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(54) Title: VACCINE AGAINST BOTULISM

(57) Abstract: The invention relates to novel DNA and protein vaccines against *Clostridium botulinum.* The DNA vaccine includes a DNA molecule that includes a first segment encoding a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin, wherein the first segment is codon-enhanced to improve expression of the isolated DNA molecule in a mammalian host, and preferably a second segment that encodes a secretion signal peptide. The chimeric protein of the present invention includes the secretion signal peptide linked N-terminal of the fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin. Use of these materials to raise antibodies, and to impart resistance against *Clostridium botulinum* to a mammal is also disclosed.

VACCINE AGAINST BOTULISM

[0001] This application claims the priority benefit of U.S. Provisional Patent application Serial No. 60/954,921, filed August 9, 2007, which is hereby incorporated by reference in its entirety.

[0002] The present invention was made with government support under grant number R21AI055946 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID/NIH). The government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] The present invention relates to novel DNA and protein vaccines for use in inducing a protective immune response against *Clostridium botulinum.*

BACKGROUND OF THE INVENTION

[0004] 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. The only exception to this general rule is serotypes C and D, which share significant cross-homology (Oguma et al., "Antigenic Similarity of Toxins Produced by Clostridium Botulinum Type C and D Strains," *Infect Immun* 30(3):656-60 (1980)). BoNTs are the most poisonous substances known in nature. They may be used as bioterrorism agents or in biological warfare (Arnon et al., "Botulinum Toxin as a Biological Weapon," *Medical and Public Health Management. JAMA* 285(8): 1059-70 (2001)). Therefore, there is an urgent need for the development of effective vaccines to protect against botulism.

[0005] Currently, a pentavalent botulinum toxoid vaccine that may protect against BoNT serotypes A-E is available as an Investigational New Drugs (IND) (Wright et al., "Studies on Immunity to Toxins of *Clostridium botulinum:* V. Detoxification of Purified

Type A and Type B Toxins, and the Antigenicity of Univalent and Bivalent Aluminium Phosphate Adsorbed Toxoids," *J Immunol* 84:384-9 (1960); Fiock et al, "Studies on Immunity to Toxins of *Clostridium botulinum:* IX. Immunologic Response of Man to Purified Pentavalent ABCDE Botulinum Toxoid," *J Immunol* 90:697-702 (1963)).

However, there are several shortcomings with the toxoid vaccines. First, 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. Second, the toxoid product for types A-E is in the form a crude extract of clostridial proteins that may influence immunogenicity or reactivity of the vaccine, and the type F toxoid is only partially purified. Third, 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 (Byrne et al., "Development of Vaccines for Prevention of Botulism," *Biochimie* 82(9-10):955-66 (2000)).

[0006] A high sequence and structural homology exists between the clostridial neurotoxins produced by *Clostridium tetani* and *C botulinum.* The successful demonstration that a C-fragment of tetanus toxin (TeNT) elicits protective immunity in animals has prompted the development of a new subunit vaccine against botulism (Helting et al., "Analysis of the Immune Response to Papain Digestion Products of Tetanus *Toxin*," *Acta Pathol Microbiol Immunol Scand [C]*, 92(l):59-63 (1984); Fairweather et al., "Immunization of Mice Against Tetanus with Fragments of Tetanus Toxin Synthesized in *Escherichia coli," Infect Immun* 55(1 1):2541-5 (1987)). Evaluation of the immunogenicity of different regions of BoNTs has confirmed that the C-fragment of the BoNTs elicits protective immunity in animals (LaPenotiere et al., "Expression of a Large, Nontoxic Fragment of Botulinum Neurotoxin Serotype A and its Use as an Immunogen," *Toxicon* 33(10): 1383-6 (1995); Clayton et al., "Protective Vaccination With a Recombinant Fragment of Clostridium Botulinum Neurotoxin Serotype A Expressed From a Synthetic Gene in Escherichia Coli," *Infect Immun* 63(7):2738-42

(1995); Dertzbaugh et al., "Mapping of Protective and Cross-Reactive Domains of the Type A Neurotoxin of Clostridium Botulinum," *Vaccine* 14(16):1538-44 (1996); Lee et al. "C Terminal Half Fragment (50 kDa) of Heavy Chain Components of Clostridium Botulinum Type C and D Neurotoxins Can Be Used as an Effective Vaccine," *Microbiol Immunol* 51(4):445-55 (2007); Webb et al., "Protection With Recombinant Clostridium Botulinum C1 and D Binding Domain Subunit (Hc) Vaccines Against C and D Neurotoxins," *Vaccine* 16:16 (2007); Boles et al., "Recombinant C Fragment of Botulinum Neurotoxin B Serotype (rBoNTB (HC)) Immune Response and Protection in the Rhesus Monkey," *Toxicon* 47(8):877-84 (2006)). Therefore, subsequent efforts to develop vaccine candidates to protect against BoNTs forthwith may focus on the Hc region of BoNTs (Baldwin et al., "Characterization of the Antibody Response to the Receptor Binding Domain of Botulinum Neurotoxin Serotypes A and E," *Infect Immun* 73(10):6998-7005 (2005)). 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 Hc region of BoNT/A. These experiments show the feasibility of developing a synthetic vaccine that could protect against botulinum neurotoxin intoxication (Byrne et al., "Development of Vaccines for Prevention of Botulism," *Biochimie* 82(9-10):955-66 (2000); Atassi et al., Mapping of the Antibody-Binding Regions on Botulinum Neurotoxin H-Chain Domain 855-1296 With Antitoxin Antibodies From Three Host Species," *J Protein Chem,* 15(7):691-700 (1996); Atassi et al., "Structure, Activity, and Immune (T and B cell) Recognition of Botulinum Neurotoxins," *Crit Rev Immunol* 19(3):219-60 (1999); Oshima et al., "Immune Recognition of Botulinum Neurotoxin Type A: Regions Recognized by T cells and Antibodies Against the Protective H(C) Fragment (Residues 855-1296) of the Toxin," *MoI Immunol* 34(14): 1031-40 (1997); Oshima et al., "Antibodies and T cells Against Synthetic Peptides of the C-Terminal Domain (Hc) of Botulinum Neurotoxin Type A and Their Cross-Reaction With Hc," *Immunol Lett* 60(l):7-12 (1998)). The employment of the non-toxic fragments of BoNTs as protective antigens also provides a significant

safety profile advantage. However, expression and purification of recombinant fragments of BoNTs, and their subsequent formulation into a vaccine is costly.

[0007] Given the need for an effective vaccine against botulism, it would be desirable to develop a single-dose vaccine that provides long-lasting protective immunity against botulism. The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

[0008] A first aspect of the present invention relates to an isolated DNA molecule that includes a first segment encoding a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin, wherein the first segment is codon-enhanced to improve expression of the isolated DNA molecule in a mammalian host.

[0009] A second aspect of the present invention relates to an expression vector or plasmid that includes an isolated DNA molecule according to the first aspect of the present invention operably coupled to one or more regulatory sequences that afford transcription of the isolated DNA molecule in the mammalian host. Also encompassed with this aspect of the present invention is a recombinant host cell that includes an expression vector or plasmid according to this aspect of the invention.

[0010] A third aspect of the present invention relates to a chimeric protein that includes a secretion signal peptide linked N-terminal of a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin.

[0011] A fourth aspect of the present invention relates to a vaccine that includes a pharmaceutically acceptable carrier and either (i) a DNA molecule according to the first aspect of the present invention or an expression vector according to the second aspect of the present invention; (ii) a chimeric protein according to the third aspect of the present invention; or a combination of (i) and (ii).

[0012] A fifth aspect of the present invention relates to a method of imparting resistance against a *Clostridium botulinum* neurotoxin to a mammal, which includes administering a vaccine according to the fourth aspect of the present invention to a mammal under conditions effective to induce a protective immune response against the

Clostridium botulinum neurotoxin. A related aspect of the invention relates to a method of neutralizing a neurotoxin of the present invention.

[0013] A sixth aspect of the present invention relates to an isolated antibody raised against a chimeric protein according to the third aspect of the present invention, or an antibody binding fragment thereof. A pharmaceutical composition containing the antibody or binding fragments thereof is also encompassed by this aspect of the present invention.

[0014] A seventh aspect of the present invention relates to a hybridomas cell that expresses a monoclonal antibody according to the sixth aspect of the present invention.

[0015] An eighth aspect of the present invention relates to a method of treating a *Clostridium botulinum* infection that includes administering to a patient an antibody or antibody fragment thereof (or a pharmaceutical composition containing the same) according to the sixth aspect of the invention, wherein the administration thereof is carried out under conditions effective to neutralize a botulism neurotoxin, and thereby treat the *Clostridium botulinum* infection.

[0016] As demonstrated in the accompanying Examples, a single dose of an adenoviral vector encoding a codon-optimized fusion (chimeric) protein, containing an N-terminal secretion signal peptide and a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin, was sufficient to induce a protective immune response against the neurotoxin from which the heavy chain region was derived. Importantly, a single dose of 2×10^7pfu of Ad/opt-BoNT/C-Hc50 was sufficient to provide long-term protective immunity via either intramuscular or intranasal vaccination. This study is the first to demonstrate that a single genetic vaccination is able to provide long-lasting protection against botulism.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figures IA-C are graphs that illustrate anti Hc50 of BoNT/C response in vaccinated mice. Mice were inoculated with different doses of Ad/opt-BoNT/C-Hc50 in week 0. Serum samples were obtained at weeks 0, 2, 4, and 6. Anti-BoNT/C-Hc50 IgG (Figure IA), IgGl (Figure IB) and IgG2a (Figure 1C) antibody concentrations were

measured by a quantitative ELISA kit (Bethyl, Montgomery, TX). Virus doses for groups I, II, and III were 10^5, 10^6, and 2 x 10^7 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.**

[0018] Figures 2A-B are graphs that illustrate serum anti-BoNT/C neutralizing antibody titer assay. 50 μl of serum from each mouse in the same group were pooled 6 weeks after vaccination with Ad/opt-BoNT/C-Hc50 (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. Figure 2A shows mice survival rates after challenge with neutralized BoNT/C. Figure 2B shows serum anti-BoNT/C neutralization titers (IU/ml, one IU is equal to 10,000 x MLD_{50}). IM: vaccination (n = 4).

[0019] Figure 3 is a graph showing 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-Hc50 in week 0 and then challenged in week 7 with 100 x MLD_{50} BoNT/C. Ad/opt-BONT/C-H_c50-vaccinated groups: I, 10^5 pfu; II, 10^6 pfu; III, 2 x 10^7 pfu; N.Con: negative control was inoculated with 2 x 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. (n for Groups III, II, I, N.Con, and P.Con are 12, 8, 8, 8, and 12, respectively.)

[0020] Figure 4 is a graph illustrating the 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 x 10^7 pfu of Ad/opt-BONT/C-Hc50 (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=X± SD (n = 7 or 8 in vaccination groups and n = 4 in control groups).

[0021] Figures 5A-C are graphs illustrating the long-lasting protective immunity in vaccinated mice against BoNT/C challenge. Mice were injected i.m. with 2 x 10^7 pfu of Ad/opt-BoNT/C-H_c50 or with Ad/Null in week 0 and then challenged with 100 x

MLD_{50} BoNT/C in week 11 (Figure 5A), week 19 (Figure 5B), and week 27 (Figure 5C). n = 8 in experiment groups; n = 4 in control groups.

[0022] Figures 6A-B are graphs illustrating the effect of pre-existing immunity to adenovirus on the efficacy of the adenovirus-vectored vaccine. Figure 6A shows 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 ± SE. IM group: the group that was subsequently vaccinated with Ad/opt-BONT/C-Hc50 in Figure 6B; Ad/Null: the group that was subsequently injected with Ad/Null in Figure 6B; Con: data were obtained from mouse sera before inoculation of WT Ad5 in Figure 6B. In figure 6B, each mouse was inoculated i.n. with 2×10^7 pfu of WT Ad5 in week 0 as described above, and then subsequently injected with 2×10^7 pfu Ad/opt-BoNT/C-Hc50 in vaccination group or with Ad/Null in control group in week 4, and challenged with 100 x MLD_{50} BoNT/C in week 11. (n = 8 in vaccination groups; n = 4 in control groups.)

[0023] Figures 7A-C are graphs illustrating the serum antibody responses against BoNT/C-Hc50 in vaccinated mice. Mice were vaccinated intranasally with different doses of Ad/opt-BoNT/C-H_c50 (1×10^5 to 2×10^7 pfu/mouse) in week 0. Serum samples were obtained in weeks 0, 2, 4, and 6 to measure anti-BoNT/C-Hc50 IgG (Figure 7A), IgGl (Figure 7B), and IgG2a (Figure 7C) antibody concentrations by quantitative ELISA. Mean = X ± SE (n = 8).

[0024] Figure 8 is a graph illustrating the sustaining of antigen-specific antibody responses after intranasal vaccination with the adenovirus-vectored vaccine in mice. Mice were intranasally inoculated with a single dose of 2×10^7 pfu of Ad/opt-BONT/C-Hc50 (Vaccination group) or with Ad/Null (Control group) in week 0. Serum samples were obtained in weeks 11, 19, and 27 before challenging with active BoNT/C. The anti-BoNT/C-Hc50 IgG antibody concentrations in sera were determined by quantitative ELISA. Mean = X ± SD (n = 7 or 8 in vaccination groups, and n = 4 in control groups).

[0025] Figures 9A-D are graphs illustrating mucosal antibody responses against BoNT/C-Hc50 in vaccinated mice. Mice were intranasally inoculated with a single dose

of 2 x 10^7 pfu of Ad/opt-BONT/C-H_c50 (Vaccination) or with Ad/Null (Control) in week 0. Saliva, nasal and vaginal wash samples were collected in weeks 2 and 4. Anti-BoNT/C-H_c50 IgG (Figure 9A), IgGl (Figure 9B), IgG2a (Figure 9C), and IgA (Figure 9D) concentrations were measured by quantitative ELISA. X = Mean ± SD (n = 8 in Vaccination group and n = 4 in Control group).

[0026] Figures 10A-B are graphs illustrating the results of serum anti-BoNT/C neutralizing antibody titer assay. 50 µl of serum from each mouse in the same group were pooled 6 weeks after vaccination intranasally with Ad/opt-BoNT/C-Hc50 (8 mice/group). Sera were 1:4 diluted initially with Dulbecco's PBS and then in two fold series for determination of anti-BoNT/C neutralization titers. Figure 1OA shows mice survival rates after challenge with neutralized BoNT/C. Figure 1OB shows serum anti-BoNT/C neutralization titers (IU/ml). One IU is equal to 10,000 x MLD_{50}. CON, control; IMM: vaccination. (n = 4).

[0027] Figure 1IA-D are graphs illustrating the protection against active BoNT/C challenge in vaccinated mice. Mice were intranasally inoculated with Ad/Null (N. Control group) or I x 10^5 - 2x 10^7 of Ad/opt-BoNT/C-H_c50 in week 0, and then challenged with 100 x MLD_{50} of BoNT/C in weeks 7 (Figure 1IA), 11(Figure 1IB), 19 (Figure HC), and 27 (Figure 1ID). Adenovirus dosages for Dose I, II, III, and N. Con are I x 10^5, I x 10^6, 2χ 10^7, and 2χ 10^7 pfu/mouse, respectively. (n = 4 for N. Control groups; n = 8 for vaccination groups.)

[0028] Figure 12 is a graph illustrating the *Botulinum* neurotoxin dose-dependent protection in mice vaccinated with adenovirus-vectored vaccine. Mice were intranasally vaccinated with 2x 10^7 pfu of Ad/opt-BoNT/C-Hc50 in week 0 and then challenged in week 4 with 10^3 - 10^5 x MLD_{50} of BoNT/C. Ad/Null, negative control, animals were inoculated intranasally with 2x 10^7 pfu of Ad/Null and challenged with 10^3 x MLD_{50} of BoNT/C. (n for Ad/Null, vaccinated groups 10^3 MLD_{50}, 10^4 MLD_{50}, 10^5 MLD_{50} are 4, 8, 8, 7, respectively.)

[0029] Figure 13 is a graph illustrating the anti-adenovirus neutralizing antibody response in mice inoculated with adenovirus pre-vaccination. Mice were intranasally inoculated with 2 x 10^7 pfu of wild-type human adenovirus serotype 5 in week 0. Serum

samples were obtained in week 4. Sera from 2 mice in the same group were pooled and the anti-Ad5 neutralizing antibody titers of the serum pools were subsequently measured. Mean = X ± SE. Ad/Hc50: the group were subsequently vaccinated with Ad/opt-BoNT/C-Hc50 (Figure 14); Ad/Null: the group were subsequently inoculated with Ad/Null (Figure 14); N. Con: data were obtained from mouse pre-inoculation of adenovirus. (n = 4.)

[0030] Figure 14 is a graph illustrating the effect of pre-existing immunity to adenovirus on the efficacy of the adenovirus-vectored mucosal vaccine. Each mouse was inoculated intranasally with or without 2 x 10^7 pfu of Ad5 in week 0 as shown in Figure 13, then subsequently inoculated intranasally with 2 x 10^7 pfu of Ad/opt-BoNT/C-Hc50 in vaccination group or with Ad/Null in control group in week 4, and challenged with 100 × MLD_{50} BoNT/C in week 11. (n = 8 in experiment groups; n = 4 in control group.)

DETAILED DESCRIPTION OF THE INVENTION

[0031] The present invention relates to DNA and/or protein vaccines against botulism neurotoxin. The vaccines of the present invention are capable of inducing, with a single vaccination, a high titer of neutralizing antibodies against the botulism neurotoxin.

[0032] Neutralizing antibody titers are defined as the maximum number of IU of antitoxin per ml of serum, resulting in 100% survival after challenge, where one IU of botulinum neurotoxin antitoxin neutralizes 10,000 ✗ MLD50 neurotoxin (Byrne et al., "Purification, Potency, and Efficacy of the Botulinum Neurotoxin Type A Binding Domain from *Pichia pastoris* as a Recombinant Vaccine Candidate." *Infect Immun* 66(10):4817-22 (1998); Nowakowski et al., "Potent Neutralization of Botulinum Neurotoxin by Recombinant Oligoclonal Antibody," *Proc Natl Acad Sci USA* 99: 11346-11350 (2002), each of which is hereby incorporated by reference in its entirety). As used herein, a high titer of neutralizing antibodies refers to at least about 1 IU/ml, more preferably at least about 6 IU/ml, and most preferably at least about 10 IU/ml.

[0033] The vaccines of the present invention are suitable for use in any mammal including, without limitation, humans and nonhuman primates, such as chimpanzees and

other apes and monkey species; farm animals including cattle, sheep, pigs, goats and horses, etc.; domestic animals including cats and dogs; laboratory animals including rodents such as mice rats, and guinea pigs, and the like. The mammal can be of any age or sex. Thus, adults and post-natal (newborn) subjects, as well as fetuses, are intended to be covered.

[0034] The DNA vaccine involves the use of a DNA molecule that contains a first nucleotide sequence encoding a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin, wherein the nucleotide sequence is codon-enhanced to improve expression of the DNA molecule in a mammalian host. The DNA molecule also preferably contains a second nucleotide sequence encoding a secretion signal peptide. The second nucleotide sequence is preferably located 5' of the first nucleotide sequence, affording expression of a chimeric protein that includes an N-terminal secretion signal peptide and the fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin (BoNT).

[0035] The BoNT from which the fragment is derived can be any one or more of neurotoxin A *(see* Genbank Accession No. X52066, which is hereby incorporated by reference in its entirety), neurotoxin B *(see* Genbank Accession No. M81 186, which is hereby incorporated by reference in its entirety), neurotoxin C *(see* Genbank Accession No. D90210, which is hereby incorporated by reference in its entirety), neurotoxin D *(see* Genbank Accession No. X54254, which is hereby incorporated by reference in its entirety), neurotoxin E *(see* Genbank Accession No. X62089, which is hereby incorporated by reference in its entirety), neurotoxin F *(see* Genbank Accession No. M92906, which is hereby incorporated by reference in its entirety), or neurotoxin G *(see* Genbank Accession No. X74162, which is hereby incorporated by reference in its entirety).

