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Oral vaccination with an adenovirus-vectored vaccine protects against botulism

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Abstract

We have previously shown that an adenovirus vectored vaccine delivered intramuscularly or intranasally was effective in protection against botulism in a mouse model. The adenoviral vector encodes a human codon-optimized heavy chain C-fragment (H_C50) of botulinum neurotoxin type C (BoNT/C). Here, we evaluate the same vaccine candidate as an oral vaccine against BoNT/C in a mouse model. To elicit protective immunity, the mice were orally vaccinated with a single dose of 1×10^4 to 1×10^7 plaque forming units (pfu) of the adenoviral vector. The immune sera, collected six weeks after oral vaccination with 2×10^7 pfu adenovirus, has shown an ability to neutralize the biological activity of BoNT/C *in vitro*. Additionally, animals receiving a single dose of 2×10^6 pfu adenovirus or greater were completely protected against challenge with $100 \times \text{MLD}_{50}$ of BoNT/C. The data demonstrated the feasibility to develop an adenovirus-based oral vaccine against botulism.

Keywords

botulinum neurotoxin; oral vaccination; protective immunity; recombinant adenovirus; toxin neutralization

1. Introduction

Botulinum neurotoxins (BoNTs) are mainly produced by the gram-positive, anaerobic bacteria *Clostridium botulinum*. These neurotoxins, with seven distinct serotypes A–G, are the most toxic substances known and are classified as Category A biological agents with a high probability for use in bioterrorism [1]. The serotypes A, B, E, and F cause naturally occurring human botulism, while serotypes C and D cause animal botulism [2]. An active BoNT consists of a 100-kDa heavy chain and a 50-kDa light chain linked by disulfide bonds. The heavy chain contains the 50-kDa cell receptor-binding (C-fragment or H_C50)

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and 50-kDa translocation (N- fragment or H_N) domains; the light chain contains the catalytic zinc endopeptidase domain.

Previously, an investigational botulism vaccine had been available. However, as of November 2011, this pentavalent botulinum toxoid (PBT) vaccine (used for workers at risk for exposure to BoNTs) has been discontinued by the CDC [3]. There were several issues with the vaccine: declining immunogenicity, decreased product potency, and increased occurrence of injection site-related adverse reactions. The CDC suspect that these issues are related to the age of the product (>30 years). Perhaps due to factors such as high cost of production, length of time needed to produce sufficient yields of product, and safety concerns in producing toxoids, the PBT vaccine stocks were not replaced more frequently. In the case of a bioterrorism event involving BoNTs, production of the PBT vaccine may be too slow and may not meet the demand. Furthermore, the PBT vaccine is inherently not feasible for a rapid civilian immunization program.

Our replication-incompetent adenovirus-vectored vaccine, containing a human codon-optimized sequence for BoNT/C-H_C50 (encoding amino acids 849–1291 in BoNT/C, GenBank accession number EU142252), addresses most of these issues: low cost of production, quick production of vaccine, and few safety concerns. Our previous studies have demonstrated that this vaccine is effective against botulism through a single intramuscular (i.m.) or intranasal (i.n.) inoculation [4, 5]. In this study, we evaluated the same vector as an oral vaccine candidate against botulinum neurotoxin type C challenge in a mouse model.

2. Material and methods

2.1. Experimental design

Six week old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed, four animals per cage, in animal facility under a specific pathogen free (SPF) condition. All experimental tests were carried out in accordance with the US Public Health Service *Guide for the Care and Use of Laboratory Animals* (NRC Publication, 1996 ed.) and other related federal statutes and regulations in Animal Welfare Act.

60 mice were split into five experimental groups and two control groups, eight mice per group (exception: positive control group). The animals were orally inoculated with the Ad/opt- BoNT/C-H_C50 vaccine at doses of 1×10⁴, 1×10⁵, 1×10⁶, 2×10⁶, and 1×10⁷ plaque forming units (pfu) per mouse. The negative control group was orally inoculated with Ad/Null (no transgene) at a dose of 1×10⁷ pfu per mouse. The positive control group (n=12) was i.m. inoculated with the PBT vaccine (Michigan Department of Public Health, Lot No PB003) at a dose of 50 µl per mouse. Six weeks after immunization, serum samples were collected for measuring toxin neutralization antibody titers. The vaccinated mice were then intraperitoneally (i.p.) challenged with 100×MLD₅₀ of purified BoNT/C (MetabioLogics Inc., Madison, WI) as previously described [4, 5].

