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Protective immunity against botulism provided by a single dose vaccination with an adenovirus-vectored vaccine

Mingtao Zeng^{a,*}, Qingfu Xu^a, MD Elias^b, Michael E. Pichichero^a, Lance L. Simpson^b, and Leonard A. Smith^c

^a Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Box 672, 601 Elmwood Avenue, Rochester, New York 14642, USA

^b Department of Medicine and Department of Biochemistry and Molecular Biology, Jefferson Medical College, Philadelphia, Pennsylvania 19107, USA

^c Integrated Toxicology Division, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, Maryland 21702, USA

Abstract

Botulinum neurotoxins cause botulism, a neuroparalytic disease in humans and animals. We constructed a replication-incompetent adenovirus encoding a synthesized codon-optimized gene for expression of the heavy chain C-fragment (H_C50) of botulinum neurotoxin type C (BoNT/C). This recombinant human serotype 5 adenoviral vector (Ad5) was evaluated as a genetic vaccine candidate against botulism caused by BoNT/C in a mouse model. A one-time intramuscular injection with 10⁵ to 2 × 10⁷ pfu of adenoviral vectors elicited robust serum antibody responses against H_C50 of BoNT/C as assessed by ELISA. Immune sera showed high potency in neutralizing the active BoNT/C *in vitro*. After a single dose of 2 × 10⁷ pfu adenoviral vectors, the animals were completely protected against intraperitoneal challenge with 100 × MLD₅₀ of active BoNT/C. The protective immunity appeared to be vaccine dose-dependent. The anti-toxin protective immunity could last for at least 7 months without a booster injection. In addition, we observed that pre-existing immunity to the wild type Ad5 in the host had no significant influence on the protective efficacy of vaccination. The data suggest that an adenovirus-vectored genetic vaccine is a highly efficient prophylaxis candidate against botulism.

Keywords

Botulism Vaccine; Protective immunity; Replication-incompetent adenovirus

1. Introduction

Botulism is a life-threatening neuroparalytic disease caused by botulinum neurotoxins (BoNTs), which are produced by one of the seven structurally similar *Clostridium botulinum* serotypes, designated A to G in which type C has two subtypes (C1 and C2). In addition, *Clostridium baratii* synthesizes only serotype F and *Clostridium butyricum* synthesizes only serotype E. As the concept of serotype implies, each of the toxins is immunologically distinct.

*Corresponding author. Mailing address: Mingtao Zeng, Ph.D., Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Box 672, Rochester, New York 14642, USA, Phone: (585) 275-1003, Fax: (585) 473-9573, E-Mail: Mingtao_Zeng@urmc.rochester.edu.

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The only exception to this general rule is serotypes C and D, which share significant cross-homology [1]. BoNTs are the most poisonous substances known in nature. They may be used as bioterrorism agents or in biological warfare [2]. Therefore, there is an urgent need for the development of effective vaccines to protect against botulism.

Currently, a pentavalent botulinum toxoid vaccine that may protect against BoNT serotypes A–E is available as an Investigational New Drugs (IND) [3,4]. However, there are shortcomings with the toxoid vaccines. For example, the cost of manufacturing is very high, because *C. botulinum* is a spore-former and a dedicated cGMP facility is required to manufacture a toxin-based product. The yields of toxin production from *C. botulinum* are relatively low; it is dangerous to produce them, as the toxoiding process involves handling large quantities of toxin, and the added safety precautions increase the cost of manufacturing. The toxoid product for types A–E consists of a crude extract of clostridial proteins that may influence immunogenicity or reactivity of the vaccine, but the type F toxoid is only partially purified; residual formaldehyde (not to exceed 0.02%) and the preservative thimerosal (0.01%), are part of final product formulation. This increases the reactogenicity of the vaccine [5].

A high sequence and structural homology exists between the clostridial neurotoxins produced by *Clostridium tetani* and *C. botulinum*. The successful demonstration that C-fragment of tetanus toxin (TeNT) elicits protective immunity in animals has prompted the development of a new subunit vaccine against botulism [6,7]. Evaluation of the immunogenicity of different regions of BoNTs has confirmed that the C-fragment of the BoNTs elicits protective immunity in animals [8–13]. Therefore, subsequent efforts to develop vaccine candidates to protect against BoNTs forthwith may focus on the H_C region of BoNTs [14]. Because the C-domains of the BoNTs are responsible for receptor binding, host immune response against these domains may prevent the BoNTs from gaining access into target cells. Research on peptide-based vaccines has shown several synthetic peptides elicit antibody and T-cell responses in two different strains of mice (BALB/c and SJL) that cross-react with the H_C region of BoNT/A. These experiments show the feasibility of developing a synthetic vaccine that could protect against botulinum neurotoxin intoxication [5,15–18]. The employment of the non-toxic fragments of BoNTs as protective antigens also provides a significant safety profile advantage.