[0036] The fragment of the BoNT heavy chain region should be non-toxic and antigenic, and capable of eliciting immunity responses against botulism. Preferably, the fragment of the heavy chain region is a C-terminal fragment that is about 50 kDa (referred to hereinafter as "Hc50" subunit or fragment). The Hc50 fragments of BoNTs are known to possess these attributes, *i.e.*, non-toxic, antigenic, and capable of eliciting

immunity responses against botulism (Byrne et al., "Development of Vaccines for Prevention of Botulism," *Biochimie* 82:955-966 (2000); Webb et al., "Protection with Recombinant *Clostridium botulinum* C1 and D Binding Domain Subunit (Hc) Vaccines Against C and D Neurotoxins," *Vaccine* 25:4273-4282 (2007); Byrne et al., "Purification, Potency, and Efficacy of the Botulinum Neurotoxin Type A Binding Domain from *Pichia pastoris* as a Recombinant Vaccine Candidate," *Infect Immun* 66:4817-4822 (1998); Atassi et al., "Structure, Activity, and Immune (T and B cell) Recognition of Botulinum Neurotoxins," *Crit Rev Immunol* 19:219-260 (1999), each of which is hereby incorporated by reference in its entirety.)

[0037] The DNA molecule can also include multiple open reading frames that afford expression of any combinations of the Hc50 fragments of BoNTs A-G, thus affording a multivalent vaccine.

[0038] As noted above, the first nucleotide sequence is preferably codon-optimized. Codon-optimization of the nucleotide sequence encoding the Hc50 fragment of BoNTs is believed to afford high expression levels of the expressed polypeptide, which in turn affords an immune response that produces a high titer of neutralizing antibodies.

[0039] According to one embodiment of the present invention, a codon-optimized DNA sequence encoding the Hc50 fragment of BoNT/C is shown in Table 1 below. The codon-optimized DNA sequence is SEQ ID NO: 2, which is compared to the native DNA sequence of SEQ ID NO: 1. Differences between these two redundant sequences is shown by nucleotide symbols in bold typeface in SEQ ID NO: 2. The encoded Hc50 fragment of BoNT/C has the amino acid sequence of SEQ ID NO: 3.

[0040] According to another embodiment of the present invention, a codon-optimized DNA sequence encoding the Hc50 fragment of BoNT/A is shown in Table 2 below. The codon-optimized DNA sequence is SEQ ID NO: 5, which is compared to the native DNA sequence of SEQ ID NO: 4. Differences between these two redundant sequences is shown by nucleotide symbols in bold typeface in SEQ ID NO: 5. The encoded Hc50 fragment of BoNT/A has the amino acid sequence of SEQ ID NO: 6.

[0041] The second nucleotide sequence can encode any suitable secretion signal peptide that affords secretion of the chimeric protein in mammalian cells. The secretion signal peptide should not interfere with the antigenicity of the encoded Hc50 BoNT fragment. Exemplary secretion signal peptides include, without limitation, human tissue plasminogen activator, human serum albumin, human IL-3, human growth hormone, etc.

[0042] The 25-amino acid secretion signal of human tissue plasminogen activator and its encoding nucleotide sequence are reported at Genbank Accession Nos. BC002795 and AAH02795, each of which is hereby incorporated by reference in its entirety.

[0043] The 24-amino acid secretion signal peptide of human serum albumin and its encoding nucleotide sequence are reported at Genbank Accession Nos. AAA98797 and M12523, each of which is hereby incorporated by reference in its entirety.

[0044] The 19-amino acid secretion signal peptide of human IL-3 and its encoding nucleotide sequence are reported at Genbank Accession Nos. NP_000579 and NM_000588, each of which is hereby incorporated by reference in its entirety.

[0045] The 26-amino acid secretion signal peptide of human growth hormone and its encoding nucleotide sequence are reported at Genbank Accession Nos. AAA72555 and M14422 (synthetic construct), each of which is hereby incorporated by reference in its entirety.

[0046] The nucleic acid molecules encoding the various polypeptide components of a chimeric protein can be ligated together along with appropriate regulatory elements that provide for expression of the chimeric protein. Typically, the nucleic acid construct encoding the chimeric protein can be inserted into any of the many available expression vectors and cell systems using reagents that are well known in the art.

[0047] For purposes of preparing the DNA vaccine of the present invention, the DNA molecule encoding the chimeric protein of the present invention is operably coupled to regulatory elements that are operable in mammalian systems. For purposes of preparing the chimeric protein, as the primary antigen of a protein-based vaccine, the DNA molecule encoding the chimeric protein can be operably coupled to regulatory elements that are operable in the desired eukaryotic or prokaryotic host cells in which recombinant expression of the chimeric protein is intended.

[0048] Referring now to the materials suitable for us in the DNA vaccine, the recombinant gene includes, operatively coupled to one another, an upstream promoter operable in mammalian cells and optionally other suitable regulatory elements *(i.e.,* enhancer or inducer elements), the coding sequence that encodes the BoNT fragment, and a downstream transcription termination region. The promoter is preferably a constitutive promoter. Common promoters operable in mammalian cells include, without limitation, SV40, MMTV, metallothionein-1, adenovirus EIa, CMV immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR promoters. Any suitable transcription termination region can be used, *e.g.,* SV40 polyadenylation signal.

[0049] The DNA sequences of these various regions can be cloned into a shuttle or transfer vector using standard cloning procedures known in the art, including restriction enzyme cleavage and ligation with DNA ligase as described by Sambrook et al, *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor Press, NY (1989), and Ausubel et al., *Current Protocols in Molecular Biology,* John Wiley & Sons, New York, N.Y. (1989), which are hereby incorporated by reference in their entirety. U.S. Patent No. 4,237,224 issued to Cohen and Boyer, which is hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. Thereafter, the recombinant gene can be similarly excised an inserted into a infective transformation vector or, alternatively, naked DNA or a recombinant plasmid can be used in combination with a non-infective delivery vehicle.

[0050] Any suitable viral or infective transformation vector can be used. Preferably, the infective transformation vector is replication-incompetent, and the vector itself is produced in a cell line that supplies any missing proteins suitable for production of the vector capable of transfecting cells with the recombinant transgene.

[0051] Exemplary viral vectors include, without limitation, adenovirus, adeno-associated virus, and retroviral vectors (including lentiviral vectors).

[0052] Adenovirus gene delivery vehicles can be readily prepared and utilized given the disclosure provided in Berkner, "Development of Adenovirus Vectors for Expression of Heterologous Genes," *Biotechniques* 6:616-627 (1988); Rosenfeld et al.,

"Adenovirus-Mediated Transfer of a Recombinant α1-Antitrypsin Gene to the Lung Epithelium *in vivo," Science* 252:431-434 (1991); PCT Publication No. WO 93/07283; PCT Publication No. WO 93/06223; and PCT Publication No. WO 93/07282, each of which is hereby incorporated by reference in its entirety. Additional types of adenovirus vectors are described in U.S. Patent No. 6,057,155 to Wickham et al; U.S. Patent No. 6,033,908 to Bout et al.; U.S. Patent No. 6,001,557 to Wilson et al.; U.S. Patent No. 5,994,132 to Chamberlain et al.; U.S. Patent No. 5,981,225 to Kochanek et al.; U.S. Patent No. 5,885,808 to Spooner et al.; and U.S. Patent No. 5,871,727 to Curiel, each of which is hereby incorporated by reference in its entirety.

[0053] Adeno-associated viral gene delivery vehicles can be constructed and used to deliver into cells a recombinant gene encoding a desired nucleic acid. The use of adeno-associated viral gene delivery vehicles *in vivo* is described in Flotte et al., "Stable *in vivo* Expression of the Cystic Fibrosis Transmembrane Conductance Regulator with an Adeno-associated Virus Vector," *Proc. Nat'I Acad. Sci. USA* 90:10613-10617 (1993); and Kaplitt et al., "Using Adeno-associated Virus Vectors in the Mammalian Brain," *Nature Genet.* 8:148-153 (1994), each of which is hereby incorporated by reference in its entirety.

[0054] Retroviral vectors which have been modified to form infective transformation systems can also be used to deliver a recombinant gene encoding a desired nucleic acid product into a target cell. One such type of retroviral vector is disclosed in U.S. Patent No. 5,849,586 to Kriegler et al., which is hereby incorporated by reference in its entirety. Lentivirus vectors can also be utilized, including those described in U.S. Patent No. 6,790,657 to Arya, and U.S. Patent Application Nos. 20040170962 to Kafri et al. and 20040147026 to Arya, each of which is hereby incorporated by reference in its entirety.

[0055] As noted above, viral vectors have been successfully employed in order to increase the efficiency of introducing a recombinant vector into suitably sensitive host cells. Therefore, viral vectors are particularly suited for use in the present invention, including any adenoviral, retroviral, lentiviral, or adeno-associated viral vectors described above or known in the art. Current research in the field of viral vectors is producing

improved viral vectors with high-titer and high-efficiency of transduction in mammalian cells *(see, e.g.*, U.S. Patent No. 6,218,187 to Finer et al., which is hereby incorporated by reference in its entirety). Such vectors are suitable in the present invention, as is any viral vector that includes a combination of desirable elements derived from one or more of the viral vectors described herein. It is not intended that the expression vector be limited to a particular viral vector.

[0056] Certain "control elements" or "regulatory sequences" are also incorporated into the vector-construct. The term "control elements" refers collectively to promoter regions, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription, and translation of a coding sequence in a recipient cell. Not all of these control elements need always be present so long as the selected coding sequence is capable of being replicated, transcribed, and translated in an appropriate host cell. Some of these control elements have been described above.

[0057] Following transfection of an appropriate host with the viral vector of the present invention, the virus is propagated in the host and collected. Generally, this involves collecting the cell supernatants at periodic intervals, and purifying the viral plaques from the crude lysate using techniques well-known in the art, for example, cesium chloride density gradient. The titer (pfu/ml) of the virus is determined, and can be adjusted up (by filtration, for example) or down (by dilution with an appropriate buffer/medium), as needed. In the present invention, typical Ad titers are in the range of 10^6-10^{12} pfu/ml.

[0058] Infective transformation vectors that contain a recombinant gene of the present invention can be presented for administration to a mammal in a pharmaceutical composition that includes a suitable carrier. Typically, DNA vaccines containing infective transformation vectors include a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents that assist in the cellular uptake of DNA, such as, but not

limited to calcium ion, may also be used to advantage. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers.

[0059] The infective transformation vectors are preferably administered in an effective amount to induce, with a single dose, a high titer of neutralizing antibodies. Dosages of the recombinant virus will depend primarily on factors, such as the condition being treated, the selected fusion protein, the age, weight, and health of the patient, and may thus vary among patients. A therapeutically effective human dosage of the viruses of the present invention is believed to be in the range of about 5 ml of saline solution containing concentrations of from about 10^6 pfu/ml to 2.5 X 10^{12} pfu/ml virus of the present invention. The dosage can be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the selected gene can be monitored to determine the selection, adjustment, or frequency of dosage administration. If required, a boost can be administered following a suitable period of delay to maximize the immune response against the botulism neurotoxin.

[0060] Any suitable mode of delivering an infective transformation vector is contemplated. Exemplary modes of delivery include, without limitation, intradermal or transdermal introduction; impression though the skin; intralesionally; via intramuscular, intraperitoneal, intravenous, intraarterial, or subcutaneous injection; via inhalation, and application to mucous membranes such as via intranasal delivery; orally; parenterally; implantation; and by intracavitary or intravesical instillation.

[0061] As noted above, non-infective DNA vaccines are also contemplated. These modes of administration encompass the use of naked DNA with or without an uptake agent, DNA bioconjugates, as well as DNA administered via a transfection agent. Preferably, the recombinant gene is present in the form of a non-infective DNA plasmid.

[0062] The DNA can be formulated or complexed with polyethylenimine *{e.g.,* linear or branched PEI) and/or polyethylenimine derivatives, including for example grafted PEIs such as galactose PEI, cholesterol PEI, antibody derivatized PEI, and polyethylene glycol PEI (PEG-PEI) derivatives thereof (*see, e.g.,* Furgeson et al., "Modified Linear Polyethylenimine—Cholesterol Conjugates for DNA Complexation," *Bioconjugate Chem.* 14:840-847 (2003); Kunath et al., "The Structure of PEG-Modified

Poly(Ethylene Imines) Influences Biodistribution and Pharmacokinetics of Their Complexes withNF- κB Decoy in Mice," *Pharmaceutical Res* 19:810-817 (2002); Choi et al., "Effect of Poly(ethylene glycol) Grafting on Polyethylenimine as a Gene Transfer Vector *in vitro*," *Bull. Korean Chem. Soc.* 22:46-52 (2001); Bettinger et al., "Size Reduction of Galactosylated PEI/DNA Complexes Improves Lectin-Mediated Gene Transfer into Hepatocytes," *Bioconjugate Chem.* 10:558-561 (1999); Peterson et al., "Polyethylenimine-grq/?-Poly(ethylene glycol) Copolymers: Influence of Copolymer Block Structure on DNA Complexation and Biological Activities as Gene Delivery System," *Bioconjugate Chem.* 13:845-854 (2002); Erbacher et al., "Transfection and Physical Properties of Various Saccharide, Poly(ethylene glycol), and Antibody-Derivatized Polyethylenimines (PEI)," *J. Gene Medicine Preprint* 1(2):2 10-222 (1999); Godbey et al., "Tracking the Intracellular Path of Poly(ethylenimine)/DNA Complexes For Gene Delivery," *Proc Natl Acad Sci USA* 96:5177-5181 (1999); Godbey et al., "Poly(ethylenimine) and Its Role in Gene Delivery," *J Controlled Release* 60:149-160 (1999); Diebold et al., *J Biol Chem* 274:19087-19094 (1999); Thomas et al., "Enhancing Polyethylenimine' s Delivery of Plasmid DNA into Mammalian Cells," *Proc Natl Acad Sci USA* 99:14640-14645 (2002); and U.S. Patent No. 6,586,524 to Sagara, each of which is hereby incorporated by reference in its entirety.

[0063] The DNA molecule can also be present in the form of a bioconjugate, for example a nucleic acid conjugate as described in U.S. Patent No. 6,528,631, U.S. Patent No. 6,335,434, U.S. Patent No. 6,235,886, U.S. Patent No. 6,153,737, U.S. Patent No. 5,214,136, or U.S. Patent No. 5,138,045, each of which is hereby incorporated by reference in its entirety.

[0064] The recombinant DNA molecule can also be administered via a liposomal delivery mechanism. Basically, this involves providing a liposome which includes the DNA molecule (or plasmid) to be delivered, and then contacting a cell with the liposome under conditions effective for delivery of the DNA (or plasmid) into the cell. The liposomal delivery system can be made to accumulate at a target organ, tissue, or cell via active targeting *(e.g.,* by incorporating an antibody or hormone on the surface of the

liposomal vehicle). This can be achieved using antibodies specific for an appropriate cell marker.

[0065] Different types of liposomes can be prepared according to Bangham et al., "Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids," *J. MoI. Biol.* 13:238-252 (1965); U.S. Patent No. 5,653,996 to Hsu et al.; U.S. Patent No. 5,643,599 to Lee et al.; U.S. Patent No. 5,885,613 to Holland et al.; U.S. Patent No. 5,631,237 to Dzau et al.; and U.S. Patent No. 5,059,421 to Loughrey et al., each of which is hereby incorporated by reference in its entirety.

[0066] These liposomes can be produced such that they contain, in addition to the DNA payload, other therapeutic agents, such as immune-enhancing agents, e.g., IL-2 or interferon alpha or GM-CSF), which would also be released at the target site (Wolff et al., "The Use of Monoclonal Anti-Thyl IgGl for the Targeting of Liposomes to AKR-A Cells *in vitro* and *in vivo," Biochem. et Biophys. Acta* 802:259 (1984), which is hereby incorporated by reference in its entirety).

[0067] The amount of expressible DNA to be introduced into a vaccine recipient will depend partially on the strength of the promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 μg to 300 μg of a plasmid vaccine vector is administered directly into tissue. The non-infective DNA vaccines are also intended to be administered in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. The DNA may also be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents that assist in the cellular uptake of DNA, such as, but not limited to calcium ion, may also be used to advantage. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers. Any suitable mode of delivering the non-infective DNA is contemplated, including those identified above for infective transformation.

[0068] Finally, the use of vaccines comprising the chimeric protein of the present invention is also contemplated. Preferably, the chimeric protein includes the N-terminal secretion signal linked as an in-frame gene fusion to the HC_{50} BoNT fragment. The DNA

encoding the chimeric protein is preferably introduced into a recombinant host cell using a suitable vector, after which the protein is expressed, and then recovered and purified before being presented in a pharmaceutical formulation suitable for administration.

[0069] Suitable vectors include, but are not limited to, the following viral vectors such as baculovirus lambda vector system gtl 1, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKClOl, SV 40, pBluescript II SK +/-or KS +/- *(see* "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, CA, which is hereby incorporated by reference in its entirety), pQE, pIH821, pGEX, pET series *(see* Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," *Gene Expression Technology* vol. 185 (1990), which is hereby incorporated by reference in its entirety), and any derivatives thereof.

[0070] The DNA sequences can be cloned into the vector using standard cloning procedures known in the art, including restriction enzyme cleavage and ligation with DNA ligase as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor Press, NY (1989), and Ausubel et al., *Current Protocols in Molecular Biology,* John Wiley & Sons, New York, N.Y. (1989), which are hereby incorporated by reference in their entirety. Recombinant molecules, including plasmids, can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. Once these recombinant plasmids are introduced into unicellular cultures, including prokaryotic organisms and eukaryotic cells, the cells are grown in tissue culture and vectors can be replicated.

[0071] Any number of vector-host combinations can **be** employed, including plasmids and bacterial host cells, yeast vectors and yeast hosts, baculovirus vectors and insect host cells, vaccinia virus vectors and mammalian host cells, etc.

[0072] As noted above, transcription of DNA is dependent upon the presence of a promoter, which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a

prokaryotic system, and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells. A number of promoters suitable for expression in eukaryotes and prokaryotes are well known in the art, any of which can be utilized.

[0073] The promoter used for expression of the above-identified proteins or polypeptide fragments thereof can be a constitutive promoter, which directs expression continually, or an inducible promoter, which is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer (whereas, in the absence of an inducer the DNA sequences or genes will not be transcribed). In addition, any enhancer or inducer elements can be included to generate the level and control over expression of the transgene.

[0074] The DNA construct can also include an operable 3' regulatory region, selected from among those which are capable of providing correct transcription termination and polyadenylation of mRNA for expression in the host cell of choice, operably linked to a DNA molecule which encodes for a protein of choice.

[0075] One alternative to the use of prokaryotic host cells is the use of eukaryotic host cells, such as yeast or mammalian cells, which can also be used to recombinantly produce the various proteins or polypeptide fragments thereof as noted above. Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573), CHOP, NS-I, NIH3T3 (ATCC No. CRL 1658), and CNSl cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcription and translation control sequences known in the art and noted above for the infective transformation systems.

[0076] Once the DNA construct of the present invention has been prepared, it is ready to be incorporated into a host cell. Recombinant molecules can be introduced into cells *via* transformation, particularly transfection, lipofection, transduction, conjugation, mobilization, electroporation, or infection (e.g., with a viral vector). Accordingly, another aspect of the present invention relates to a method of making a recombinant host cell. Basically, this method is carried out by transforming a host cell with a DNA

construct of the present invention under conditions effective to yield transcription of the DNA molecule in the host cell.

[0077] Once the host cell has been prepared, the chimeric can be expressed and recovered in a substantially pure form. In a particular embodiment, the substantially pure chimeric protein is at least about 80% pure, more preferably at least 90% pure, most preferably at least 95% pure. A substantially pure chimeric protein can be obtained by conventional techniques well known in the art. Given its secretion signal, the substantially pure chimeric protein is secreted into the growth medium of recombinant host cells. The medium can be recovered and then subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the chimeric protein from debris and other secreted proteins. If necessary, a protein fraction containing the substantially pure chimeric protein may be further purified by high performance liquid chromatography ("HPLC").