2.2. Adenoviral vector encoding codon-optimized H_C50 of BoNT/C

A replication-incompetent human adenovirus serotype-5 vector Ad/opt-BoNT/C-H_C50 and a control vector Ad/Null were constructed using the AdEasy System (Agilent, Stratagene Products Division, La Jolla, CA) as described previously [4, 5]. The Ad/opt-BoNT/C- H_C50 vector contained a synthesized human codon-optimized gene encoding the H_C50 fragment of BoNT type C1 [6] and a native gene encoding the signal peptide of human tissue plasminogen activator (GenBank accession number BC002795).

The recombinant adenoviruses were produced in AD293 cells (Agilent) and purified by centrifugation in a CsCl gradient. Virus layers were collected and pooled, and the cesium

chloride was removed via dialysis. The resulting product was sterilized by filtration, then stored in a 1.0 M sucrose solution in a -86°C freezer until use. Viral titers, in pfu, were determined by plaque assay on AD293 cells.

2.3. Measurement of neutralization titer to active BoNT/C

Additional 56 female BALB/c mice were used to test neutralizing antibody titers. Two groups of mice (8/group) were inoculated with either 2×10^7 pfu per mouse Ad/opt-BoNT/C-H_C50 or 1×10^7 pfu per mouse Ad/Null. Serum samples were obtained 6 weeks after vaccination for measuring neutralizing antibody titers. The remaining 40 mice were split into 10 dilution groups, 4 mice per group.

Neutralization titers of mouse sera to BoNT/C was determined as previously described [5, 7]. Briefly, a volume of 25 μl sera from each mouse, collected six weeks after inoculation with 2×10^7 pfu vaccine, was pooled and serially diluted two-fold in phosphate buffered saline (PBS). $100 \times \text{MLD}_{50}$ of active BoNT/C was added into each serum dilution, followed by incubation at room temperature for 1 hour. Four mice per dilution group were i.p. injected with the corresponding BoNT/sera mixture. The animals were monitored, and the number of deaths was recorded. Neutralizing antibody titers was defined as the maximum number of international unit (IU) of antitoxin per ml of serum, resulting in 100% survival after challenge. One IU of antitoxin neutralizes $10,000 \times \text{MLD}_{50}$ toxin [7, 8].

3. Results

3.1. In vitro neutralization of BoNT/C by immune sera

We evaluated the bioactive capability of the sera from mice after oral vaccination with Ad/opt-BoNT/C-H_C50. 32-fold diluted sera, collected six weeks after vaccination, was sufficient to neutralize $100 \times \text{MLD}_{50}$ of active BoNT/C and resulted in a 100% survival rate in the mouse bioassay (Fig. 1A). Further dilutions of sera resulted in 50% survival rate at 64-fold dilution and 0% survival rate at further dilutions (Fig. 1A). This translated neutralization titer was 3.2 IU/ml (Fig. 1B). Serum from the control mice receiving a single dose of Ad/Null did not neutralize the neurotoxin.

3.2. Protective immunity against challenge with active BoNT/C

To evaluate vaccine efficacy, mice were orally inoculated with different doses (1×10^4 pfu to 1×10^7 pfu) of Ad/opt-BoNT/C-H_C50 in week 0. In week 7, the mice were challenged with $100 \times \text{MLD}_{50}$ active BoNT/C. The protective rates increased in a dose-dependent manner (Fig. 2). The mice were 100% protected against BoNT/C challenge at the $100 \times \text{MLD}_{50}$ level when a single dose of 2×10^6 pfu adenovirus vector or greater was administered. None of the mice receiving control vector Ad/Null survived the toxin challenge, and 83% (10/12) of mice receiving one dose (50 μl /mouse) of PBT vaccine survived toxin challenge.

4. Discussion

We have assessed the efficacy of oral vaccination with the adenoviral vector encoding a human codon-optimized BoNT/C(H_C50) against the active toxin challenge in a mouse model. Our results showed both protective immunity and functional neutralizing antibody response against active BoNT/C. Due to the potential of BoNTs to be used in bioterrorism, our goal is to develop a vaccine that can be easily and quickly produced, rapidly deployed, and effortlessly used by vaccine recipients without the requirement of medically trained personnel.