Replication-incompetent adenoviruses are currently available efficient gene transfer vehicles for both *in vitro* and *in vivo* approaches [19,20]. Adenovirus-vectored recombinant vaccines expressing a wide array of antigens have been constructed and protective immunity against different pathogens has been demonstrated in animal models [21–25]. In our research, we demonstrated the efficacy of using an adenovirus-based vaccine for single-time genetic vaccination that provided long-lasting protective immunity against botulism caused by botulinum neurotoxin type C.

2. Materials and methods

2.1. Construction of adenoviral vector encoding codon-optimized H_C50 of BoNT/C

Replication-incompetent recombinant adenoviral vectors were constructed using the AdEasy™ System (Stratagene, La Jolla, CA) [26,27]. The adenoviral vector is derived from human adenovirus serotype 5 rendered replication-incompetent by the deletion of the E1 and E3 regions. To construct the Ad/opt-BoNT/C-H_C50, the nucleotides encoding the 50-kDa C-terminal fragment of heavy chain of botulinum neurotoxin type C1 [28] was optimized with human codon preference by the DNAworks program [29]. The nucleotides encoding the signal peptide of human tissue plasminogen activator (PLAT) (amino acids 1–25, GenBank Acc# BC002795) plus 2 serines followed with the codon-optimized BoNT/C-H_C50 (encoding amino acids 849–1291 in BoNT/C, GenBank Acc# D90210) were then synthesized by a PCR-based method [30]. The synthesized DNA was subsequently cloned into a shuttle vector pShuttle-

CMV (Stratagene, La Jolla, CA) at its *SaI*I site. The DNA sequence of the synthesized gene was further confirmed by DNA sequencing analysis. The new opt-BoNT/C-H_C50 sequence will be assigned an access number #X (pending submission) in the GenBank. Table 1 shows the native and codon-optimized opt-BoNT/C-H_C50 sequences. The adenoviral vector was then constructed according to the standard procedures as described previously [26,27]. In the adenovirus, the transgene expression is under control of human cytomegalovirus (CMV) immediate early promoter/enhancer and then followed with a simian virus 40 (SV40) stop/polyadenylation signal [26]. Similarly, the Ad/Null vector without transgene was also constructed. Adenoviruses isolated from single plaques were then produced in AD293 cells (Stratagene, La Jolla, CA) and purified by CsCl gradient purification and dialyzed with adenovirus storage buffer containing 10 mM Tris pH 7.5, 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂. The purified adenoviruses were stored in 1.0 M sucrose in a -80°C freezer until use and their titers (pfu) were determined by plaque assay before vaccinating animals.

2.2. Animal vaccination and sample collection

Six to eight-week old, female Balb/c mice, were purchased from Taconic Farms (Hudson, NY), and housed in the animal facility of University of Rochester (4 animals per cage). They were maintained in a controlled environment (22 ± 2°C; 12 h light/12 h dark cycles) in accordance with the U.S. Public Health Service "Guide for the Care and Use of Laboratory Animals." The animals were provided Laboratory Rodent Diet 5001 with *ad libitum* access to food and water. The research was conducted in compliance with the Animal Welfare Act and other federal and state statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition.

Experiment 1—Mice were allotted into 5 groups (8 mice/group). They were injected i.m. into the hind-leg quadriceps with different doses of Ad/opt-BoNT/C-H_C50 vector (10⁵, 10⁶, or 2 × 10⁷ pfu/mouse), Ad/Null (2 × 10⁷ pfu/mouse), and the Botulinum Toxoid Adsorbed Pentavalent (ABCDE) (0.05 ml/mouse), an IND vaccine which was produced by the Michigan Department of Public Health. Animals were inoculated once in week 0. Animal sera were obtained by retro-orbital bleeding every 2 weeks (in week 0, 2, 4, and 6) and stored at -20°C until further assays. Vaccinated mice were intraperitoneally (i.p.) challenged with 100 × MLD₅₀ BoNT/C 1 week after last bleeding described in the following section.