[0078] The chimeric protein, once it has been recovered in substantially pure form, can be formulated into a vaccine that includes a pharmaceutically acceptable carrier, typically sterile saline or sterile buffered saline, and any suitable adjuvants such as those described above. In general, an immunologically or prophylactically effective dose of about 10 μg to 100 mg, and preferably about 100 μg to 1 mg of chimeric protein is administered directly into tissue. Any suitable mode of delivering the chimeric protein vaccine is contemplated, including those identified above for DNA-based vaccines.

[0079] From the foregoing, it should be appreciated that the present invention also relates to a method of imparting resistance against *Clostridium botulinum* (or botulism neurotoxin) to a mammal. This method is carried out by administering to a mammal a DNA or chimeric protein vaccine of the present invention under conditions effective to induce a protective immune response against *Clostridium botulinum.* As demonstrated by the accompanying Examples, a single dose of an infective adeno-virus vector of the present invention is capable of inducing long-term protection (for more than 6 months) against BoNT C. Thus, it is contemplated that the vaccines of the present invention can be administered once to a mammal, or repeated boost vaccinations can be administered following a sufficient delay following a first dosage.

Table 1: Comparison of Native (SEQ ID NO: 1) and Codon-optimized (SEQ ID NO: 2) BoNT/C-HcSO DNA and Protein (SEQ ID NO: 3) Sequences

	Position	Sequence											
Native	1 - **36**	GAT	ATA	ATT	AAT	GAA	TAT	TTC	AAT	AAT	ATT	AAT	GAT
Optimized	1 - **36**	GAC	ATT	ATC	AAC	GAG	TAC	TTC	AAT	AAC	ATC	AAT	GAC
Protein	1 - 12	D	I	I	N	E	Y	F	N	N	I	N	D
Native	**37** - **72**	TCA	AAA	ATT	TTG	AGC	CTA	CAA	AAC	AGA	AAA	AAT	ACT
Optimized	**37** - **72**	AGC	AAG	ATC	CTG	TCC	CTG	CAA	AAT	CGG	AAA	AAC	ACT
Protein	13 - **24**	S	K	I	L	S	L	Q	N	R	K	N	T
Native	**73** - 108	TTA	GTG	GAT	ACA	TCA	GGA	TAT	AAT	GCA	GAA	GTG	AGT
Optimized	**73** - 108	CTG	GTG	GAC	ACC	AGC	GGA	TAT	AAC	GCT	GAG	GTG	AGC
Protein	**25** - **36**	L	V	D	T	S	G	Y	N	A	E	V	S
Native	109 - 144	GAA	GAA	GGC	GAT	GTT	CAG	CTT	AAT	CCA	ATA	TTT	CCA
Optimized	109 - 144	GAA	GAA	GGC	GAT	GTG	CAA	CTG	AAC	CCA	ATC	TTC	CCC
Protein	**37** - **48**	E	E	G	D	V	Q	L	N	P	I	F	P
Native	145 - 180	TTT	GAC	TTT	AAA	TTA	GGT	AGT	TCA	GGG	GAG	GAT	AGA
Optimized	145 - 180	TTT	GAT	TTT	AAG	CTG	GGC	TCC	TCC	GGC	GAG	GAT	AGG
Protein	**49** - **60**	F	D	F	K	L	G	S	S	G	E	D	R
Native	181 - 216	GGT	AAA	GTT	ATA	GTA	ACC	CAG	AAT	GAA	AAT	ATT	GTA
Optimized	181 - 216	GGG	AAA	GTC	ATC	GTC	ACC	CAG	AAT	GAA	AAC	ATC	GTC
Protein	61 - **72**	G	K	V	I	V	T	Q	N	E	N	I	V
Native	217 - 252	TAT	AAT	TCT	ATG	TAT	GAA	AGT	TTT	AGC	ATT	AGT	TTT
Optimized	217 - 252	TAC	AAT	AGC	ATG	TAC	GAG	AGC	TTC	AGC	ATC	TCC	TTC
Protein	**73** - **84**	Y	N	S	M	Y	E	S	F	S	I	S	F
Native	253 - 288	TGG	ATT	AGA	ATA	AAT	AAA	TGG	GTA	AGT	AAT	TTA	CCT
Optimized	253 - 288	TGG	ATC	AGA	ATT	AAC	AAA	TGG	GTC	AGC	AAC	CTG	CCA
Protein	**85** - **96**	W	I	R	I	N	K	W	V	S	N	L	P
Native	289 - 324	GGA	TAT	ACT	ATA	ATT	GAT	AGT	GTT	AAA	AAT	AAC	TCA
Optimized	289 - 324	GGA	TAT	ACC	ATC	ATC	GAC	AGC	GTG	AAG	AAC	AAC	TCC
Protein	**97** - 108	G	Y	T	I	I	D	S	V	K	N	N	S
Native	325 - 360	GGT	TGG	AGT	ATA	GGT	ATT	ATT	AGT	AAT	TTT	TTA	GTA
Optimized	325 - 360	GGG	TGG	TCC	ATC	GGG	ATT	ATC	TCC	AAT	TTT	CTG	GTG
Protein	109 - 120	G	W	S	I	G	I	I	S	N	F	L	V
Native	361 - 396	TTT	ACT	TTA	AAA	CAA	AAT	GAA	GAT	AGT	GAA	CAA	AGT
Optimized	361 - 396	TTC	ACT	CTG	AAA	CAA	AAC	GAA	GAT	AGC	GAA	CAG	AGC
Protein	121 - 132	F	T	L	K	Q	N	E	D	S	E	Q	S
Native	397 - 432	ATA	AAT	TTT	AGT	TAT	GAT	ATA	TCA	AAT	AAT	GCT	CCT
Optimized	397 - 432	ATC	AAT	TTC	TCC	TAC	GAC	ATT	TCC	AAC	AAT	GCA	CCA
Protein	133 - 144	I	N	F	S	Y	D	I	S	N	N	A	P

Table 1 cont.

	Position	Sequence											
Native	**433** - 468	GGA	TAC	AAT	AAA	TGG	TTT	TTT	GTA	ACT	GTT	ACT	AAC
Optimized	**433** - 468	GGG	TAT	AAC	AAG	TGG	TTC	TTT	GTC	ACT	GTC	ACC	AAC
Protein	145 - 156	G	Y	N	K	W	F	F	V	T	V	T	N
Native	469 - **504**	AAT	ATG	ATG	GGA	AAT	ATG	AAG	ATT	TAT	ATA	AAT	GGA
Optimized	469 - **504**	AAC	ATG	ATG	GGG	AAC	ATG	AAG	ATT	TAC	ATC	AAC	GGA
Protein	157 - 168	N	M	M	G	N	M	K	I	Y	I	N	G
Native	**505** - **540**	AAA	TTA	ATA	GAT	ACT	ATA	AAA	GTT	AAA	GAA	CTA	ACT
Optimized	**505** - **540**	AAA	CTG	ATT	GAT	ACT	ATT	AAG	GTC	AAG	GAA	CTC	ACC
Protein	169 - 180	K	L	I	D	T	I	K	V	K	E	L	T
Native	541 - 576	GGA	ATT	AAT	TTT	AGC	AAA	ACT	ATA	ACA	TTT	GAA	ATA
Optimized	541 - 576	GGC	ATT	AAC	TTC	TCC	AAG	ACA	ATT	ACA	TTT	GAG	ATC
Protein	181 - 192	G	I	N	F	S	K	T	I	T	F	E	I
Native	577 - 612	AAT	AAA	ATT	CCA	GAT	ACC	GGT	TTG	ATT	ACT	TCA	GAT
Optimized	577 - 612	AAT	AAG	ATT	CCA	GAC	ACC	GGA	CTC	ATT	ACT	AGC	GAC
Protein	193 - 204	N	K	I	P	D	T	G	L	I	T	S	D
Native	613 - 648	TCT	GAT	AAC	ATC	AAT	ATG	TGG	ATA	AGA	GAT	TTT	TAT
Optimized	613 - 648	TCC	GAC	AAT	ATC	AAT	ATG	TGG	ATT	AGG	GAC	TTC	TAC
Protein	205 - 216	S	D	N	I	N	M	W	I	R	D	F	Y
Native	649 - 684	ATA	TTT	GCT	AAA	GAA	TTA	GAT	GGT	AAA	GAT	ATT	AAT
Optimized	649 - 684	ATC	TTT	GCT	AAA	GAA	CTG	GAT	GGC	AAG	GAT	ATT	AAC
Protein	217 - 228	I	F	A	K	E	L	D	G	K	D	I	N
Native	685 - 720	ATA	TTA	TTT	AAT	AGC	TTG	CAA	TAT	ACT	AAT	GTT	GTA
Optimized	685 - 720	ATT	CTC	TTC	AAC	TCC	CTC	CAA	TAC	ACA	AAC	GTC	GTC
Protein	229 - 240	I	L	F	N	S	L	Q	Y	T	N	V	V
Native	721 - 756	AAA	GAT	TAT	TGG	GGA	AAT	GAT	TTA	AGA	TAT	AAT	AAA
Optimized	721 - 756	AAA	GAC	TAT	TGG	GGC	AAC	GAC	CTG	AGA	TAC	AAC	AAA
Protein	241 - 252	K	D	Y	W	G	N	D	L	R	Y	N	K
Native	757 - 792	GAA	TAT	TAT	ATG	GTT	AAT	ATA	GAT	TAT	TTA	AAT	AGA
Optimized	757 - 792	GAG	TAT	TAC	ATG	GTC	AAC	ATC	GAT	TAC	CTG	AAC	AGA
Protein	253 - 264	E	Y	Y	M	V	N	I	D	Y	L	N	R
Native	793 - 828	TAT	ATG	TAT	GCG	AAC	TCA	CGA	CAA	ATT	GTT	TTT	AAT
Optimized	793 - 828	TAT	ATG	TAC	GCC	AAC	AGC	AGG	CAA	ATT	GTG	TTC	AAC
Protein	265 - 276	Y	M	Y	A	N	S	R	Q	I	V	F	N
Native	829 - 864	ACA	CGT	AGA	AAT	AAT	AAT	GAC	TTC	AAT	GAA	GGA	TAT
Optimized	829 - 864	ACA	CGG	AGG	AAT	AAC	AAT	GAT	TTC	AAC	GAA	GGC	TAT
Protein	277 - 288	T	R	R	N	N	N	D	F	N	E	G	Y

Table 1 cont.

	Position	Sequence											
Native	865 - 900	AAA	ATT	ATA	ATA	AAA	AGA	ATC	AGA	GGA	AAT	ACA	AAT
Optimized	865 - 900	AAG	ATC	ATC	ATC	AAA	AGA	ATC	AGG	GGA	AAC	ACT	AAT
Protein	289 - 300	K	I	I	I	K	R	I	R	G	N	T	N
Native	901 - 936	GAT	ACT	AGA	GTA	CGA	GGA	GGA	GAT	ATT	TTA	TAT	TTT
Optimized	901 - 936	GAC	ACT	AGG	GTC	AGA	GGC	GGC	GAC	ATT	CTG	TAT	TTT
Protein	301 - 312	D	T	R	V	R	G	G	D	I	L	Y	F
Native	937 - 972	GAT	ATG	ACA	ATT	AAT	AAC	AAA	GCA	TAT	AAT	TTG	TTT
Optimized	937 - 972	GAC	ATG	ACT	ATC	AAC	AAT	AAG	GCC	TAC	AAC	CTG	TTT
Protein	313 - 324	D	M	T	I	N	N	K	A	Y	N	L	F
Native	973 -1008	ATG	AAG	AAT	GAA	ACT	ATG	TAT	GCA	GAT	AAT	CAT	AGT
Optimized	973 -1008	ATG	AAA	AAC	GAG	ACA	ATG	TAT	GCT	GAT	AAC	CAC	AGC
Protein	325 - 336	M	K	N	E	T	M	Y	A	D	N	H	S
Native	1009-1044	ACT	GAA	GAT	ATA	TAT	GCT	ATA	GGT	TTA	AGA	GAA	CAA
Optimized	1009-1044	ACA	GAA	GAT	ATT	TAC	GCA	ATC	GGC	CTG	AGG	GAG	CAA
Protein	337 - 348	T	E	D	I	Y	A	I	G	L	R	E	Q
Native	1045-1080	ACA	AAG	GAT	ATA	AAT	GAT	AAT	ATT	ATA	TTT	CAA	ATA
Optimized	1045-1080	ACC	AAA	GAC	ATT	AAC	GAT	AAT	ATC	ATT	TTC	CAG	ATC
Protein	349 - 360	T	K	D	I	N	D	N	I	I	F	Q	I
Native	1081-1116	CAA	CCA	ATG	AAT	AAT	ACT	TAT	TAT	TAC	GCA	TCT	CAA
Optimized	1081-1116	CAG	CCA	ATG	AAT	AAT	ACC	TAC	TAC	TAC	GCA	AGC	CAA
Protein	361 - 372	Q	P	M	N	N	T	Y	Y	Y	A	S	Q
Native	1117-1152	ATA	TTT	AAA	TCA	AAT	TTT	AAT	GGA	GAA	AAT	ATT	TCT
Optimized	1117-1152	ATT	TTC	AAG	AGC	AAC	TTT	AAC	GGA	GAG	AAC	ATC	AGC
Protein	373 - 384	I	F	K	S	N	F	N	G	E	N	I	S
Native	1153-1188	GGA	ATA	TGT	TCA	ATA	GGT	ACT	TAT	CGT	TTT	AGA	CTT
Optimized	1153-1188	GGA	ATC	TGC	AGC	ATT	GGG	ACC	TAC	AGG	TTT	AGA	CTC
Protein	385 - 396	G	I	C	S	I	G	T	Y	R	F	R	L
Native	1189-1224	GGA	GGT	GAT	TGG	TAT	AGA	CAC	AAT	TAT	TTG	GTG	CCT
Optimized	1189-1224	GGG	GGA	GAC	TGG	TAT	AGA	CAT	AAT	TAC	CTC	GTG	CCT
Protein	397 - 408	G	G	D	W	Y	R	H	N	Y	L	V	P
Native	1225-1260	ACT	GTG	AAG	CAA	GGA	AAT	TAT	GCT	TCA	TTA	TTA	GAA
Optimized	1225-1260	ACC	GTC	AAG	CAG	GGA	AAT	TAT	GCC	AGC	CTC	CTC	GAA
Protein	409 - 420	T	V	K	Q	G	N	Y	A	S	L	L	E
Native	1261-1296	TCA	ACA	TCA	ACT	CAT	TGG	GGT	TTT	GTA	CCT	GTA	AGT
Optimized	1261-1296	AGC	ACT	TCC	ACC	CAT	TGG	GGA	TTT	GTC	CCC	GTC	TCC
Protein	421 - 432	S	T	S	T	H	W	G	F	V	P	V	S
Native	1297-1299	GAA											
Optimized	1297-1302	GAG	TGA										
Protein	433	E	*										

Table 2: Comparison of Native (SEQ ID NO: 4) and Codon-optimized (SEQ ID NO: 5) BoNT/A-HcSO DNA and Protein (SEQ ID NO: 6) Sequences

	Position	Sequence											
Native	1 - 36	AGA	TTA	TTA	TCT	ACA	TTT	ACT	GAA	TAT	ATT	AAG	AAT
Optimized	1 - 36	CGG	CTC	CTG	TCC	ACT	TTC	ACA	GAA	TAT	ATC	AAA	AAC
Protein	1 - 12	R	L	L	S	T	F	T	E	Y	I	K	N
Native	37 - 72	ATT	ATT	AAT	ACT	TCT	ATA	TTG	AAT	TTA	AGA	TAT	GAA
Optimized	37 - 72	ATT	ATC	AAT	ACT	AGC	ATC	CTG	AAT	CTC	CGG	TAT	GAG
Protein	13 - 24	I	I	N	T	S	I	L	N	L	R	Y	E
Native	73 - 108	AGT	AAT	CAT	TTA	ATA	GAC	TTA	TCT	AGG	TAT	GCA	TCA
Optimized	73 - 108	AGC	AAC	CAC	CTG	ATT	GAC	CTG	AGC	AGG	TAC	GCA	AGC
Protein	25 - 36	S	N	H	L	I	D	L	S	R	Y	A	S
Native	109 - 144	AAA	ATA	AAT	ATT	GGT	AGT	AAA	GTA	AAT	TTT	GAT	CCA
Optimized	109 - 144	AAA	ATC	AAC	ATC	GGC	TCC	AAG	GTG	AAC	TTT	GAC	CCC
Protein	37 - 48	K	I	N	I	G	S	K	V	N	F	D	P
Native	145 - 180	ATA	GAT	AAA	AAT	CAA	ATT	CAA	TTA	TTT	AAT	TTA	GAA
Optimized	145 - 180	ATC	GAT	AAG	AAC	CAA	ATT	CAA	CTC	TTT	AAT	CTC	GAA
Protein	49 - 60	I	D	K	N	Q	I	Q	L	F	N	L	E
Native	181 - 216	AGT	AGT	AAA	ATT	GAG	GTA	ATT	TTA	AAA	AAT	GCT	ATT
Optimized	181 - 216	TCC	AGC	AAG	ATT	GAG	GTC	ATT	CTG	AAA	AAC	GCT	ATC
Protein	61 - 72	S	S	K	I	E	V	I	L	K	N	A	I
Native	217 - 252	GTA	TAT	AAT	AGT	ATG	TAT	GAA	AAT	TTT	AGT	ACT	AGC
Optimized	217 - 252	GTG	TAC	AAC	TCC	ATG	TAC	GAG	AAC	TTT	TCC	ACC	AGC
Protein	73 - 84	V	Y	N	S	M	Y	E	N	F	S	T	S
Native	253 - 288	TTT	TGG	ATA	AGA	ATT	CCT	AAG	TAT	TTT	AAC	AGT	ATA
Optimized	253 - 288	TTC	TGG	ATT	AGG	ATC	CCA	AAA	TAC	TTC	AAT	AGC	ATT
Protein	85 - 96	F	W	I	R	I	P	K	Y	F	N	S	I
Native	289 - 324	AGT	CTA	AAT	AAT	GAA	TAT	ACA	ATA	ATA	AAT	TGT	ATG
Optimized	289 - 324	TCC	CTC	AAT	AAC	GAG	TAT	ACC	ATC	ATC	AAT	TGT	ATG
Protein	97 - 108	S	L	N	N	E	Y	T	I	I	N	C	M
Native	325 - 360	GAA	AAT	AAT	TCA	GGA	TGG	AAA	GTA	TCA	CTT	AAT	TAT
Optimized	325 - 360	GAA	AAC	AAT	AGC	GGC	TGG	AAG	GTG	TCC	CTG	AAT	TAC
Protein	109 - 120	E	N	N	S	G	W	K	V	S	L	N	Y
Native	361 - 396	GGT	GAA	ATA	ATC	TGG	ACT	TTA	CAG	GAT	ACT	CAG	GAA
Optimized	361 - 396	GGA	GAG	ATC	ATC	TGG	ACT	CTG	CAA	GAC	ACC	CAG	GAG
Protein	121 - 132	G	E	I	I	W	T	L	Q	D	T	Q	E
Native	397 - 432	ATA	AAA	CAA	AGA	GTA	GTT	TTT	AAA	TAC	AGT	CAA	ATG
Optimized	397 - 432	ATC	AAA	CAG	AGA	GTC	GTG	TTC	AAA	TAC	TCC	CAA	ATG
Protein	133 - 144	I	K	Q	R	V	V	F	K	Y	S	Q	M

Table 2 cont.

