resuspended in 300 μL PBS containing 0.5% FBS and 2 mM EDTA for 5 min on ice. 200 μL of Anti-Biotin microbeads were added to the mix to remove non-memory B cells using LD columns. Anti-APC microbeads were introduced in the flow through of LD columns to further purify memory B cells from the cellular suspension using MS columns. The isolated cells were counted to prepare a cellular suspension containing 10^6 cells/100 μL. Memory B cells (10^6 cells/mouse) were then intraperitoneally transferred into recipient naïve BALB/c mice. One month later, recipient mice were intoxicated with BoNT/A complex (1μg/mouse). Mouse survival was monitored.

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