Experiment 2—To determine long-term protective immunity, mice were allotted into another 3 experimental groups (8 mice/group) and 3 control groups (4 mice/group). Animals were intramuscularly (i.m.) vaccinated with Ad/opt-BoNT/C-H_C50 vector (2 × 10⁷ pfu/mouse) in experimental groups and with Ad/Null (2 × 10⁷ pfu/mouse) in control groups in week 0. Animal sera were obtained by retro-orbital bleeding in weeks 0, 10, 18, and 26. One experiment and one control group were challenged i.p. with 100 × MLD₅₀ BoNT/C in weeks 11, 19, and 27.

Experiment 3—To investigate the effect of pre-existing immunity to adenovirus on the vaccination efficacy, 12 mice were allotted into one experiment (8 mice/group) and one control group (4 mice/group). Animals were intranasally (i.n.) inoculated with wide-type human adenovirus serotype 5 (2 × 10⁷ pfu/mouse) (ATCC, VA) in week 0 and bled in week 4. They were subsequently inoculated with Ad/opt-BoNT/C-H_C50 vector (2 × 10⁷ pfu/mouse) in the experimental group or with Ad/Null (2 × 10⁷ pfu/mouse) in the control group in week 4 and challenged i.p. with 100 × MLD₅₀ BoNT/C in week 11.

2.3. Challenge with botulinum neurotoxin in mice

All animals were challenged by i.p injection with 100 × MLD₅₀ botulinum neurotoxin type C (Metabolics Inc, Madison, WI) at the time indicated in each experiment. The challenged

animals were monitored for 7 days. They were observed every 4 h for the first two days and twice a day thereafter. The number of deaths for each group was recorded as the endpoint [31].

2.4. ELISA for determination of antibody concentration

Serum anti- BoNT/C-H_C50 IgG, IgG1, and IgG2a antibody concentrations were determined using an ELISA Quantization kit (Bethel Lab. Inc., Montgomery TX) with a modified procedure. Briefly, 96-well flat-bottom immuno plates (Nagle Nunc International, Rochester, NY) were coated with 0.5 µg/well of either His-tagged BoNT/C-H_C50 recombinant protein produced in *Escherichia coli* or capture antibodies (goat anti-mouse IgG-, or IgG1-, or IgG2a -affinity purified, Bethel Lab, Montgomery, TX, for standard curve) in 100 µl coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) at 4°C overnight. The plates were washed 5 times with washing buffer (0.05% Tween 20 in PBS) and nonspecific binding sites were blocked with 200 µl PBS (pH 7.4) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. After five washes, 100-µl serial dilutions of reference serum containing given amounts of mouse antibodies (for standard curve) or 1:100 dilutions of mouse serum samples in PBS (pH 7.4) containing 0.05% Tween 20 and 1% BSA were added. After 2 h further incubation at 37°C, the plates were washed with washing buffer 5 times and incubated with 100 µl/well of 1:10,000 dilution of goat anti-mouse IgG, IgG1 or IgG2a conjugated to alkaline phosphatase for 1 h at room temperature. Unbound antibodies were removed by washing 5 times with washing buffer, and the bound antibody was detected after incubation with p-nitrophenylphosphate phosphatase substrate system (KPL, Gaithersburg, Maryland) for 30 min. The color reaction was terminated by adding 100 µl 0.5 M EDTA and the absorbance values were obtained using a Dynatech MR4000 model microplate reader at 405 nm. A standard curve was generated for each set of samples and serum antibody concentrations were calculated in accordance with a standard curve as previously described [32].

2.5. Botulinum neurotoxin neutralization assay

Neutralizing antibody titers to BoNT/C were measured by the ability of sera to neutralize the neurotoxin *in vitro* in combination with the mouse lethality assay. 200 µl of pooled sera from 8 mice 6 weeks after vaccination with 2×10^7 pfu of Ad/opt-BoNT/C-H_C50 vector or with Ad/Null were initially diluted 1:4 and then diluted in twofold series (1:4 to 1:1052) in DPBS (Dulbecco's PBS). $400 \times \text{MLD}_{50}$ BoNT/C in 200 µl of DPBS was added into each dilution. After incubation at room temperature for 1 h, the anti-serum and the BoNT/C mixture was injected i.p. into mice, 100 µl (corresponding to $100 \times \text{MLD}_{50}$ of BoNT/C before neutralization) per mouse, 4 mice were tested for each dilution. The mice were monitored for 4 days, and the number of deaths at each sample dilution was recorded. If the toxin was neutralized, the mice were protected from the challenge with neutralized toxin. The detection limit for this assay was 0.04 IU/ml due to the limited amount of serum available. Neutralizing antibody titers were defined as the maximum number of IU of antitoxin per ml of serum, resulting in 100% survival after challenge. One IU of botulinum neurotoxin antitoxin neutralized $10,000 \times \text{MLD}_{50}$ [33].