	Position	Sequence											
Native	**433** - 468	ATT	AAT	ATA	TCA	GAT	TAT	ATA	AAC	AGA	TGG	ATT	TTT
Optimized	**433** - 468	ATT	AAC	ATT	AGC	GAC	TAC	ATC	AAC	AGA	TGG	ATC	TTC
Protein	145 - 156	I	N	I	S	D	Y	I	N	R	W	I	F
Native	469 - **504**	GTA	ACT	ATC	ACT	AAT	AAT	AGA	TTA	AAT	AAC	TCT	AAA
Optimized	469 - **504**	GTG	ACA	ATT	ACA	AAC	AAC	AGG	CTG	AAT	AAC	TCC	AAG
Protein	157 - 168	V	T	I	T	N	N	R	L	N	N	S	K
Native	**505** - **540**	ATT	TAT	ATA	AAT	GGA	AGA	TTA	ATA	GAT	CAA	AAA	CCA
Optimized	**505** - **540**	ATT	TAC	ATT	AAC	GGC	AGG	CTC	ATC	GAT	CAG	AAG	CCT
Protein	169 - 180	I	Y	I	N	G	R	L	I	D	Q	K	P
Native	541 - 576	ATT	TCA	AAT	TTA	GGT	AAT	ATT	CAT	GCT	AGT	AAT	AAT
Optimized	541 - 576	ATT	AGC	AAC	CTC	GGC	AAT	ATT	CAT	GCC	AGC	AAT	AAC
Protein	181 - 192	I	S	N	L	G	N	I	H	A	S	N	N
Native	577 - 612	ATA	ATG	TTT	AAA	TTA	GAT	GGT	TGT	AGA	GAT	ACA	CAT
Optimized	577 - 612	ATC	ATG	TTT	AAG	CTC	GAC	GGC	TGT	AGG	GAT	ACC	CAC
Protein	193 - 204	I	M	F	K	L	D	G	C	R	D	T	H
Native	613 - 648	AGA	TAT	ATT	TGG	ATA	AAA	TAT	TTT	AAT	CTT	TTT	GAT
Optimized	613 - 648	AGG	TAC	ATT	TGG	ATC	AAG	TAC	TTT	AAC	CTC	TTT	GAC
Protein	205 - 216	R	Y	I	W	I	K	Y	F	N	L	F	D
Native	649 - 684	AAG	GAA	TTA	AAT	GAA	AAA	GAA	ATC	AAA	GAT	TTA	TAT
Optimized	649 - 684	AAG	GAA	CTG	AAC	GAA	AAA	GAA	ATC	AAA	GAT	CTC	TAC
Protein	217 - 228	K	E	L	N	E	K	E	I	K	D	L	Y
Native	685 - 720	GAT	AAT	CAA	TCA	AAT	TCA	GGT	ATT	TTA	AAA	GAC	TTT
Optimized	685 - 720	GAT	AAC	CAA	AGC	AAC	TCC	GGA	ATT	CTC	AAA	GAC	TTT
Protein	229 - 240	D	N	Q	S	N	S	G	I	L	K	D	F
Native	721 - 756	TGG	GGT	GAT	TAT	TTA	CAA	TAT	GAT	AAA	CCA	TAC	TAT
Optimized	721 - 756	TGG	GGC	GAT	TAC	CTG	CAA	TAC	GAC	AAG	CCA	TAC	TAC
Protein	241 - 252	W	G	D	Y	L	Q	Y	D	K	P	Y	Y
Native	757 - 792	ATG	TTA	AAT	TTA	TAT	GAT	CCA	AAT	AAA	TAT	GTC	GAT
Optimized	757 - 792	ATG	CTC	AAC	CTG	TAT	GAC	CCT	AAC	AAA	TAC	GTG	GAT
Protein	253 - 264	M	L	N	L	Y	D	P	N	K	Y	V	D
Native	793 - 828	GTA	AAT	AAT	GTA	GGT	ATT	AGA	GGT	TAT	ATG	TAT	CTT
Optimized	793 - 828	GTC	AAT	AAT	GTG	GGC	ATC	CGG	GGG	TAT	ATG	TAT	CTC
Protein	265 - 276	V	N	N	V	G	I	R	G	Y	M	Y	L
Native	829 - 864	AAA	GGG	CCT	AGA	GGT	AGC	GTA	ATG	ACT	ACA	AAC	ATT
Optimized	829 - 864	AAG	GGA	CCT	CGG	GGA	AGC	GTG	ATG	ACC	ACA	AAT	ATC
Protein	277 - 288	K	G	P	R	G	S	V	M	T	T	N	I

Table 2 cont.

	Position	Sequence											
Native	865 - 900	TAT	TTA	AAT	TCA	AGT	TTG	TAT	AGG	GGG	ACA	AAA	TTT
Optimized	865 - 900	TAC	CTG	AAC	AGC	TCC	CTG	TAT	AGA	GGC	ACT	AAA	TTC
Protein	289 - 300	Y	L	N	S	S	L	Y	R	G	T	K	F
Native	901 - 936	ATT	ATA	AAA	AAA	TAT	GCT	TCT	GGA	AAT	AAA	GAT	AAT
Optimized	901 - 936	ATC	ATT	AAG	AAG	TAT	GCT	AGC	GGA	AAC	AAG	GAT	AAC
Protein	301 - 312	I	I	K	K	Y	A	S	G	N	K	D	N
Native	937 - 972	ATT	GTT	AGA	AAT	AAT	GAT	CGT	GTA	TAT	ATT	AAT	GTA
Optimized	937 - 972	ATT	GTG	CGG	AAC	AAC	GAC	AGA	GTC	TAT	ATT	AAC	GTC
Protein	313 - 324	I	V	R	N	N	D	R	V	Y	I	N	V
Native	973 -1008	GTA	GTT	AAA	AAT	AAA	GAA	TAT	AGG	TTA	GCT	ACT	AAT
Optimized	973 -1008	GTC	GTC	AAG	AAC	AAA	GAG	TAC	AGA	CTG	GCA	ACA	AAC
Protein	325 - 336	V	V	K	N	K	E	Y	R	L	A	T	N
Native	1009-1044	GCA	TCA	CAG	GCA	GGC	GTA	GAA	AAA	ATA	CTA	AGT	GCA
Optimized	1009-1044	GCA	TCC	CAG	GCT	GGA	GTC	GAA	AAG	ATT	CTC	AGC	GCT
Protein	337 - 348	A	S	Q	A	G	V	E	K	I	L	S	A
Native	1045-1080	TTA	GAA	ATA	CCT	GAT	GTA	GGA	AAT	CTA	AGT	CAA	GTA
Optimized	1045-1080	CTG	GAA	ATT	CCT	GAC	GTG	GGC	AAT	CTC	AGC	CAG	GTC
Protein	349 - 360	L	E	I	P	D	V	G	N	L	S	Q	V
Native	1081-1116	GTA	GTA	ATG	AAG	TCA	AAA	AAT	GAT	CAA	GGA	ATA	ACA
Optimized	1081-1116	GTG	GTG	ATG	AAA	AGC	AAG	AAC	GAT	CAG	GGC	ATC	ACT
Protein	361 - 372	V	V	M	K	S	K	N	D	Q	G	I	T
Native	1117-1152	AAT	AAA	TGC	AAA	ATG	AAT	TTA	CAA	GAT	AAT	AAT	GGG
Optimized	1117-1152	AAT	AAG	TGT	AAG	ATG	AAC	CTC	CAG	GAC	AAC	AAC	GGG
Protein	373 - 384	N	K	C	K	M	N	L	Q	D	N	N	G
Native	1153-1188	AAT	GAT	ATA	GGC	TTT	ATA	GGA	TTT	CAT	CAG	TTT	AAT
Optimized	1153-1188	AAT	GAT	ATC	GGA	TTC	ATT	GGG	TTT	CAC	CAG	TTT	AAC
Protein	385 - 396	N	D	I	G	F	I	G	F	H	Q	F	N
Native	1189-1224	AAT	ATA	GCT	AAA	CTA	GTA	GCA	AGT	AAT	TGG	TAT	AAT
Optimized	1189-1224	AAC	ATT	GCT	AAA	CTC	GTC	GCC	TCC	AAC	TGG	TAC	AAT
Protein	397 - 408	N	I	A	K	L	V	A	S	N	W	Y	N
Native	1225-1260	AGA	CAA	ATA	GAA	AGA	TCT	AGT	AGG	ACT	TTG	GGT	TGC
Optimized	1225-1260	AGG	CAG	ATC	GAG	AGA	AGC	AGC	AGA	ACA	CTC	GGG	TGT
Protein	409 - 420	R	Q	I	E	R	S	S	R	T	L	G	C
Native	1261-1296	TCA	TGG	GAA	TTT	ATT	CCT	GTA	GAT	GAT	GGA	TGG	GGA
Optimized	1261-1296	AGC	TGG	GAG	TTC	ATC	CCC	GTC	GAT	GAC	GGG	TGG	GGA
Protein	421 - 432	S	W	E	F	I	P	V	D	D	G	W	G
Native	1297-1311	GAA	AGG	CCA	CTG	TAA							
Optimized	1297-1311	GAA	CGG	CCT	CTC	TGA							
Protein	**433** - 436	E	R	P	L	*							

[0080] A further aspect of the present invention relates an isolated antibody raised against a chimeric protein of the present invention, or antibody fragment thereof, which antibody or antibody fragment is capable of specifically binding and neutralizing a *Clostridium botulinum* neurotoxin.

[0081] The antibodies of the present invention can be polyclonal antibodies or monoclonal antibodies, although monoclonal antibodies are preferred because of their specificity. The antibody can also be a polyclonal preparation rendered monospecific.

[0082] Various methods of producing antibodies with a known antigen are well-known to those ordinarily skilled in the art (ANTIBODIES: A LABORATORY MANUAL (Harlow & Lane eds., 1988), which is hereby incorporated by reference in its entirety). In particular, suitable antibodies may be produced by chemical synthesis, by intracellular immunization *{i.e.,* intrabody technology), or preferably, by recombinant expression techniques. Methods of producing antibodies may further include the hybridoma technology well-known in the art.

[0083] In particular, monoclonal antibody production may be effected by techniques which are well-known in the art. Basically, the process involves first obtaining immune cells (lymphocytes) from the spleen of a mammal *{e.g.,* mouse) which has been previously immunized with the chimeric protein either *in vivo* or *in vitro.* The antibody-secreting lymphocytes are then fused with (mouse) myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured, and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, and grown either *in vivo* or *in vitro* to produce large quantities of antibody. A description of the theoretical basis and practical methodology of fusing such cells is set forth in Kohler & Milstein, "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity," *Nature,* 256:495-497 (1975), which is hereby incorporated by reference in its entirety.

[0084] Mammalian lymphocytes are immunized by *in vivo* immunization of the animal *(e.g.,* a mouse) with the chimeric protein or DNA vaccine of the present invention. Following a sufficient number of immunizations *(i.e.,* one or more), the animals are sacrificed and spleen cells removed.

[0085] Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol ("PEG") or other fusing agents *(see* Milstein & Kohler, "Derivation of Antibody-producing Tissue Culture and Tumor Lines by Cell Fusion," *Eur. J. Immunol.* 6:5 11-5 19 (1976), which is hereby incorporated by reference in its entirety). This immortal cell line, which is preferably murine, but may also be derived from cells of other mammalian species, including but not limited to rats and humans, is selected to be deficient in enzymes necessary for the utilization of certain nutrients, to be capable of rapid growth, and to have good fusion capability. Many such cell lines are known to those skilled in the art, and others are regularly described.

[0086] Procedures for raising polyclonal antibodies are also well known. Typically, such antibodies can be raised by administering the chimeric protein or DNA vaccine of the present invention subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be administered at a total volume of 100 μl per site at multiple sites. Each injected material may contain synthetic surfactant adjuvant pluronic polyols, or pulverized acrylamide gel containing the purified chimeric protein or DNA vaccine. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. Boosting may not be required with the DNA vaccine. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding chimeric protein to capture the antibody. Ultimately, the rabbits are euthanized with pentobarbital 150 mg/Kg IV. This and other procedures for raising polyclonal antibodies are disclosed in ANTIBODIES: A LABORATORY MANUAL (Harlow & Lane eds., 1988), which is hereby incorporated by reference in its entirety.

[0087] In addition to utilizing whole antibodies, the present invention also encompasses use of binding portions of such antibodies. Such binding portions include Fab fragments, $F(ab)_2$ fragments, Fab' fragments, $F(ab')_2$ fragments, Fd fragments, Fd' fragments, Fv fragments, and minibodies, *e.g.*, 61-residue subdomains of the antibody heavy-chain variable domain (Pessi et al., "A Designed Metal-binding Protein with a Novel Fold," *Nature* 362:367-369 (1993), which is hereby incorporated by reference in its entirety). Domain antibodies (dAbs) are also suitable for the methods of the present invention (Holt et al., "Domain Antibodies: Proteins for Therapy," *Trends Biotechnol.* 21:484-90 (2003), which is hereby incorporated by reference in its entirety). These antibody fragments can be made by conventional procedures, such as proteolytic fragmentation procedures, as described in J. Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE 98-118 (1984), which is hereby incorporated by reference in its entirety.

[0088] The antibodies may be from humans, or from animals other than humans, preferably mammals, such as rat, mouse, guinea pig, rabbit, goat, sheep, and pig, or avian species such as chicken. Preferred are mouse monoclonal antibodies and antigen-binding fragments or portions thereof. In addition, chimeric antibodies and hybrid antibodies are embraced by the present invention. Techniques for the production of chimeric antibodies are described in, *e.g.*, Morrison et al., "Chimeric Human Antibody Molecules: Mouse Antigen-binding Domains with Human Constant Region Domains," *Proc. Nat 7 Acad. Sci. USA* 81:6851-5 (1984), Neuberger et al., "Recombinant Antibodies Possessing Novel Effector Functions," *Nature* 312:604-8 (1984), and Takeda et al., "Construction of Chimaeric Processed Immunoglobulin Genes Containing Mouse Variable and Human Constant Region Sequences," *Nature* 314:452-4 (1985), each of which is hereby incorporated by reference in its entirety. For human therapeutic purposes, humanized antibodies or fragments are preferred.

[0089] Further, single chain antibodies are also suitable for the present invention *(e.g.,* U.S. Patent Nos. 5,476,786 to Huston and 5,132,405 to Huston & Oppermann; Huston et al., "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-digoxin Single-chain Fv Analogue Produced in *Escherichia coli,"* *Proc. Nat'lAcad. Sci. USA* 85:5879-83 (1988); U.S. Patent No. 4,946,778 to Ladner et al.; Bird et al., "Single-chain Antigen-binding Proteins," *Science* 242:423-6 (1988); Ward et al., "Binding Activities of a Repertoire of Single Immunoglobulin Variable Domains Secreted from *Escherichia coli," Nature* 341 :544-6 (1989), each of which is hereby incorporated by reference in its entirety). Single chain antibodies are formed by linking the heavy and light immunoglobulin chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Univalent antibodies are also embraced by the present invention.

[0090] A pharmaceutical composition comprising the antibodies or antibody fragments of the present invention can be administered to an individual to provide passive immunity against a botulism neurotoxin. The pharmaceutical composition can include antibodies or antibody fragments against a single botulism neurotoxin, or the composition can contain antibodies or antibody fragments against any two or more botulism neurotoxin *(e.g.,* neurotoxin A, neurotoxin B, neurotoxin C, neurotoxin D, neurotoxin E, neurotoxin F, and neurotoxin G).

[0091] The antibodies or antibody fragments can be administered to a patient exposed to *Clostridium botulinum* to afford passive immunity against a botulism neurotoxin. Thus, a further aspect of the present invention relates to treatment of a *Clostridium botulinum* infection by administering to a patient the antibodies or antibody fragments (or composition containing the same) under conditions effective to neutralize the botulism neurotoxin. Administration can be carried out by any suitable means, but preferably parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intracavitary or intravesical instillation, intraarterially, intralesionally, by application to mucous membranes, or directly to a site of infection. The amount of antiserum administered should be sufficient to neutralize the neurotoxin, *i.e.,* in excess. This

method of treatment is typically carried out in combination with other therapeutic agents, *e.g.,* antibiotics, sufficient to destroy the *Clostridium botulinum* population.

EXAMPLES

[0092] The following examples are provided to illustrate embodiments of the present invention but they are by no means intended to limit its scope.

Materials and Methods

Animals

[0093] 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.

Saliva, Nasal Wash, and Vagina Wash Specimens

[0094] For these experiments, 16 mice were divided into 2 test groups and 2 control groups, 8 mice per group. The animals were vaccinated with 2 x 10^7 pfu/mouse of Ad/opt-BoNT/C-Hc50 in the test groups and same amount of Ad/Null in the control groups at week 0. Saliva, nasal wash, and vaginal wash samples were collected at week 2 in one test group and one control group, and week 4 in one test group and one control group for each time point. The mice were anesthetized by i.p. injection with 2 mg of ketamine HCl (Bedford Laboratories, Bedford, OH) plus 0.2 mg of xylazine (Butler Company, Columbus, OH) in 100 µl. Vaginal washes were collected by flushing the vagina with 100 µl PBS by repeated aspiration using a pipette with an animal feeding needle (with a ball head) until turbid (Singh et al, "Mucosal Immunization with

Recombinant MOMP Genetically Linked with Modified Cholera Toxin Confers Protection Against Chlamydia Trachomatis Infection," *Vaccine* 24:1213-1224 (2006), which is hereby incorporated by reference in its entirety). The saliva samples were collected using a 200-µl pipette fitted with a plastic tip, after i.p. injection of carbachol (Sigma Chemical Co., St. Louis, Mo.; 10 µg in 0.1 ml) to stimulate salivation as described previously (Russell et al, "Distribution, Persistence, and Recall of Serum and Salivary Antibody Responses to Peroral Immunization with Protein Antigen I/II of *Streptococcus mutans* Coupled to the Cholera Toxin B Subunit," *Infect Immun* 59:4061-4070 (1991); Zeng et al., "Protection Against Anthrax by needle-Free Mucosal Immunization with Human Anthrax Vaccine," *Vaccine* 25:3558-3594 (2007), each of which is hereby incorporated by reference in its entirety). The mice were then incised ventrally along the median line from the xiphoid process to the chin, the heads were removed, and the lower jaws were excised. A hypodermic needle was inserted into the posterior opening of the nasopharynx and 200 µl of PBS was injected repeatedly to collect the nasal wash samples (Watanabe et al., "Characterization of Protective Immune Responses Induced by Nasal Influenza Vaccine Containing Mutant Cholera Toxin as a Safe Adjuvant (CTl 12K)," *Vaccine* 20:3443-3455 (2002), which is hereby incorporated by reference in its entirety).

Challenge with Botulinum Neurotoxin

[0095] All animals were challenged by i.p injection with 10^2 - 10^5 x MLD_{50} of C. *botulinum* neurotoxin BoNT/C (Metabiolgics Inc, Madison, WI) per mouse as specified 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 (Arimitsu et al., "Vaccination With Recombinant Whole Heavy Chain Fragments of Clostridium Botulinum Type C and D Neurotoxins." *Clin Diagn Lab Immunol* 11(3):496-502 (2004), which is hereby incorporated by reference in its entirety).

ELISA for Determination of Antibody Concentration

[0096] Anti-BoNT/C-H_c50 IgG, IgGl , IgG2a, and IgA antibody concentrations in serum, saliva, nasal wash and/or vaginal wash samples 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-Hc50 recombinant protein produced in *Escherichia coli* or capture antibodies (goat anti-mouse IgG-, or IgGl-, IgG2a -, or IgA-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, IgGl, IgG2a, or IgA 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 for antibody quantitation was generated in parallel to allow antibody concentration calculations, as previously described *(see* Zeng et al., "Protection Against Anthrax by Needle-Free Mucosal Immunization With Human Anthrax Vaccine," *Vaccine* 25(18):3558-94 (2007), which is hereby incorporated by reference in its entirety).

Anti-adenovirus neutralizing antibody titer assay

[0097] Anti-adenovirus neutralizing antibodies were determined according to previously described methods with some modifications (Zabner et al., "Repeat Administration of an Adenovirus Vector Encoding Cystic Fibrosis Transmembrane Conductance Regulator to the Nasal Epithelium of Patients With Cystic Fibrosis." *J Clin Invest* 97(6):1504-l 1 (1996); Harvey et al., "Variability of Human Systemic Humoral

Immune Responses to Adenovirus Gene Transfer Vectors Administered to Different Organs," *J Virol* 73(8):6729-42 (1999); Hashimoto et al, "Induction of Protective Immunity to Anthrax Lethal Toxin With a Nonhuman Primate Adenovirus-Based Vaccine in the Presence of Preexisting Anti-Human Adenovirus Immunity," *Infect Immun* 73(10):6885-91 (2005), each of which is hereby incorporated by reference in its entirety). 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% CO_2 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 µl of MEME containing 2% FBS was mixed with 50 µl of diluted serum samples. After incubation for 1 h at 37°C to allow neutralization to occur, 100 µl 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 µl 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 (Zabner et al., "Repeat Administration of an Adenovirus Vector Encoding Cystic Fibrosis Transmembrane Conductance Regulator to the Nasal Epithelium of Patients With Cystic Fibrosis," *J Clin Invest* 97(6): 1504-1 1 (1996); Harvey et al., "Variability of Human Systemic Humoral Immune Responses to Adenovirus Gene Transfer Vectors Administered to Different Organs," *J Virol* 73(8):6729-42 (1999); Hashimoto et al., "Induction of Protective Immunity to Anthrax Lethal Toxin With a Nonhuman Primate Adenovirus-Based Vaccine in the Presence of Preexisting Anti-Human Adenovirus Immunity," *Infect Immun* 73(10):6885-91 (2005), each of which is hereby incorporated by reference in its entirety).