Numerous studies have shown that H_C50 protein or DNA is capable of eliciting protective immunity against botulism [9–12]. Recombinant adenovirus, carrying the antigen transgene

to the host, will induce potent antigen-specific protective antibodies and T-cell responses [13–16]. This adenoviral vector is safe because it lacks the E1 gene necessary for replication and can only be produced in E1 complementing cells such as AD293 cells under laboratory condition. A single adenovirus amplifies exponentially and thus, is quickly produced in case of emergency. Our vaccine candidate has advantages in production speed compared to a protein vaccine.

Initially, we tested our vaccine candidate via i.m. and i.n. inoculations. With robust antibody responses, we evaluated the oral route for vaccination. Our previous data [4, 5], along with data from this study, show different neutralizing antibody titers: i.m. > i.n. > oral vaccination. Historically, oral vaccinations have been regarded to be less effective than i.m. vaccination [16, 17]. Despite lower titers of neutralizing antibodies produced, 2×10^6 pfu Ad/opt-BoNT/C-H_C50 was sufficient to provide 100% protection against challenge with 100×MLD₅₀ of the toxin. The PBT vaccine requires trained medical staff to administer by needle injection. A single dose oral vaccination has clear advantages as it does not require medically trained personnel. Furthermore, administration of an oral vaccine, lacking painful needle sticks, encourages patient compliance in a rapid vaccination program.

There are many difficulties involved in moving a vaccine from successful mouse studies into human clinical trials, and our study did not address some aspects. We have not assessed the longevity of protection from the oral vaccine in this study, but our previous studies have shown that a single i.m. and i.n. dose can provide full protection for at least 27 weeks post vaccination [4, 5]. Because our vaccinated mice are housed in a clean environment, our studies also have not accounted for the influence of pre-existing anti-adenovirus on efficacy of oral vaccination. However, previous research has shown that efficacies of oral vaccination with adenoviral vector were not impaired by pre-existing immunity to the vaccine carrier [18]. Furthermore, additional serotypes of adenoviruses exist and may be used as alternative vectors for vaccine antigen delivery.

In a bioterrorism event, time and resources are scarce. Our vaccine design is optimized to address these conditions. Future studies may focus on ways to improve on the vaccine: a lyophilized version of the adenovirus may increase shelf life and eliminate the need for refrigeration, adding non-infectious adenoviral particles may boost antibody titers, etc. Although we cannot conclude that an adenoviral-vectored vaccine will solve all shortcomings of protein-based vaccines, our studies suggest that the current adenovirus-vectored vaccine is a promising oral vaccine candidate against botulism.

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Highlights

- An oral vaccine can elicit neutralizing antibody response against botulinum neurotoxin
- The candidate oral vaccine can protect against lethal challenge with botulinum neurotoxin
- The protective immunity against botulinum neurotoxin is vaccine dose dependent

Fig. 1.

Anti-BoNT/C neutralizing antibody titers in sera from vaccinated mice. Mice were vaccinated orally in week 0 with 2×10^7 pfu of Ad/opt-BoNT/C-H_C50 per mouse in the experimental group (n=8) and Ad/Null in the control group (n=8). Six weeks after vaccination, a volume of 25 μ l of serum from each mouse in the same group was pooled. The pooled sera were 1:4 diluted initially with Dulbecco's PBS and then, in two-fold, serially diluted to determine anti-BoNT/C neutralizing antibody titers. **A:** survival rates of mice after challenge with neutralized BoNT/C (n=4 for each dilution). **B:** serum anti-BoNT/C neutralization titers (IU/ml). 1 IU = 10,000 $\times$ MLD₅₀. IMM, vaccination group; CON, control group.

Fig. 2.

Protective immunity against active BoNT challenge in vaccinated mice. Mice were orally vaccinated with different doses (1×10^4 to 1×10^7 pfu per mouse) of Ad/opt-BoNT/C-HC50 in week 0, then challenged with $100 \times \text{MLD}_{50}$ of active BoNT/C in week 7. A positive control (P. Con, 50 μl of PBT vaccine, i.m. route) and a negative control (N. Con, 1×10^7 pfu per mouse of Ad/Null, oral route) groups were carried out in parallel. $n=8$ for vaccination and negative control groups. $n=12$ for positive control group.