2.6. Anti-adenovirus neutralizing antibody titer assay

Anti-adenovirus neutralizing antibodies were determined according to previously described methods with some modifications [34–36]. Briefly, AD293 cells (Stratagene, CA) were seeded in 96-well plates at a density of 10^4 cells/well in 200 µl of Eagle's minimum essential medium (EMEM) containing 10% FBS, 2 mM glutamine, 50 U of penicillin per ml, and 50 µg of streptomycin per ml and incubated at 37°C, 5% CO₂ overnight. Mouse serum samples were heat-inactivated at 55°C for 45 min and then serially twofold diluted in MEME containing 2% FBS in a new 96-well culture plate. Approximately 10^4 pfu of wild-type human adenovirus

Ad5 in 50 μ l of MEME containing 2% FBS was mixed with 50 μ l of diluted serum samples. After incubation for 1 h at 37°C to allow neutralization to occur, 100 μ l of virus-serum mixture was subsequently added to AD293 cells, and incubated for 2 h at 37°C. After incubation, the virus-serum medium was replaced with 200 μ l of EMEM containing 10% FBS. The cells were incubated at 37°C until the negative wells exhibited 90% cytopathic effect. The neutralizing antibody titer was determined to be the highest dilution wells showing <50% cytopathic effect as previously described [34–36].

2.7. Statistical analysis

Serum antibody concentration titers among different groups at different time points were compared and analyzed using the LSD test and ANOVA/MANOVA with STATISTICA 7.1 software (StatSoft, Tulsa, OK); in comparing groups, those with P -values < 0.05 and < 0.01 were considered to be significant and very significant, respectively.

3. Results

3.1. Antibody response to H_C50 of BoNT/C after vaccination

Groups (8 mice/group) of female Balb/c mice were inoculated intramuscularly (i.m.) with recombinant adenovirus Ad/opt-BoNT/C-H_C50. After a single dose of 10^5 , 10^6 , 2×10^7 plaque-formation units (pfu) of Ad/opt-BoNT/C-H_C50 vector in week 0, antibody responses in animal sera were measured by quantitative ELISA. Fig. 1 shows BoNT/C-H_C50-specific antibody responses in sera 2, 4, and 6 weeks after vaccination. The data indicate that the lowest vaccine dosage 10^5 pfu tested was sufficient to elicit significant IgG1 and IgG2a antibody responses in Week 6 compared with the control group injected with Ad/Null (an Ad5 vector with no transgene) (P values < 0.05). The rise in antigen-specific IgG1 (Fig. 1B) and IgG2a (Fig. 1C) after vaccination suggested that both Th2 and Th1 immune responses were elicited. More specifically, the IgG antibody response could be characterized as a predominant Th2 response (values of IgG2a/IgG1 < 1.0). In addition, serum antibody responses were clearly vaccine-dose dependent.

3.2. In vitro neutralization of botulinum neurotoxin

In order to determine whether anti-H_C50 antibodies in the sera were capable of neutralizing active BoNT/C, an *in vitro* neurotoxin neutralization combined with the mouse lethality assay (MLA) were performed. Pooled sera from vaccinated animals were used for *in vitro* toxin neutralization. As shown in Fig. 2A, up to 128-fold diluted sera from animals 6 weeks after a single injection of 2×10^7 pfu of Ad/opt-BoNT/C-H_C50 completely neutralized $100 \times$ MLD₅₀ BoNT/C under experimental conditions and resulted in a 100% survival rate after administration of the neutralized toxin in mice. This translated to a 13 IU/ml anti-BoNT/C neutralization titer (Fig. 2B). These data suggested that parental inoculation with the adenoviral vector could elicit functional antibody responses that neutralized active botulinum neurotoxin.