Statistical analysis

[0098] Serum and mucosal antibody concentrations 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.

Example 1 — Construction of Nucleic Acid Molecule Encoding Codon-optimized H_c50 of BoNT/C Chimeric Protein, and Insertion in Adeno-viral Vector

[0099] Replication-incompetent recombinant adenoviral vectors were constructed using the AdEasy™ System (Stratagene, La Jolla, CA) (He et al., "A Simplified System for Generating Recombinant Adenoviruses," *Proc Natl Acad Sci USA* 95(5):2509-14 (1998); Zeng et al., "AdEasy System Made Easier by Selecting the Viral Backbone Plasmid Preceding Homologous Recombination," *Biotechniques* 31(2):260-2 (2001); which are hereby incorporated by reference in their entirety). The adenoviral vector is derived from human adenovirus serotype 5 rendered replication-incompetent by the deletion of the E1 and E3 regions.

[0100] 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 (Kimura et al., "**The** Complete Nucleotide Sequence of the Gene Coding for Botulinum Type C1 Toxin in the C-ST Phage Genome," *Biochem Biophys Res Commun* 171(3): 1304-1 1 (1990), which is hereby incorporated by reference in their entirety) was optimized with human codon preference by the DNAworks program (Hoover et al., "DNA Works: An Automated Method for Designing Oligonucleotides for PCR-Based Gene Synthesis," *Nucleic Acids Res* 30(10):e43 (2002), which is hereby incorporated by reference in its entirety). The encoded 50-kDa C-terminal fragment of heavy chain of botulinum neurotoxin type C1 corresponds to amino acids 849-1291 of the BoNT/C recited in Genbank Accession No. D90210, which is hereby incorporated by reference in its entirety. The codon-optimized nucleotide sequence is shown in Table 1, *supra.*

[0101] The 50-kDa fragment of BoNT/C was prepared as an in-frame gene fusion with a 25 residue signal peptide of human tissue plasminogen activator (PLAT) plus two serine residues followed with the codon-optimized BoNT/C-Hc50. The nucleotide sequence encoding amino acids 1-25 of PLAT

(MDAMKRGLCCVLLLCGAVFVSPSQE, SEQ ID NO: 7) was obtained from GenBank Accession No. BC002795, which is hereby incorporated by reference in its entirety. The nucleotide sequence corresponding to the PLAT secretion signal (with Ser-Ser linker) corresponds to SEQ ID NO: 8 as follows:

atggatgcaatgaagagagggctctgctgtgtgctgctgctgtggagcagtcttcgtttcgcccagccaggaaagcagc.

The chimeric open reading frame was then synthesized by a PCR-based method (Gao et al., "Thermodynamically Balanced Inside-Out (TBIO) PCR-Based Gene Synthesis: A Novel Method of Primer Design for High-Fidelity Assembly of Longer Gene Sequences," *Nucleic Acids Res* 31(22):el43 (2003), which is hereby incorporated by reference in its entirety).

[0102] The synthesized DNA was subsequently cloned into a shuttle vector pShuttle-CMV (Stratagene, La Jolla, CA) at its *Sail* site. The DNA sequence of the synthesized gene was further confirmed by DNA sequencing analysis. The adenoviral vector was then constructed according to the standard procedures as described previously (He et al., "A Simplified System for Generating Recombinant Adenoviruses," *Proc Natl Acad Sci USA* 95(5):2509-14 (1998); Zeng et al., "AdEasy System Made Easier by Selecting the Viral Backbone Plasmid Preceding Homologous Recombination," *Biotechniques* 31(2):260-2 (2001), each of which is hereby incorporated by reference in its entirety). 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 (He et al., "A Simplified System for Generating Recombinant Adenoviruses," *Proc Natl Acad Sci USA* 95(5):2509-14 (1998), which is hereby incorporated by reference in its entirety). 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 $MgCl_2$. The purified adenoviruses were stored in 1.0 M sucrose in a -80^{0}C freezer until use and their titers (pfu) were determined by plaque assay before vaccinating animals.

Example 2 — Antibody **Response to H_c50 of BoNT/C after Intramuscular Vaccination**

[0103] Mice were allotted into five groups (8 mice/group). They were injected i.m. into the hind-leg quadriceps with different doses of Ad/opt-BoNT/C-Hc50 vector prepared in Example 1 (10^5, 10^6, or 2 x 10^7 pfu/mouse), Ad/Null (2 x 10^7 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.

[0104] Antibody responses in animal sera were measured by quantitative ELISA. Figures IA-C show BoNT/C-Hc50-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 IgGl 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 IgGl (Figure IB) and IgG2a (Figure 1C) after vaccination suggested that both Th2 and ThI immune responses were elicited. More specifically, the IgG antibody response could be characterized as a predominant Th2 response (values of IgG2a/IgGl < 1.0). In addition, serum antibody responses were clearly vaccine-dose dependent.

Example 3 — ***In vitro*** **Neutralization of Botulinum Neurotoxin**

[0105] 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 x 10^7 pfu of Ad/opt-BoNT/C-Hc50 vector or with Ad/Null (both obtained from inoculated mice of Example 2). The sera were initially diluted 1:4, and then diluted in twofold series (1:4 to 1:1052) in DPBS (Dulbecco's PBS). 400 x 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 x MLD_{5O} of BoNT/C before neutralization) per mouse, 4 mice were tested for each dilution.

[0106] 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 x MLD50 neurotoxin (Byrne et al., "Purification, Potency, and Efficacy of the Botulinum Neurotoxin Type A Binding Domain from *Pichia pastoris* as a Recombinant Vaccine Candidate." *Infect Immun* 66(10):4817-22 (1998); Nowakowski et al., "Potent Neutralization of Botulinum Neurotoxin by Recombinant Oligoclonal Antibody," *Proc NatlAcadSci USA* 99:1 1346-1 1350 (2002), each of which is hereby incorporated by reference in its entirety).

[0107] As shown in Figure 2A, up to 128-fold diluted sera from animals 6 weeks after a single injection of 2 x 10^7 pfu of Ad/opt-BoNT/C-Hc50 completely neutralized 100 x MLD_{50} 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 (Figure 2B). These data indicate that parental inoculation with the adenoviral vector could elicit functional antibody responses that neutralized active botulinum neurotoxin.

Example 4 — Protective Immunity Elicited by the Adenovirus-vectored Vaccine

[0108] To explore whether the Hc50-based adenovirus-vectored vaccine protect against botulinum neurotoxin intoxication, the vaccinated mice (from Example 2) were intraperitoneally challenged (i.p.) with 100 50% mouse lethal dose (MLD_{50}) units of active BoNT/C 7 weeks after injection (i.e., one week after last bleeding). The results from the challenge experiments showed that vaccination with 10^5 pfu of Ad/opt-BoNT/C-Hc50 could protect 12.5 % of the animals against the toxin challenge (Figure 3). However, the protection rates rose to 75% and 100% when the vaccine dose was increased to 10^6 and 2 x 10^7 pfu, respectively. This indicates that vaccine dose-dependent protective immunity was achieved after vaccination.

[0109] A separate experiment was also performed to determine whether a single dose of Ad/opt-BoNT/C-Hc50 could provide long-term immunity against botulinum neurotoxin. Mice were allotted into three experimental groups (8 mice/group) and three control groups (4 mice/group). Animals were intramuscularly (i.m.) vaccinated with Ad/opt-BoNT/C-Hc50 vector (2 x 10^7 pfu/mouse) in experimental groups and with Ad/Null (2 x 10^7 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 x MLD_{50} BoNT/C in weeks 11, 19, and 27.

[0110] As shown in Figure 4, BoNT/C-Hc50-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 BoNT/C neurotoxin in weeks 11, 19, and 27. As indicated in Figures 5A-C, a single dose of Ad/opt-BoNT/C-Hc50 administered i.m. completely protected animals against botulism at all time points examined. This indicates that long-lasting memory immunity against botulinum neurotoxin was elicited after a single dose of the adenovirus-vectored vaccine.

Example 5 — Influence of Pre-existing Anti-adenovirus Immunity **on the** Efficacy **of Vaccination**

[0111] An investigation was also made to assess whether host pre-existing immunity to the adenoviral vector could limit the efficacy of the vaccination with this adenovirus-based vaccine.

[0112] Twelve 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 x 10^7 pfu/mouse) (WT Ad5) (ATCC, VA) in week 0 and bled in week 4. They were subsequently inoculated with Ad/opt-BoNT/C-Hc50 vector (2 x 10^7 pfu/mouse) in the experimental group or with Ad/Null (2 x 10^7 pfu/mouse) in the control group in week 4, and challenged i.p. with 100 χ MLD50 BoNTVC in week 11.

[0113] Serum-neutralizing antibody titers against WT Ad5 were assessed by a 96-well neutralization antibody assay described above. The results showed all animals had

anti-adenovirus neutralizing antibody responses, with average viral neutralization titers ranging from 96 to 112 (Figure 6A) 4 weeks after inoculation of WT Ad5. The animals with pre-existing immunity to adenovirus were subsequently inoculated i.m. with the single dose Ad/opt-BoNT/C-Hc50 or with control vector Ad/Null in week 4, and challenged in week 11 with BoNT/C neurotoxin. The results in Figure 6B show that all animals inoculated with Ad/opt-BoNT/C-Hc50 survived the toxin challenge and none of the control mice were protected. These data indicate that pre-existing immunity to adenovirus in the host did not affect the protective efficacy of the vaccination with this adenovirus-based botulism vaccine.

Example 6 — Serum Antibody **Responses Against BoNT7C-H_c50 Following Intranasal Vaccination**

[0114] To evaluate an optimal intranasal dose, 40 mice were allotted into 4 experimental groups and one control group, 8 mice per group. The animals were vaccinated by intranasal inhalation with Ad/opt-BoNT/C-Hc50 adenoviral vector at doses of $1x10^4$, $1x10^5$, $1x10^6$, and $2 x 10^7$ pfu/mouse, respectively, in the experimental groups, and Ad/Null at a dose of $2 x 10^7$ pfu/mouse in the control group. Sera samples were obtained at weeks 0, 2, 4, 6 and stored at -20 ^{0}C until further assayed. The experiment mice were i.p. challenged with botulinum neurotoxin one week after the last bleeding as described above.

[0115] The immune response in sera after a single i.n. vaccination with varying doses adenoviral vector Ad/opt-BoNT/C-Hc50 is shown in Figures 7A-C. The pre-immune sera (week 0) and sera from mice vaccinated with negative control vector (Ad/Null, adenovirus vector without transgene) were negative. IgG, IgGl and IgG2a responses to BoNT/C-Hc50 were detectable at week 2 for all three doses evaluated. Even at the lowest dose of Ad/opt-BoNT/C-H_c50 ($1 x 10^5$ pfu) serum, IgG levels were significantly higher than those of control mice receiving Ad/null ($P < 0.01$). The time-course of the response in serum for IgG, IgGl, and IgG2a to BoNT/C-Hc50 in the vaccinated mice is also shown in Figures IA-C. Overall, the dose ranging study showed that the serum IgG concentration in mice receiving $2 x 10^7$ pfu Ad/opt-BoNT/C-Hc50 was the highest (Figure 7A).

[0116] The mice receiving 1 x 10^5, 1 x 10^6 or 2 x 10^7 pfu/mouse of Ad/opt-BoNT/C-H_c50 achieved sera anti-BoNT/C-H_c50 IgG concentrations of 6.83 ± 2.61, 24.14 ± 7.32, 28.86 ± 6.81 μg/ml respectively at week 2, 9.61 ± 4.87, 50.16 ±19.1 1, 68.49 ± 5.58 μg/ml at week 4, 22.59 ± 6.67, 67.20 ± 24.83, 104.98 ± 9.63 μg/ml at week 6. Serum IgG antibodies against BoNT/C-Hc50 continued to rise with time from 2 to 6 weeks after vaccination (Figure 7A).

[0117] The IgGl responses to BoNT/C-Hc50 in the mice 6 weeks after vaccination with Ad/opt-BoNT/C-Hc50 accounted for about three-fourths of the total IgG. IgGl concentrations against BoNT/C-Hc50 in serum of vaccinated mice were significantly increased by week 2 compared with those in week 0 (Figure 7B). IgG2a antibody also was produced after vaccination although of lower magnitude than IgGl (Figure 7C).

Example 7 — Persistence of Antibodies after Mucosal Vaccination

[0118] In these experiments, 48 mice were allotted into 3 test groups and 3 control groups, 8 mice per group. The animals were i.n. vaccinated at week 0 with Ad/opt-BoNT/C-Hc50 vector at doses of 2 x 10^7 pfu/mouse in test groups, and Ad/Null at the same dosage in the control groups. One test group and one control group mice were challenged with BoNT/C at weeks 11, 19, and 27, respectively. The serum samples were obtained at week 0 and one week before challenge.

[0119] Antibody levels for IgG, IgGl, and IgG2a were measured at 11, 19, and 27 weeks post vaccination (Figure 8). The results demonstrate that antibody levels persisted at levels similar to post week 6 following vaccination, and they did not significantly decline between week 11 and 27. The same IgGl > IgG2a predominance as observed at post week 6 was observed throughout the experiment. These results for i.n administration are consistent with the results observed in Example 4 for i.p. administration (Figure 4)

Example 8 — Antibody **Responses against BoNTVC-Hc50 in Mucosal Secretions**

[0120] To evaluate the mucosal immune response, specific IgG and IgA antibody concentrations were measured in saliva, nasal wash, and vaginal wash following i.n.

vaccination with Ad/opt-BoNT/C-HcSO. A single i.n. vaccination with 2 x 10^7 pfu/mouse of Ad/opt-BoNT/C-Hc50 resulted in significant IgG and IgA antibody responses (Figures 9A-D). Two weeks post-vaccination, high local antibody responses were measurable, while no specific antibodies were detectable in the samples from control mice. All the examined antibody levels in the saliva, nasal wash and vaginal wash samples from vaccinated mice at week 4 were significantly higher than those at week 2. The BoNT/C-Hc50-specific IgG, IgGl, IgG2a antibody concentrations in mucosal samples were lower than those in sera (P <0.01), and the ratio of IgG2a/IgGl was reversed compared to that in serum (P <0.01). Mucosal anti- BoNT/C-H_c50 IgA in saliva reached 160.4 ± 50 ng/ml at week 2 and 393 ± 132 ng/ml at week 4 (Figure 9D), while sera anti-BoNT/C-H_c50 IgA was not detectable.

Example 9 — Neutralizing Capacity **of Anti-sera to Botulinum Neurotoxin**

[0121] Neutralizing antibody titers to BoNT/C were measured by the ability of anti-sera from mice i.n. vaccinated with Ad/opt-BoNT/C-Hc50 to neutralize the neurotoxin *in vitro.* The neutralization capacity of was determined using a mouse bioassay as described in Example 3 above. Due to the limited amount of serum available, the sera from 8 vaccinated mice were pooled.

[0122] The results of the assay are shown in Figures 10A-B. The neutralization titer from the mice receiving single i.n. doses of 2 x 10^7 pfu/mouse of Ad/opt-BoNT/C-Hc50 was 6.4 IU/ml 6 weeks after vaccination (Figure 10b). The antiserum from these mice, diluted by 64-fold or less, completely neutralized 100 x MLD50 of BoNT/C resulting in a 100% survival (Figure 10A). Serum from control mice did not neutralize the neurotoxin.

Example 10 — Protection against Active BoNT/C in Vaccinated Mice

[0123] After vaccination intranasally with a single dose of Ad vector, mice were challenged intraperitoneally (i.p.) with 100 x MLD_{50} of active BoNT/C. The results are summarized in Figures 1IA-D. Seven weeks after a single vaccination none of the mice that received control vector Ad/Null survived the toxin challenge, whereas all mice (8/8, or 100%) that received 2 x 10^7 pfu of Ad/opt-BoNT/C-H_c50 and 92% mice (11/12) that

received 1 x 10^6 pfu/mouse of Ad/opt-BoNT/C-H_c50 survived 100 x MLD_{50} challenge with no botulism symptoms (Figure 1IA). Fifty-percent (4 of 8 mice) at the lowest vaccine dose studied (1 x 10^5 pfu of Ad/opt-BoNT/C-H_c50) died (Figure 1IA), and one of the four surviving mice showed botulism symptoms. As shown in Figures 1IB-D, 11, 19 and 27 weeks after immunization, mice given 2 x 10^7 pfu of Ad/opt-BoNT/C-Hc50 also were completely protected from 100 x MLD50 of BoNT/C toxin.

[0124] To further assess the vaccine potency for protection, higher doses of active BoNT/C up 10^5 x MLD_{50} were also used in toxin challenge. Figure 12 shows that animals i.n. vaccinated with 2 x 10^7 pfu of Ad/opt-BoNT/C-Hc50 could be completely protected against challenge with 10^4 χ MLD50 of BoNT/C—a 100-fold increase in dosage—four weeks after vaccination. However, the protection rate decreased to 14% (1 of 7 mice) when the BoNT/C toxin challenge dose rose to 10^5 χ MLD_{50}. This shows the protective immunity, though significant, is also toxin challenge dose dependent.

Example 11 — Effect of Preexisting Anti-Ad5 Immunity **on Vaccination**

[0125] As in Example 5 above, an assessment was made as to the effect of preexisting anti-human Ad5 neutralization antibody on the efficacy of i.n. vaccination with Ad/opt-BoNT/C-Hc50. Twelve mice were allotted into one experimental group (8 mice) and one control group (4 mice). All animals were i.n. inoculated with WT Ad5 at a dose of 2 x 10^7 pfu/mouse, and then vaccinated by nasal inhalation at week 4 with Ad/opt-BoNT/C-Hc50 vector at a dose of 2 x 10^7 pfu/mouse in the experimental group and Ad/Null at the same dose in the control group. The serum samples were obtained at weeks 0, 4, prior to inoculation with WT Ad5 and i.n. vaccination with Ad/opt-BoNT/C-Hc50, respectively. The vaccinated animals were subsequently challenged i.p. with 100χ MLD_{50} of BoNT/C at week 11.

[0126] Significant serum anti-Ad5 neutralizing antibody titers were produced (Figure 13). The results show that all the vaccinated animals were fully protected against 100 x MLD_{50} BoNT/C challenge, whereas none of the control mice survived the toxin challenge (Figure 14). These data indicate that the Ad/opt-BoNT/C-Hc50 vector provides

protection against BoNT/C neurotoxin despite pre-existing immunity to adenovirus in the host.

Discussion of Examples 1-11

[0127] The neurotoxins produced by *C. botulinum* are among the most potent poisons known and there is a need to prepare for their use in a bioterrorism attack (Villar et al., "Botulism: The Many Faces of Botulinum Toxin and Its Potential for Bioterrorism," *Infect Dis Clin North Am* 20:3 13-327 ix, (2006); Atlas RM., "Bioterriorism: From Threat to Reality," *Annu Rev Microbiol* 56: 167-185 (2002), each of which is hereby incorporated by reference in its entirety). Botulinum neurotoxins can be lethal by ingestion of minute amounts in food and/or by inhalation. The latter delivery mode is the strongest bioterrorism-related threat (Arnon et al., "Botulinum Toxin as a Biological Weapon," in Henderson, eds., *Bioterrorism : Guidelinesfor Medical and Public Health Management,* Chicago, IL: AMA Press, pp. 141-165 (2002); Caya et al., *"Clostridium botulinum* and the Clinical Laboratorian: A Detailed Review of Botulism, Including Biological Warfare Ramifications of Botulinum Toxin," *Arch Pathol Lab Med* 128:653-662 (2004), each of which is hereby incorporated by reference in its entirety). The mucosal immune system is the first line of defense against botulism. However the current injection-type botulism toxoid vaccine only provides protective immunity in the systemic compartment. Clearly, the development of a safe and effective mucosal vaccine should be a high priority against bioterrorism-related botulism (Fujihashi et al., "Mucosal Vaccine Development for Botulinum Intoxication," *Expert Rev Vaccines* 6:35-45 (2007); which is hereby incorporated by reference in its entirety).