3.3. Protective immunity elicited by the adenovirus-vectored vaccine

To explore whether the H_C50-based adenovirus-vectored vaccine protect against botulinum neurotoxin intoxication, the vaccinated mice were intraperitoneally challenged (i.p.) with 100 50% mouse lethal dose (MLD₅₀) units of active BoNT/C 7 weeks after injection. The results from the challenge experiments showed that vaccination with 10^5 pfu of Ad/opt-BoNT/C-H_C50 could protect 12.5 % of the animals against the toxin challenge (Fig. 3). However, the protection rates rose to 75% and 100% when the vaccine dose was increased to 10^6 and 2×10^7 pfu, respectively. This suggested that vaccine dose-dependent protective immunity was achieved after vaccination.

In order to determine if a single dose of Ad/opt-BoNT/C-H_C50 could provide long-term immunity against botulinum neurotoxin, another 6 groups of mice were inoculated with 2×10^7 pfu of Ad/opt-BoNT/C-H_C50 or with control vector Ad/Null. Serum samples were collected in weeks 0, 11, 19, and 27. As shown in Fig. 4, BoNT/C-H_C50-specific antibody titers in sera were sustained until at least 27 weeks after vaccination ($p < 0.05$). To evaluate protective immunity at different time points, the vaccinated animals were subsequently challenged with $100 \times \text{MLD}_{50}$ of active BoNT/C in week 11, 19, and 27. As indicated in Fig. 5, a single dose of Ad/opt-BoNT/C-H_C50 administered i.m. completely protected animals against botulism at all time points examined. This suggests the long-lasting memory immunity against botulinum neurotoxin was elicited after a single dose of the adenovirus-vectored vaccine.

3.4. Influence of pre-existing anti-adenovirus immunity on the efficacy of vaccination

To investigate if host pre-existing immunity to the adenoviral vector could limit the efficacy of the vaccination with this adenovirus-based vaccine, we performed an additional experiment. Mice were first inoculated intranasally (i.n.) with 2×10^7 pfu of wild-type 5 human adenovirus (WT Ad5). Serum-neutralizing antibody titers against WT Ad5 were assessed by a 96-well neutralization antibody assay. The results showed all animals had anti-adenovirus neutralizing antibody responses, with average viral neutralization titers ranging from 96 to 112 (Fig. 6A) 4 weeks after inoculation of WT Ad5. The animals with pre-existing immunity to adenovirus were subsequently inoculated i.m. with a single dose of 2×10^7 pfu of Ad/opt-BoNT/C-H_C50 or with control vector Ad/Null in week 4 and challenged i.p. with $100 \times \text{MLD}_{50}$ of active BoNT/C in week 11. The results in Fig. 6B show that all animals inoculated with Ad/opt-BoNT/C-H_C50 survived the toxin challenge and none of the control mice were protected. These data suggest that pre-existing immunity to adenovirus in the host did not affect the protective efficacy of the vaccination with this adenovirus-based botulism vaccine.

4. Discussion

The genetic vaccination strategy was previously attempted in botulism vaccine development. Clayton and Middlebrook constructed a plasmid DNA encoding the nontoxic H_C50 region of BoNT/A and showed it to partially protect against toxin challenge after up to 4 booster injections [37]. Jathoul et al. constructed plasmid DNA expressing BoNT/F H_C under the control of human ubiquitin gene (UbC) promoter and found that two i.m. injections afforded 90% protection against BoNT/F challenge [38]. In addition, Lee and coworkers introduced the H_C50 of BoNT/A or BoNT/C into the Venezuelan equine encephalitis (VEE) virus replicon vector. These constructs not only yielded high levels of H_C fragments, as judged by immunofluorescence and immunoblotting analysis, but also protected mice against BoNT/A or BoNT/C challenge after 2 or 3 injections with 10^7 infectious units (i.u.) of VEE [39–41]. However, a single dose of genetic vaccine was not fully protective against botulism in these studies.

In our research, we demonstrated that it is possible to develop a highly efficient genetic vaccine against botulism using an adenoviral vector encoding the H_C50 fragment of BoNT/C. In the recombinant adenovirus we constructed, the DNA sequence encoding the H_C50 fragment antigen was codon-optimized with human codon preference. This resulted in high-level expression of H_C50. It might be secreted from host cells using the signal peptide of human tissue plasminogen activator (PLAT) [42,43], and the secretory H_C50 might be presented to antigen-presenting cells (APCs) more efficiently after vaccination. In any case, the vaccine construct was more potent at inducing host immune responses than was an adenoviral vector encoding the native H_C50 sequence (data not shown).

A single i.m. dose of the Ad/opt-BoNT/C-H_C50 was capable of eliciting significant Th2 and Th1 immune responses against H_C50 fragment of BoNT/C (Fig. 1) and the serum antigen-specific antibodies were capable of neutralizing active BoNT/C (Fig. 2). The protective antibodies against H_C50 of BoNT/C were sustained for long-term (up to 27 weeks) (Fig. 4). Host immune responses and protective immunity appeared to be vaccine dose-dependent. Most importantly, a single dose of 2×10^7 pfu of Ad/opt-BoNT/C-H_C50 was sufficient to provide long-term protective immunity (Fig. 5). This study is the first to demonstrate that a single genetic vaccination is able to provide long-lasting protection against botulism.

Because we used the human Ad5-derived replication-incompetent adenovirus for vaccine delivery, the issue of host pre-existing immunity against adenoviruses needed to be addressed. The data from our experiments showed that even with pre-existing anti-adenovirus neutralizing antibody in the host, the protective efficacy of the vaccination was sustained (Fig. 6). However, this result may need to be interpreted with caution as the ability for replication of human adenovirus is limited in murine cells [44], and human immune response to adenovirus may differ from that of mouse.

Another important issue in using adenoviruses as vaccines is to maintain the adenoviral vectors non-infective in the host. There is possible contamination of replication-competent adenovirus (RCA) in the vaccines using currently available HEK293 cells for adenovirus propagation. The 293 cells are human embryonic kidney cells originally transformed with the right arm of adenovirus serotype 5. When the E1/E3 defective adenovirus is produced with this cell line, it is possible that the vector could acquire an E1 gene from the cellular genome by homologous recombination to generate RCA. To avoid RCA occurrence, new suitable packaging cell lines such as the Per.C6 and UR cell lines were developed recently [45–47], and these cell lines could be worthwhile if a clinical trial were to be pursued.

In summary, our research demonstrated for the first time that an adenovirus-based vector encoding a humanized H_C 50-kDa fragment of BoNT/C was capable of eliciting robust host immunity against botulism caused by BoNT/C after a single dose. The anti-BoNT/C protective immunity was sustained for a prolonged time period. This strategy could be used to further develop a multivalent vaccine against all serotypes of botulinum neurotoxins.

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Fig. 1.

Anti H_C50 of BoNT/C response in vaccinated mice. Mice were inoculated with different doses of Ad/opt-BoNT/C-H_C50 in week 0. Serum samples were obtained at weeks 0, 2, 4, and 6. Anti-BoNT/C-H_C50 IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) antibody concentrations were measured by a quantitative ELISA kit (Bethyl, Montgomery, TX). Virus doses for groups I, II, and III were 10⁵, 10⁶, and 2 × 10⁷ pfu, respectively. Mean = X ± SE (n = 8). Values without the same letters (a, b, c, d) differ significantly in the same dosage groups (*P* < 0.05). * *P* < 0.05, ** *P* < 0.01.

Fig. 2.

Serum anti-BoNT/C neutralizing antibody titer assay. 50 μ l of serum from each mouse in the same group were pooled 6 weeks after vaccination with Ad/opt-BoNT/C-H_C50 (8 mice/group). Sera were 1:4 diluted initially with Dulbecco's PBS and then in twofold series for determination of anti-BoNT/C neutralization titers. Panel A: mice survival rates after challenge with neutralized BoNT/C; Panel B: serum anti-BoNT/C neutralization titers (IU/ml, one IU is equal to 10,000 $\times$ MLD₅₀). IM: vaccination (n = 4).

Fig. 3.

Protection against active BoNT/C in mice vaccinated with adenoviral vector. Mice were vaccinated with different dosages of adenovirus-vectored vaccine Ad/opt-BoNT/C-H_C50 in week 0 and then challenged in week 7 with $100 \times \text{MLD}_{50}$ BoNT/C. Ad/opt-BONT/C-H_C50-vaccinated groups: I, 10^5 pfu; II, 10^6 pfu; III, 2×10^7 pfu; N.Con: negative control was inoculated with 2×10^7 pfu of Ad/Null; P.Con: positive control group was vaccinated i.m. with 50 μl of the pentavalent (ABCDE) botulinum toxoid vaccine.