[0128] Protection against botulism neurotoxin is expected to be antibody-mediated and antibody levels have been correlated with protection (Byrne et al., "Fermentation, Purification, and Efficacy of a recombinant Vaccine Candidate against Botulinum Neurotoxin Type F from *Pichia pastoris," Protein Expr Purif* 18:327-337 (2000); Holley et al., "Cloning, Expression and Evaluation of a Recombinant Sub-Unit Vaccine Against *Clostridium botulinum* Type F Toxin," *Vaccine* 19:288-297 (2000), each of which is hereby incorporated by reference in its entirety).

[0129] The genetic vaccination strategy was previously attempted in botulism vaccine development. Clayton and Middlebrook constructed a plasmid DNA encoding the nontoxic Hc50 region of BoNT/A and showed it to partially protect against toxin challenge after up to 4 booster injections (Clayton et al., "Vaccination of Mice with DNA Encoding a Large Fragment of Botulinum Neurotoxin Serotype A," *Vaccine* 18(17): 1855-62 (2000), which is hereby incorporated by reference in its entirety). Subsequently, in another study using constructed plasmid DNA expressing BoNT/F H_c under the control of human ubiquitin gene (UbC) promoter, it was found that two i.m. injections afforded 90% protection against BoNT/F challenge (Jathoul et al., "Efficacy of DNA Vaccines Expressing the Type F Botulinum Toxin Hc Fragment Using Different Promoters," *Vaccine* 22(29-30):3942-6 (2004), which is hereby incorporated by reference in its entirety). In addition, Lee and coworkers introduced the Hc50 of BoNT/A or BoNT/C into the Venezuelan equine encephalitis (VEE) virus replicon vector, which not only yielded high levels of Hc fragments, as judged by immunofluorescence and immunoblotting analysis, but also protected mice against BoNT/A or BoNT/C challenge after two or three injections with 10^7 infectious units (i.u.) of VEE (Pushko et al., "Replicon-Helper Systems From Attenuated Venezuelan Equine Encephalitis Virus: Expression of Heterologous Genes In Vitro and Immunization Against Heterologous Pathogens *in vivo*," *Virology* 239(2):389-401 (1997); Lee et al., "Candidate Vaccine Against Botulinum Neurotoxin Serotype A Derived From a Venezuelan Equine Encephalitis Virus Vector System," *Infect Immun* 69(9):5709-15 (2001); Lee et al., "Multiagent Vaccines Vectored by Venezuelan Equine Encephalitis Virus Replicon Elicits Immune Responses to Marburg Virus and Protection Against Anthrax and Botulinum Neurotoxin in Mice," *Vaccine* 24(47-48):6886-92 (2006), each of which is hereby incorporated by reference in its entirety). However, a single dose of genetic vaccine was not fully protective against botulism in these studies.

[0130] In contrast to these other studies, the Examples presented above demonstrate that it is possible to develop a highly efficient genetic vaccine against botulism using an adenoviral vector encoding the Hc50 fragment of BoNT/C. In the recombinant adenovirus constructed in Example 1, the DNA sequence encoding the Hc50

fragment antigen was codon-optimized with human codon preference. This resulted in high-level expression of Hc50 in mice.

[0131] Because of the presence of a signal peptide from human tissue plasminogen activator (PLAT), it is believed that the Hc50 fragment antigen was secreted efficiently from adenoviral vector transformed host cells (Ertl et al., "Technical Issues in Construction of Nucleic Acid Vaccines," *Methods* 31(3): 199-206 (2003); Hermanson et al., "A Cationic Lipid-Formulated Plasmid DNA Vaccine Confers Sustained Antibody-Mediated Protection Against Aerosolized Anthrax Spores," *Proc Natl Acad Sci USA* 101(37): 13601-6 (2004), each of which is hereby incorporated by reference in its entirety). Consequently, the secretory Hc50 was likely presented to antigen-presenting cells (APCs) more efficiently after vaccination.

[0132] Examples 1-5 demonstrate that a single i.m. dose of the Ad/opt-BoNT/C-Hc50 vector was capable of eliciting significant Th2 and ThI immune responses against Hc50 fragment of BoNT/C (Figures IA-C) and the serum antigen-specific antibodies were capable of neutralizing active BoNT/C (Figures 2A-B). The protective antibodies against Hc50 of BoNT/C were sustained for long-term, at least up to 27 weeks (Figure 4) and likely for much longer. Host immune responses and protective immunity appeared to be vaccine dose-dependent. Most importantly, a single dose of 2 x 10^7pfu of Ad/opt-BoNT/C-Hc50 was sufficient to provide long-term protective immunity (Figures 5A-C). This study is the first to demonstrate that a single genetic vaccination is able to provide long-lasting protection against botulism.

[0133] Examples 6-1 1 extend these initial results, demonstrating that a single i.n. dose of the Ad/opt-BoNT/C-Hc50 vector was also capable of eliciting a significant immune response against the Hc50 fragment of BoNT/C (Figures 7A-C) and the serum antigen-specific antibodies were capable of neutralizing active BoNT/C (Figures 1OA-B). The protective antibodies against Hc50 of BoNT/C were sustained for long-term, at least up to 27 weeks (Figure 8) and likely for much longer. Host immune responses and protective immunity appeared to be vaccine dose-dependent. Most importantly, a single i.n. dose of 2 x 10^7pfu of Ad/opt-BoNT/C-Hc50 was sufficient to provide long-term protective immunity even against high doses of neurotoxin (Figures 1IA-D, 12).

[0134] Because of the simple intranasal route of vaccination, this vaccine can be self-administered to protect the population in the event of terrorist attack with *C. botulism* or neurotoxins. In addition to the ease of administration and rapid onset of protection demonstrated in the Examples, the vaccine can be produced inexpensively, in high quantity, and in a short time frame. The Hc50 fragment of botulinum neurotoxin type C was selected, because the Hc50 subunits of BoNTs are non-toxic and antigenic and capable of eliciting immunity responses against botulism (Byrne et al., "Development of Vaccines for Prevention of Botulism," *Biochimie* 82:955-966 (2000); Webb et al., "Protection with Recombinant *Clostridium botulinum* C1 and D Binding Domain Subunit (Hc) Vaccines Against C and D Neurotoxins," *Vaccine* 25:4273-4282 (2007); Byrne et al., "Purification, Potency, and Efficacy of the Botulinum Neurotoxin Type A Binding Domain from *Pichia pastoris* as a Recombinant Vaccine Candidate," *Infect Immun* 66:4817-4822 (1998); Atassi et al., "Structure, Activity, and Immune (T and B cell) Recognition of Botulinum Neurotoxins," *Crit Rev Immunol* 19:219-260 (1999), each of which is hereby incorporated by reference in its entirety).

[0135] Adenoviruses invade their host naturally via the mucosa surface, notably in the respiratory or gastrointestinal tract (Lemiale et al., "Enhanced Mucosal Immunoglobulin A Response of intranasal Adenoviral Vector Human Immunodeficiency Virus Vaccine and Localization in the Central Nervous System," *J Virol* 77: 10078-10087 (2003), which is hereby incorporated by reference in its entirety). Adenoviral vector vaccines can be effectively delivered by intranasal mucosal route and can induce strong adaptive immune responses in mammalian hosts (Bangari et al., "Development of Nonhuman Adenoviruses as Vaccine Vectors," *Vaccine;* 24:849-862 (2006); Tatsis et al., "Adenoviruses as Vaccine Vectors," *MoI Ther* 10:616-629 (2004), each of which is hereby incorporated by reference in its entirety.)

[0136] Other studies have established that preexisting anti-adenovirus antibody may drastically reduce the take of adenovirus vectored vaccines (Bangari et al., "Development of Nonhuman Adenoviruses as Vaccine Vectors," *Vaccine;* 24:849-862 (2006); Casimiro et al., "Comparative Immunogenicity in Rhesus Monkeys of DNA Plasmid, Recombinant Vaccinia Virus, and Replication-Defective Adenovirus Vectors

Expressing a Human Immunodeficiency Virus Type 1 Gag Gene," *J Virol* 77:6305-63 13 (2003); Yang et al., "Cellular and Humoral Immune Responses to Viral Antigens Create Barriers to Lung-Directed Gene Therapy with Recombinant Adenoviruses," *J Virol* 69:2004-2015 (1995); Barouch et al., "Plasmid Chemokines and Colony-Stimulating Factors Enhance the Immunogenicity of DNA Priming-Viral Vector Boosting Human Immunodeficiency Virus Type 1 Vaccines," *J Virol* 77:8729-8735 (2003), each of which is hereby incorporated by reference in its entirety), but immunity to the vector has been overcome in some situations (Babiuk et al., "Adenoviruses as Vectors for Delivering Vaccines to Mucosal Surfaces," *J Biotechnol* 83:105-1 13 (2000); Papp et al., "The Effect of Pre-Existing Adenovirus-Specific Immunity on Immune Responses Induced by Recombinant Adenovirus Expressing Glycoprotein D of Bovine Herpesvirus Type 1," *Vaccine* 17:933-943 (1999); Fischer et al., "Vaccination of Puppies Born to immune Dams with a Canine Adenovirus-Based Vaccine Protects Against a Canine Distemper Virus Challenge," *Vaccine* 20:3485-3497 (2002), each of which is hereby incorporated by reference in its entirety. The data from Examples 5 and 11 demonstrate that even with pre-existing anti-adenovirus neutralizing antibody in the host, the protective efficacy of the vaccination was sustained (Figures 6A-B; 13-14). This will, of course, need to be further evaluated in human trials, because the ability for replication of human adenovirus is limited in murine cells (Duncan et al., "Infection of Mouse Liver by Human Adenovirus Type 5," *J Gen Virol* 40(l):45-61 (1978); which is hereby incorporated by reference in their entirety) and human immune response to adenovirus may differ from that of mouse.

[0137] To avoid the possibility of contamination of replication-competent adenovirus, in future human clinical trials the vector will be prepared using new suitable packaging cell lines such as the Per.C6 and UR cell lines that were developed recently (Fallaux et al., "New Helper Cells and Matched Early Region 1-Deleted Adenovirus Vectors Prevent Generation of Replication-Competent Adenoviruses," *Hum Gene Ther* 9(13): 1909-17 (1998); Schiedner et al., "Efficient Transformation of Primary Human Amniocytes by E1 Functions of Ad5: Generation of New Cell Lines for Adenoviral Vector Production," *Hum Gene Ther* 11(15):2105-16 (2000); Xu et al., "A New

Complementing Cell Line for Replication-Incompetent E1-Deleted Adenovirus Propagation," *Cytotechnology* 51:133-40 (2006), each of which is hereby incorporated by reference in its entirety).

[0138] In summary, the preceding Examples demonstrate for the first time that an adenovirus-based vector encoding a humanized Hc 50-kDa fragment of BoNT/C is capable of eliciting robust host immunity against botulism caused by BoNT/C after a single dose regardless of the mode of administration. Both intramuscular and intranasal administration elicited high serum antibody response to BoNTC/Hc50. The anti-BoNT/C protective immunity generated by these vaccinations was sustained for a prolonged time period. This strategy can also be applied for the development of a multivalent vaccine against all serotypes of botulinum neurotoxins.

Example 12 — Construction of Nucleic Acid Molecule Encoding Codon-optimized H_c50 of BoNT/A Chimeric Protein, and Insertion in Adeno-viral Vector

[0139] An adenoviral vector encoding human codon-optimized Hc50 of BoNT/A was synthesized using the same strategy described above for construction of Ad/opt-BoNT/C-H_c50. The codon-optimized H_c50 of BoNT/A nucleotide sequence (SEQ ID NO: 5) is shown in Table 2 above.

[0140] The adenoviral vector prepared above will be screened via intramuscular injection and intranasal instillation to mice for the generation of an immune response against BoNT/A. The antibody titer generated and the ability of the antisera to neutralize BoNT/A toxicity will be assessed as demonstrated in the preceding Examples. The generation of long term protection (longer than 6 months) will also be assessed.

[0141] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

WHAT IS CLAIMED:

1. An isolated DNA molecule comprising a first segment encoding a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin, wherein the first segment is codon-enhanced to improve expression of the isolated DNA molecule in a mammalian host.

2. The isolated DNA molecule according to claim 1, wherein the mammalian host is a human or a non-human primate.

3. The isolated DNA molecule according to claim 1, wherein the *Clostridium botulinum* neurotoxin is neurotoxin A, neurotoxin B, neurotoxin C, neurotoxin D, neurotoxin E, or neurotoxin F, or neurotoxin G.

4. The isolated DNA molecule according to claim 1, wherein the *Clostridium botulinum* neurotoxin is neurotoxin C.

5. The isolated DNA molecule according to claim 4, wherein the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin C comprises a C-terminal fragment that is about 50 kDa.

6. The isolated DNA molecule according to claim 4, wherein the nucleotide sequence of the first segment is SEQ ID NO: 2.

7. The isolated DNA molecule according to claim 1, wherein the *Clostridium botulinum* neurotoxin is neurotoxin A.

8. The isolated DNA molecule according to claim 7, wherein the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin A comprises a C-terminal fragment that is about 50 kDa.

9. The isolated DNA molecule according to claim 7, wherein the nucleotide sequence of the first segment is SEQ ID NO: 5.

10. The isolated DNA molecule according to claim 1, wherein the DNA molecule includes a second segment located 5' to the first segment, the second segment encoding a secretion signal peptide.

11. The isolated DNA molecule according to claim 10, wherein the secretion signal peptide comprises a secretion signal from human tissue plasminogen activator, human serum albumin, human IL-3, or human growth hormone.

12. An expression vector comprising the isolated DNA molecule according to any one of claims 1 to 11 operably coupled to one or more regulatory sequences that afford transcription of the isolated DNA molecule in the mammalian host.

13. The expression vector according to claim 12, wherein the one or more regulatory sequences comprises a promoter sequence located 5' to the isolated DNA molecule and a transcription termination sequence located 3' to the isolated DNA molecule.

14. The expression vector according to claim 13, wherein the promoter sequence is a constitutive promoter.

15. The expression vector according to claim 13, wherein the promoter sequence is a human cytomegalovirus immediate early promoter/enhancer.

16. The expression vector according to claim 13, wherein the transcription termination sequence encodes a simian virus 40 polyadenylation signal.

17. The expression vector according to claim 12, wherein the expression vector is a replication-defective adenoviral vector.

18. The expression vector according to claim 12, wherein the expression vector comprises two or more isolated DNA molecules encoding fragments of different *Clostridium botulinum* neurotoxins.

19. A host cell comprising the expression vector according to any one of claims 12 to 18.

20. The host cell according to claim 19, wherein the host cell is *in vitro.*

21. The host cell according to claim 19, wherein the host cell is *in vivo.*

22. The host cell according to claim 19, wherein the host cell is a mammalian cell.

23. The host cell according to claim 22, wherein the mammalian cell is a human cell.

24. A chimeric protein comprising a secretion signal peptide linked N-terminal of a fragment of a heavy chain region of a *Clostridium botulinum* neurotoxin.

25. The chimeric protein according to claim 24, wherein the secretion signal peptide comprises a secretion signal from human tissue plasminogen activator, human serum albumin, human IL-3, or human growth hormone.

26. The chimeric protein according to claim 24, wherein the *Clostridium botulinum* neurotoxin is neurotoxin A, neurotoxin B, neurotoxin C, neurotoxin D, neurotoxin E, or neurotoxin F, or neurotoxin G.

27. The chimeric protein according to claim 24, wherein the *Clostridium botulinum* neurotoxin is neurotoxin C.

28. The chimeric protein according to claim 27, wherein the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin C comprises a C-terminal fragment that is about 50 kDa.

29. The chimeric protein according to claim 28, wherein the C-terminal fragment of the heavy chain region of *Clostridium botulinum* neurotoxin C comprises the amino acid sequence of SEQ ID NO: 3.

30. The chimeric protein according to claim 24, wherein the *Clostridium botulinum* neurotoxin is neurotoxin A.

31. The chimeric protein according to claim 30, wherein the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin A comprises a C-terminal fragment that is about 50 kDa.

32. The chimeric protein according to claim 31, wherein the C-terminal fragment of the heavy chain region of *Clostridium botulinum* neurotoxin A comprises the amino acid sequence of SEQ ID NO: 6.

33. The chimeric protein according to claim 24, wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 7 and the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin comprises the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 6.

34. The chimeric protein according to claim 33, wherein the chimeric protein further comprises a linker sequence between the secretion signal peptide and the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin.

35. The chimeric protein according to claim 34, wherein the linker sequence is Ser-Ser.

36. A vaccine comprising a pharmaceutically acceptable carrier and either the DNA molecule according to any one of claims 1 to 11, an expression vector according to any one of claims 12 to 18, a chimeric protein according to any one of claims 24 to 35, or a combination thereof.

37. The vaccine according to claim 36 further comprising an adjuvant.

38. A method of imparting resistance against a *Clostridium botulinum* neurotoxin to a mammal comprising:

administering a vaccine according to claims 36 or 37 to a mammal under conditions effective to induce a protective immune response against a *Clostridium botulinum* neurotoxin.

39. The method according to claim 38, wherein said administering is carried out orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal instillation, by implantation, by intracavitary or intravesical instillation, intraarterially, intralesionally, transdermally, by application to mucous membranes.

40. The method according to claim 38, wherein the vaccine comprises the chimeric protein.

41. The method according to claim 38, wherein the vaccine comprises the expression vector.

42. The method according to claim 41, wherein the expression vector encodes the chimeric protein.

43. The method according to claim 40 or 42, wherein the chimeric protein comprises a secretion signal peptide comprising the amino acid sequence of SEQ ID NO: 7 and a fragment of the heavy chain region of *Clostridium botulinum* neurotoxin comprising the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 6.

44. The method according to claim 43, wherein the chimeric protein further comprises a linker sequence between the secretion signal peptide and the fragment of the heavy chain region of *Clostridium botulinum* neurotoxin C.

45. The method according to claim 44, wherein the linker sequence is Ser-Ser.

46. The method according to claim 38, wherein said administering is effective to induce immunity for more than 6 months.

47. The method according to claim 38 further comprising repeating said administering after a delay.

48. An isolated antibody raised against a chimeric protein according to any one of claims 24 to 35, or binding fragment thereof.

49. The isolated antibody according to claim 48, wherein the antibody is present in a polyclonal antiserum.

50. The isolated antibody according to claim 48, wherein the antibody is present in a monoclonal preparation.

51. The isolated antibody binding fragment according to claim 48, wherein the antibody fragment is selected from the group consisting of include Fab fragments, $F(ab)_2$ fragments, Fab' fragments, $F(ab')_2$ fragments, Fd fragments, Fd' fragments, Fv fragments, minibodies, and single-chain antibodies.

52. A hybridoma cell that expresses the monoclonal antibody of claim **50.**

53. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an antibody, or binding fragment thereof, according to any one of claims 48 to 51.

54. The pharmaceutical composition according to claim 53, wherein the composition comprises antibodies, or binding fragments thereof, that specifically bind and neutralize two or more botulism neurotoxins.

55. A method of treating a *Clostridium botulinum* infection comprising administering to a patient an antibody or antibody fragment thereof according to any one of claims 48 to 51, or a pharmaceutical composition according to claims 53 or 54, under conditions effective to neutralize a botulism neurotoxin, and thereby treat the *Clostridium botulinum* infection.