Fig. 4.

Sustaining of antigen specific antibody responses after vaccination with the adenovirus-vectored vaccine in mice. Mice were inoculated i.m. with a single dose of 2×10^7 pfu of Ad/opt-BONT/C-H_C50 (vaccination) or with Ad/Null (control) in week 0. Serum samples were obtained in weeks 11, 19, and 27 before challenging with BoNT/C. The anti-BoNT/C-H_C50 IgG antibody concentrations in sera were determined using a quantitative ELISA kit (Bethyl, Montgomery, TX). Mean = $\bar{X} \pm SD$ (n = 7 or 8 in vaccination groups and n = 4 in control groups).

Fig. 5.

Long-lasting protective immunity in vaccinated mice against BoNT/C challenge. Mice were injected i.m. with 2×10^7 pfu of Ad/opt-BoNT/C-HC50 or with Ad/Null in week 0 and then challenged with $100 \times \text{MLD}_{50}$ BoNT/C in week 11 (Panel a), week 19 (Panel b) and week 27 (Panel c). $n = 8$ in experiment groups; $n = 4$ in control groups.

Fig. 6.

Effect of pre-existing immunity to adenovirus on the efficacy of the adenovirus-vectored vaccine. Panel A: Anti-adenovirus neutralizing antibodies in animals inoculated with adenovirus pre-vaccination. Mice were inoculated i.n. with 2×10^7 pfu/mouse of wild-type human adenovirus serotype 5 in week 0. Serum samples were obtained in week 4 and the anti-Ad5 neutralizing antibody titers were subsequently measured. Mean = $X \pm SE$. IM group: the group that was subsequently vaccinated with Ad/opt-BONT/C-H_C50 in Panel B; Ad/Null: the group that was subsequently injected with Ad/Null in Panel B; Con: data were obtained from mouse sera before inoculation of WT Ad5. Panel B: Protective immunity. Each mouse was inoculated i.n. with 2×10^7 pfu of WT Ad5 in week 0 as shown in Panel A, then subsequently injected with 2×10^7 pfu Ad/opt-BoNT/C-H_C50 in vaccination group or with Ad/Null in control group in week 4, and challenged with $100 \times MLD_{50}$ BoNT/C in week 11. (n = 8 in vaccination groups; n = 4 in control groups)