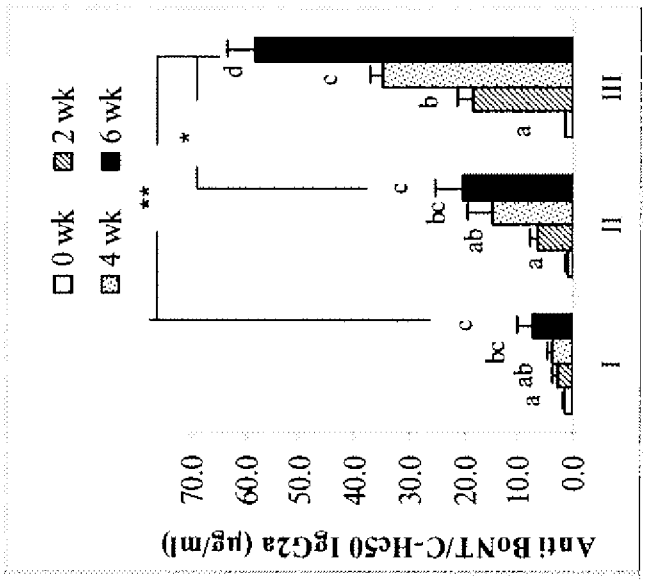


Figure 1C

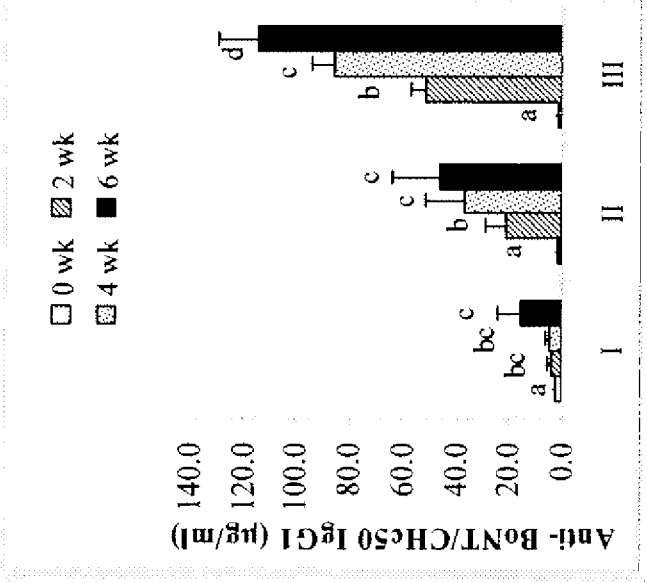


Figure 1B

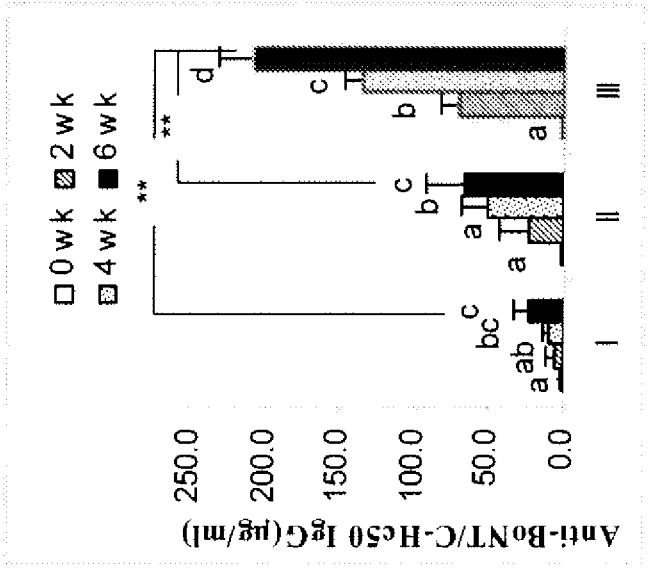


Figure 1A

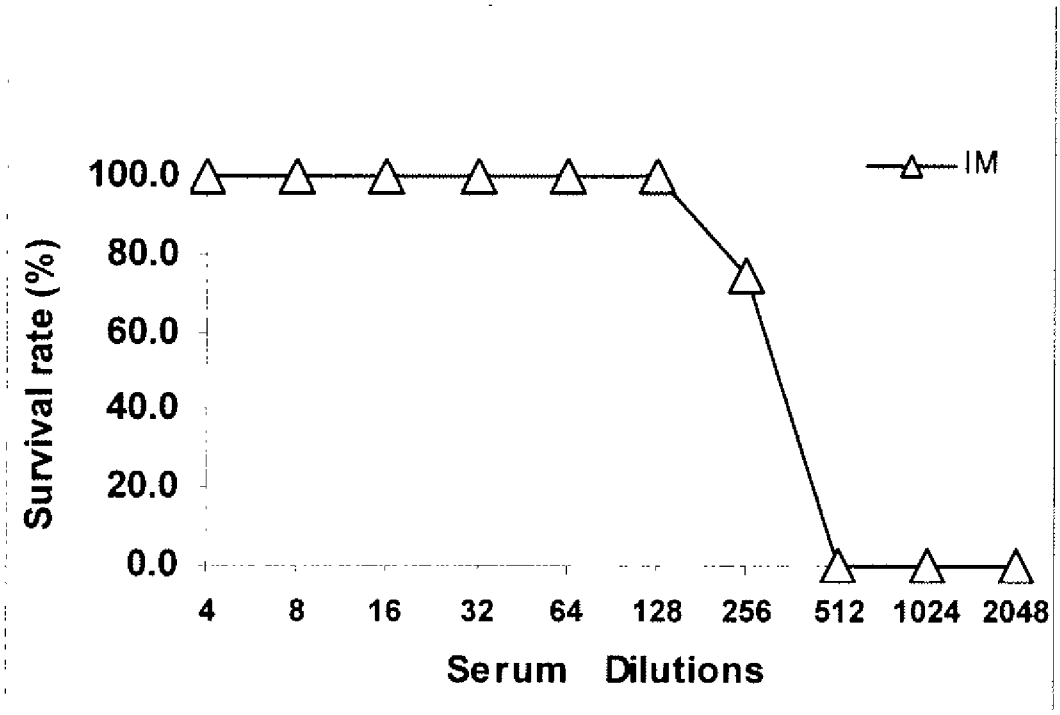


Figure 2A

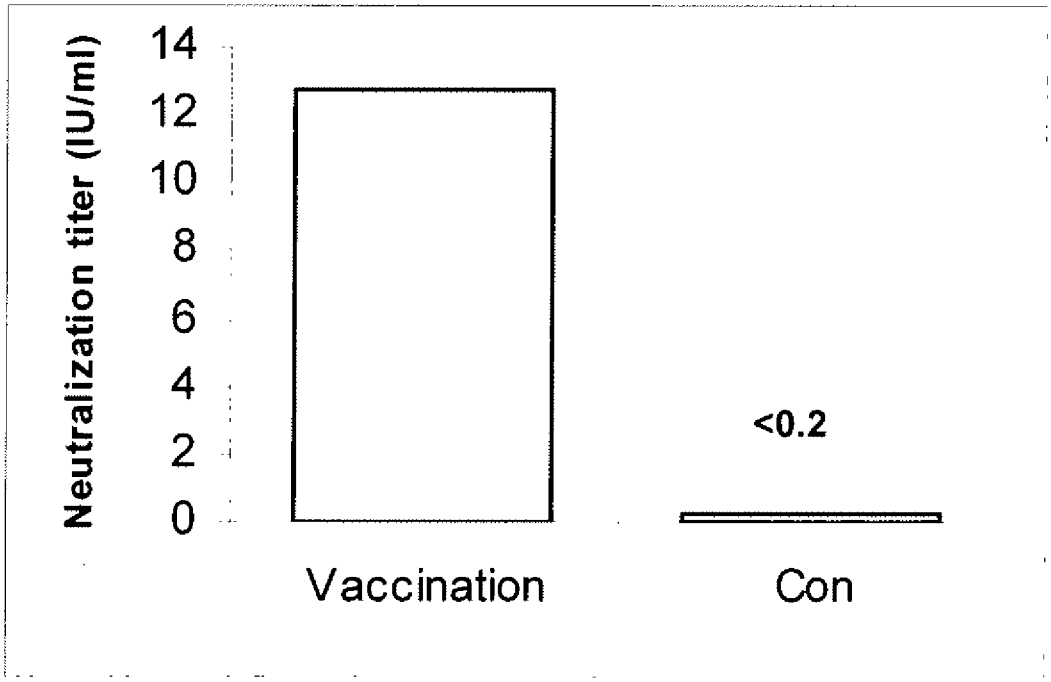


Figure 2B

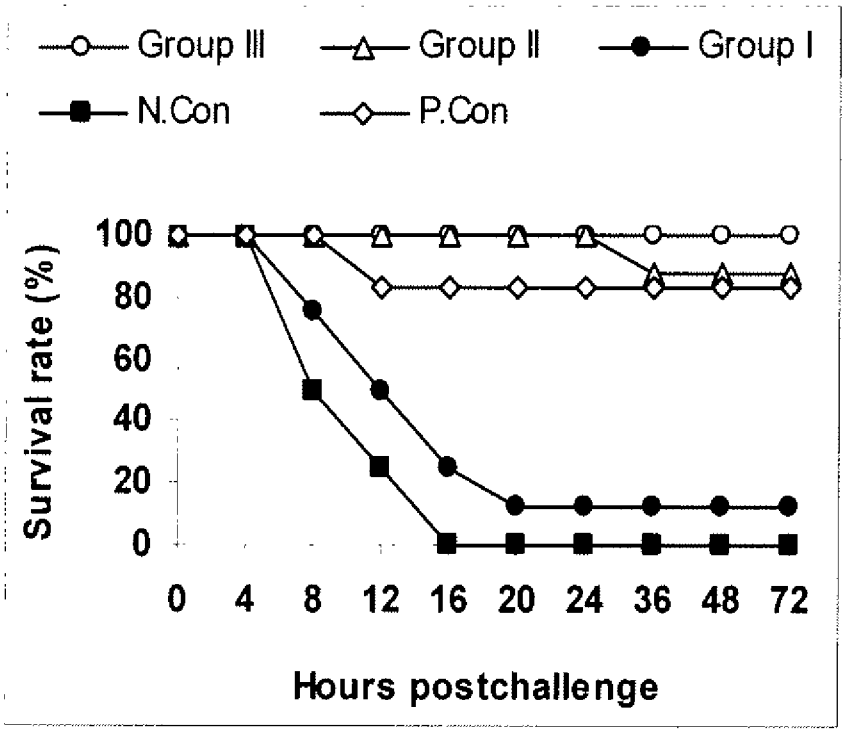


Figure 3

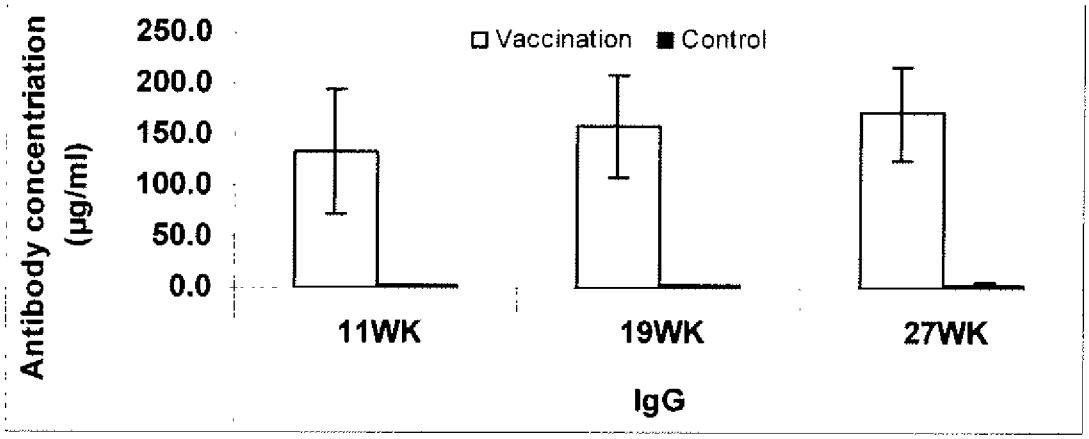


Figure 4

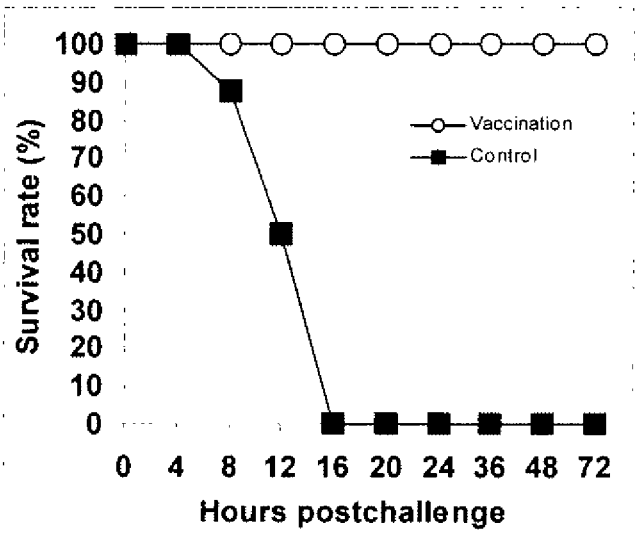


Figure 5A

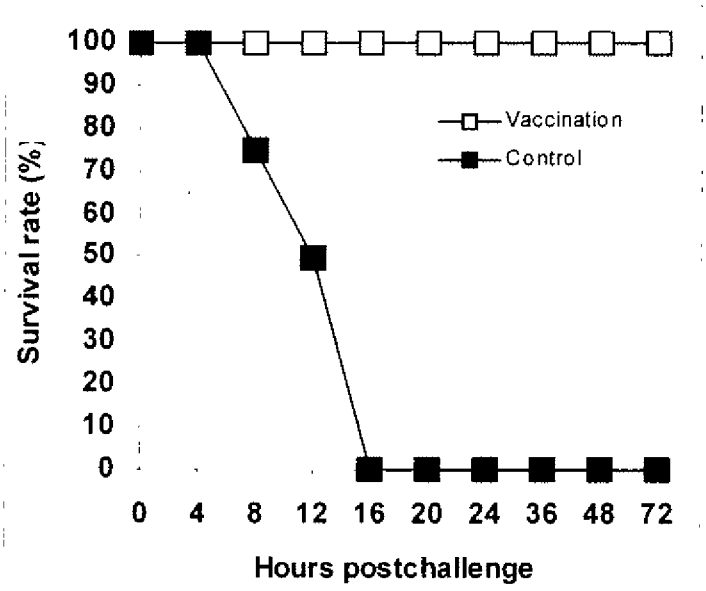


Figure 5B

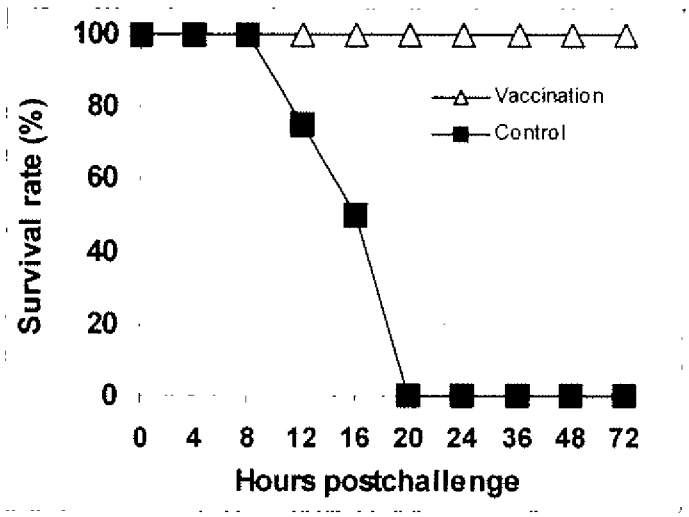


Figure 5C

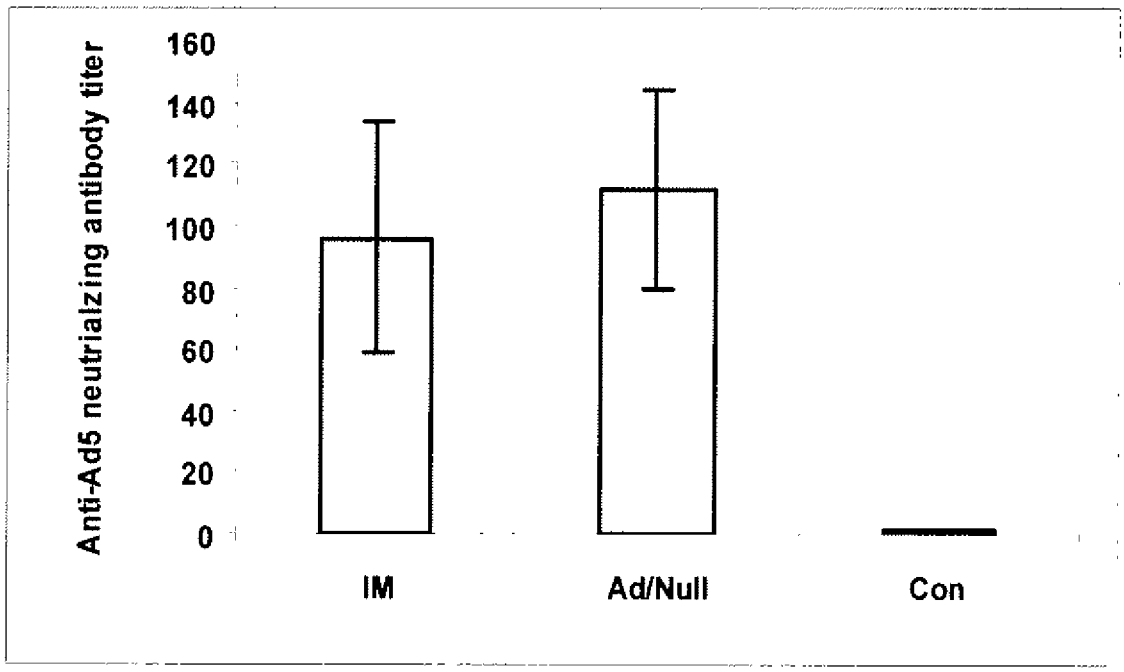


Figure 6A

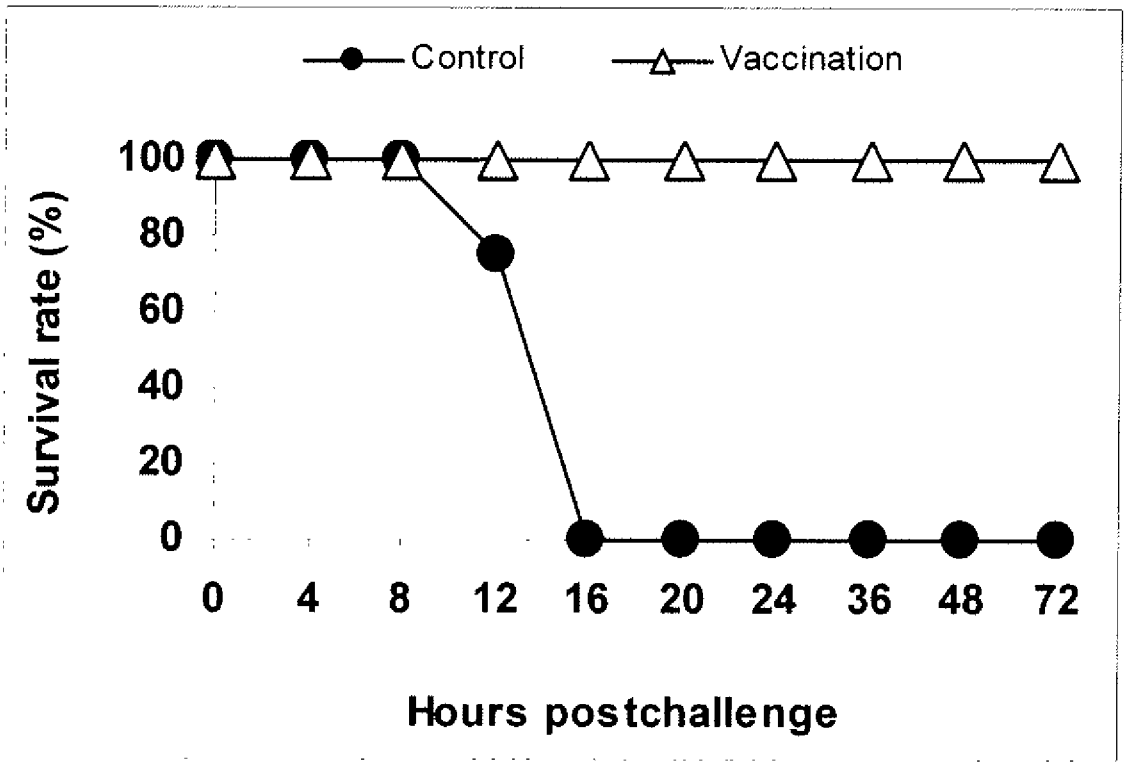


Figure 6B