Table 1

Position	Sequence
1	GATATAATTAAATGATATTTGAGCCTACAAAATTTTGTGGATACATCGGATATAATGCA
1	GACATTAAACGAGTACTCAATAACAGTACAGCAATCTGTCCCTGCAAAATCGGAAAAACATCTGGTGGACACAGCGGATATAACCGCT
1	DIINEYFNINDSKILLSQNRKNTLVDSYNA
100	GAAATGAGTGAAGAGCGGATGTCACTTAATCTCAATATTCATTGACTTTAAATAGGTAGTTCAGGGAGGATAGAGGTAAGAGTTATAGTAAACC
100	GAGGTAGCGAAGAGCGGATGTCACTGAACCAATCTTCCCTTTGATTTAAAGCTGGGCTCCTCCGGCGAGGATAGGGGAAAGTCAATCGTCACCC
34	EVSEEGDVQLNPFPDFKLKSSGEDRGKIVIT
199	CAGAAATGAAATATGTATATATCTATGAAATTTAGCATTAAGTTTGGATTAGAATAAAATGGGTAAAGTAAATTTACCTGGGATATACT
199	CAGAAATGAAATATGTATATATCTATGAAATTTAGCATTAAGTTTGGATTAGAATAAAATGGGTAAAGTAAATTTACCTGGGATATACT
67	QENIVYSMYESFSFWIRINKVVSNLPGYT
298	ATAATTGATGTAAAAATAAATCACTAGGTGGATATAGGTATTAAGTAATTTTTAGTATTTTAACTGTACTAACAATATGATGGGAAATATGAAGATTTAT
298	ATCATCGACGGTGAAGAACAACTCCGGGTGCTCATCGGATTAATCTCCAATTTTCTGGTGTCACTCTGAAACAAACGAAAGATAGCGAACAGAGC
100	IIDSVKNSGSWSIGHSNLFVFLTKQNEDEQS
397	ATAAATTTAGTATGATCAATTAATGCTGCTGGAACAATAAATAAATGGTTTTTGTAACTGTACTAACAATATGATGGGAAATATGAAGATTTAT
133	ATCAATTTCTCTACGACATTTCCAACATGACACAGGTATAACAAGTGGTCTTTGTCACTGTCACCAACAACATGATGGGAAACATGAAGATTTAC
397	INFSYDISNNAAPYNNKWFVTVTNMGMNMIKY
496	ATAAATGGAAATTAATAGATATATAAAGATTAAGAACTAACTCGAATTAATTTAGCAAAACTATAAACATTTGAAATAAATAAATTTCCAGATACC
496	ATCAACGGAAACTGATTGATACATTAAGGTCAAGGAACCTACCCGGCATTAACCTCTCCAAGACAATTAATTTGAGATCAATAAGATTTCCAGACACC
166	INGKLIDTIKVKELTGINFSKTITFEINKIPDIT
595	GGTTGTATTACTCAGACTCTGATAACATCAATATGTGGATAAGAGATTTTATATATTTGCTAAAGAATTAGATGGTAAAGATTAATATATATATTTT
595	GGACTATTACTAGCGACTCCGACATATCAATATGTGGATTAGGCACTTCTACATCTTTGCTAAAGAACTGGATGGCAAGGATATTAACATTCTCTTC
199	GLITSDSDINMWRDIFYFAKELDGKDINILF
694	AATAGCTTTCGAATATACATGTTGTAAGAATATTGGGGAATGATTTAAGATATAATAAGAAATATTATATGGTTAATAATAGATTATTTAAATAGA
694	AACTCCCTCCAATACACAAACGTCGTCAAAGACTATTGGGGCAACGCACTGAGATACAAACAAGAGTATTACATGGTCAACATCGATTACTGAAACAGA
232	NSLQTYNNKVDYNDLRYNKEYMVNIDYLN
793	TATATGTATGCAACTCAGCAAAATGTGTTTTAATACACGTAGAAATAATAATGACTTCAATGAAGGATATAAAATTTAATAATAAAAGAAATCAGAGGA
793	TATATGTAGCCCAACAGCAGGCAATGTGTCTCAACACGCGGAGGAATAACAATGATTTCAACGAAGGCTATAAGATCATCATCAAAAGAAATCAGGCGGA
265	YMYANSRQIVFNTRNNNDNEYKIIIKIRIG
892	AATACAAATGATATAGTACGAGGAGATATTTATATTTGATATGACAAATTAATAACAAGACATATAATTTGTTTATGAAGAAATGAACACTATG
298	AGACATAATGACACTAGGTCAGAGGGCGGACATCTGTATTTTGACATGACTATCAACAATAAGGCTCAACACTGTTTATGAAACACGAGACAATG
991	NTNDTRVRGDILYFDMTNKAYLFMKNETM
991	TATGCGATAATCATAGTACATGAAGATATATGCTATAGGTTAAGAGACACAAACAAAGACATAACGATAATATATTTTCAATATACAAACCAATG
991	TATGCTGATAACACAGCAGACAGATATTACGCAATCGGCCTGAGGGAGCAACCAAGACATTAACGATAATATATTTTCAATATACAAACCAATG
331	YADNHSTEDIYAIGRETKINDINIIQPIM
1090	AATAATCTATTATTACCGCATCTCAATATTAATCAAAATTTAATGGAGAAATATTTCTGGAAATATTTCTGGAAATAGGTACTTATCTGTTTATAGACTT
1090	AATAATCTACTACTACGCAAGCAAAATTTTCAAGACAACTTTAACGGAGAGAACATCAGCGGAACTCGCAGCATTTGGGAACTACAGGTTTAGACTTC
364	NNTYASYASQIFKSNFNGENISGICSIGTYRFL
1189	GGAGGTGATTTGGTATAGACAAATATTCTTGGTGCCTCTGTAAGCAAGGAAATATGCTTCATTATTAGAATCAACATCAACTCATTTGGGGTTTTGTGA
1189	GGGGAGACTGGTATAGACAAATATTCTTGGTGCCTCTGTAAGCAAGGAAATATGCTTCATTATTAGAATCAACATCAACTCATTTGGGGTTTTGTGA
397	GGDWYRHNLYLPTVKQNYASLLESTSTHWGFV
1288	CCTGTAAGTGAA
1288	CCCGTCTCCGAGTGA
430	PVSE*