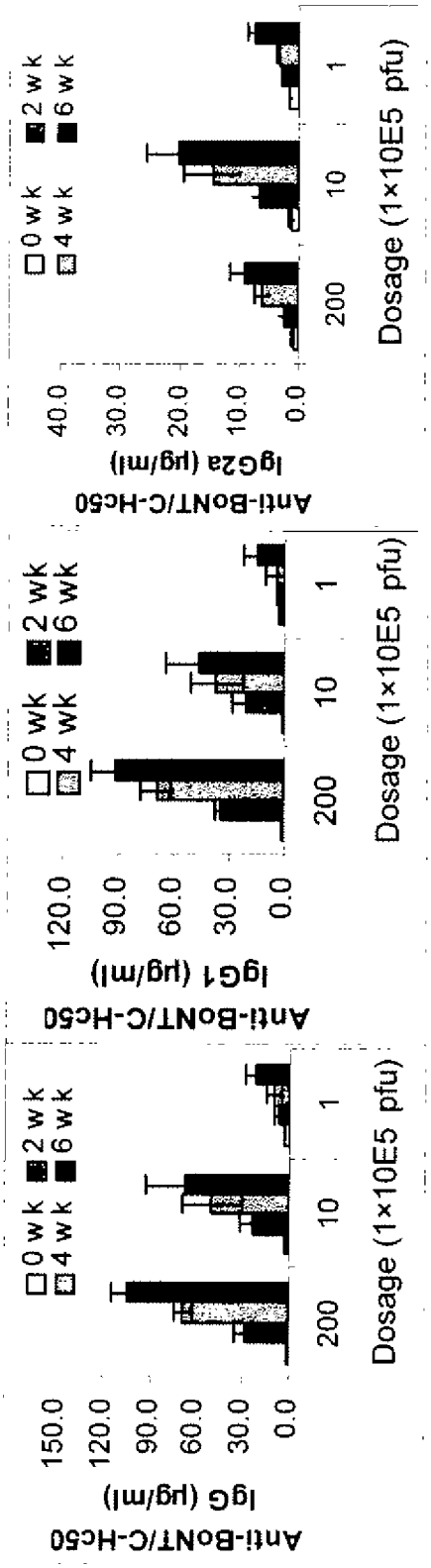


Figure 7A

Figure 7B

Figure 7C

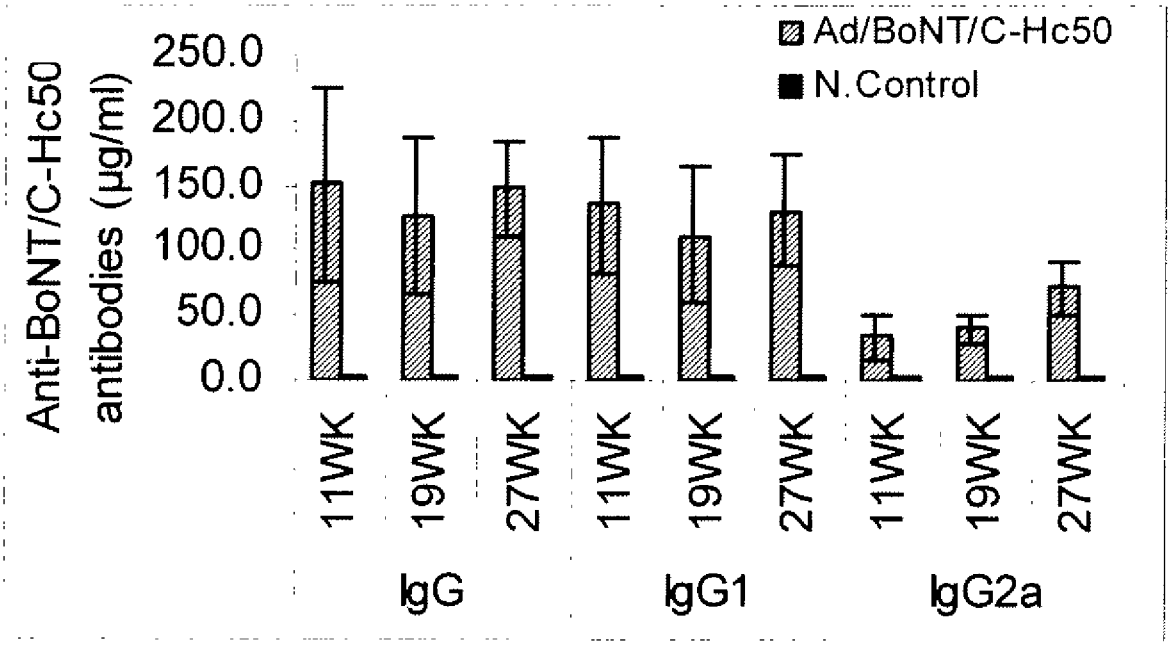
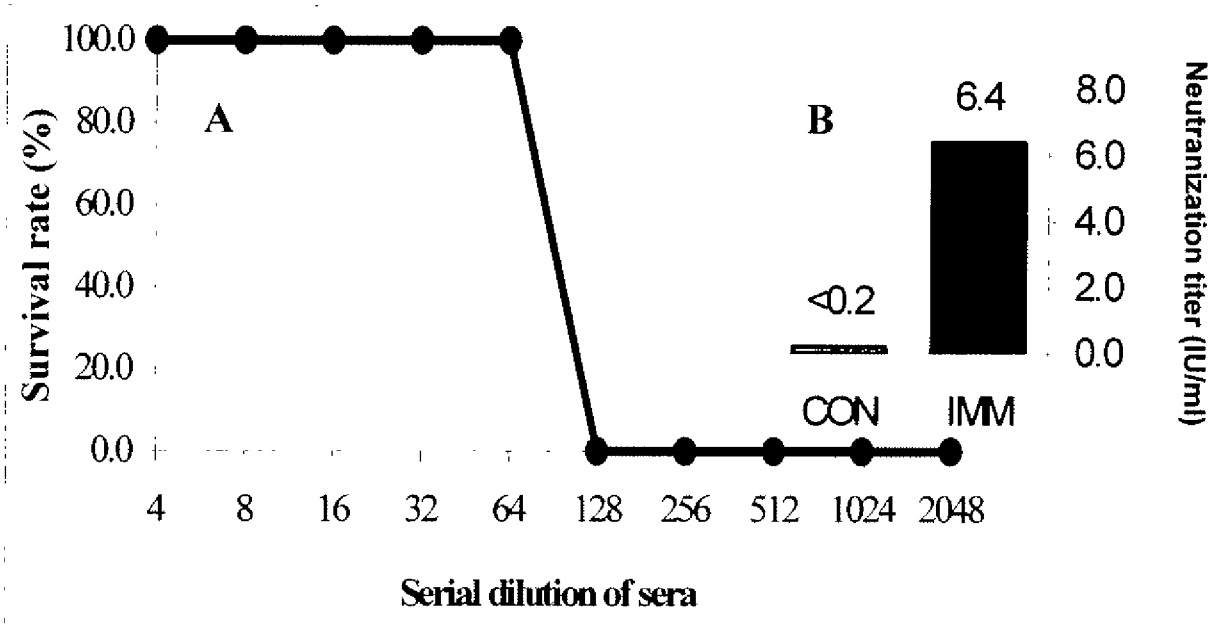


Figure 8



Figures 10A-B

Figure 9A

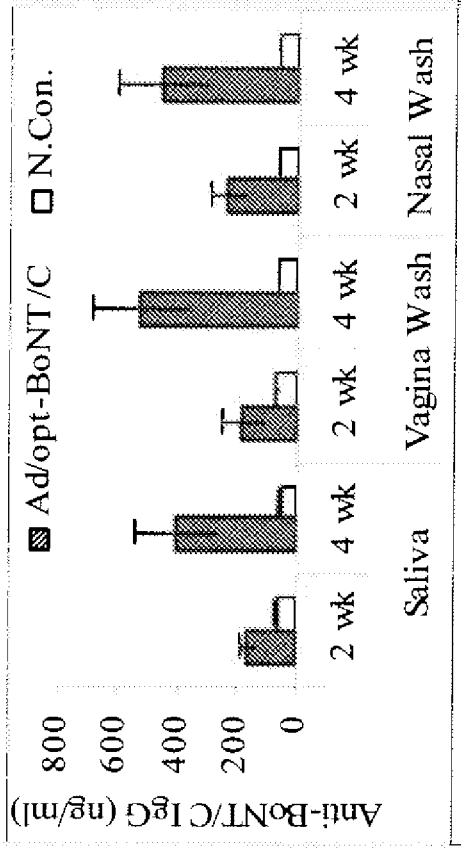


Figure 9B

Figure 9C

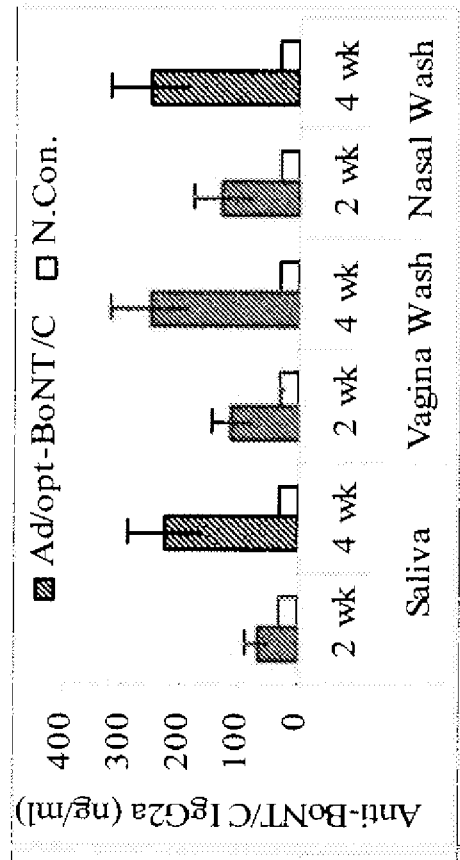


Figure 9D

Figure 11A

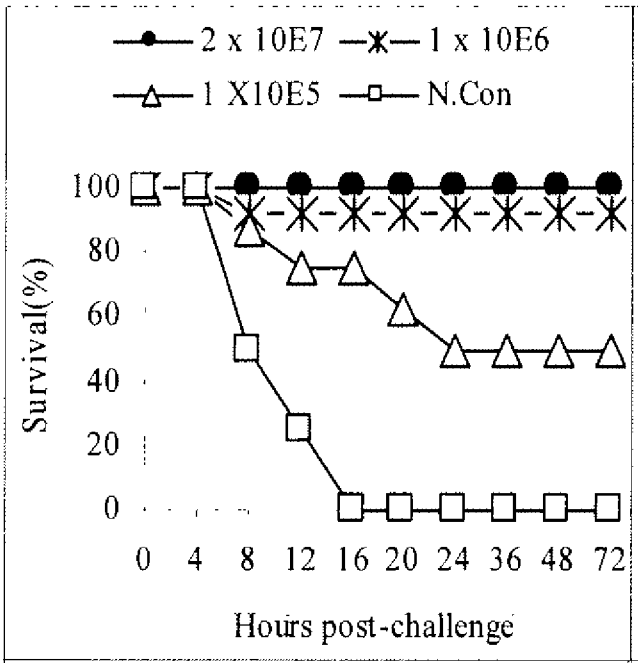
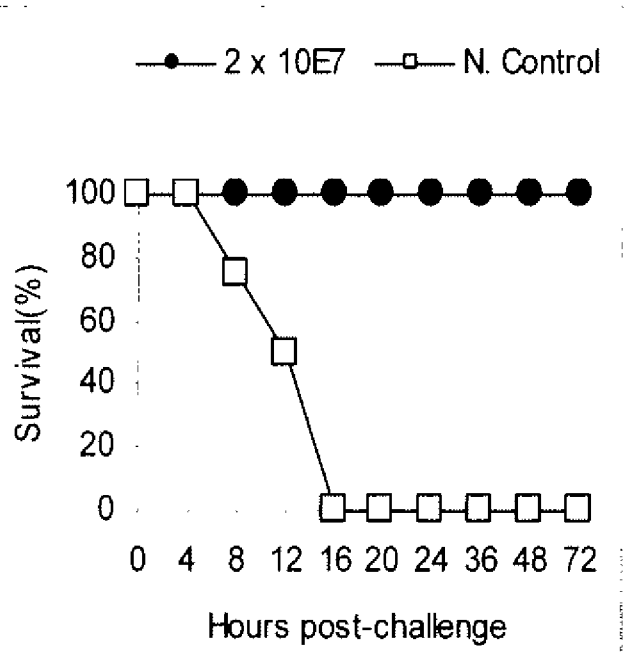


Figure 11C



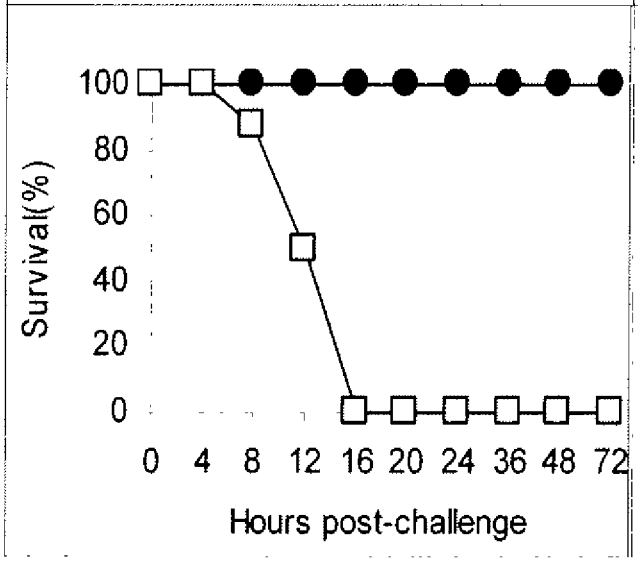


Figure 11B

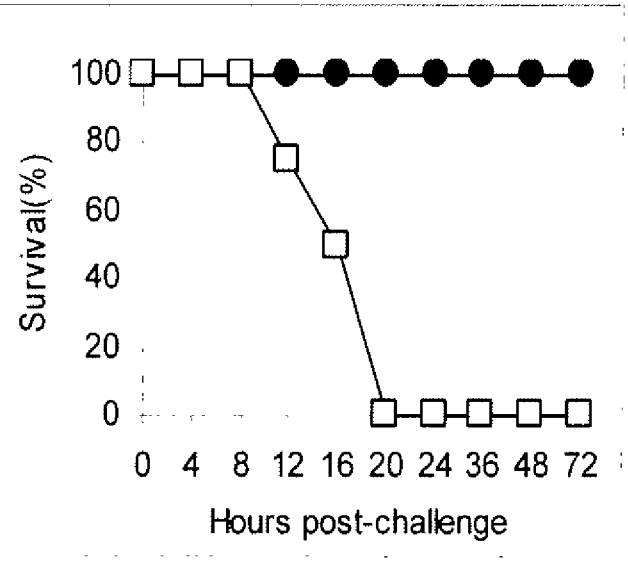


Figure 11D

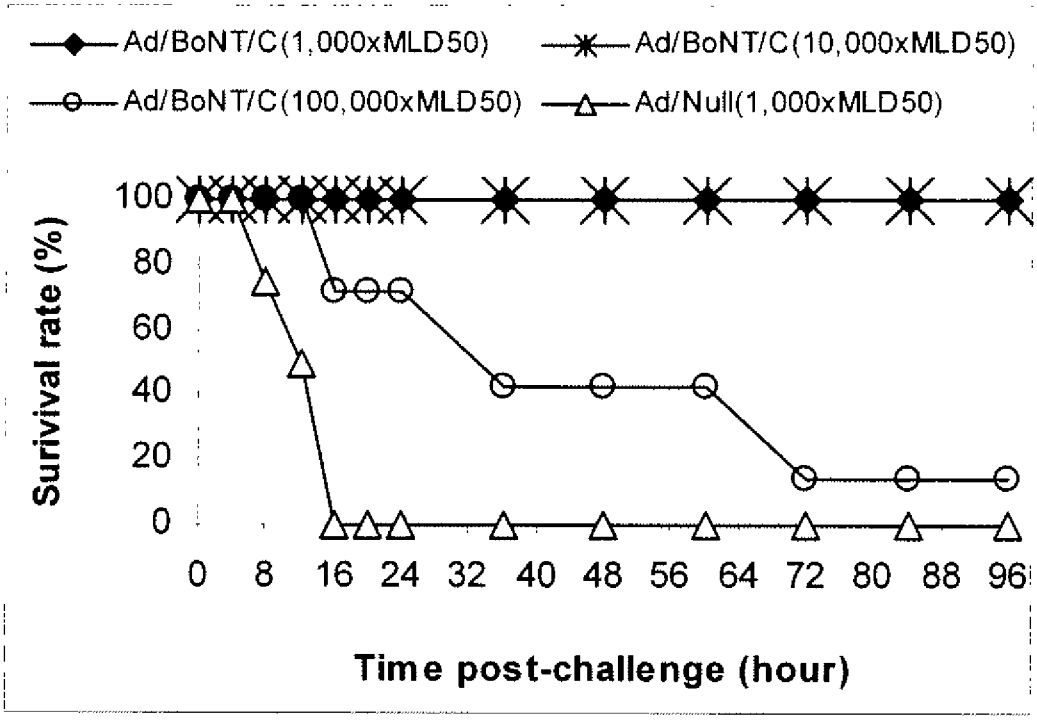


Figure 12

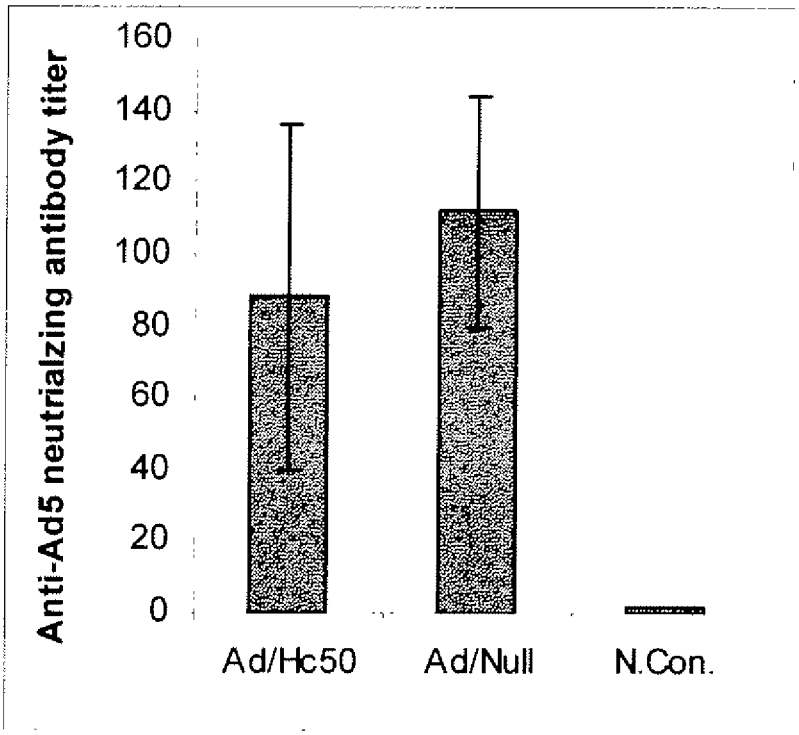


Figure 13

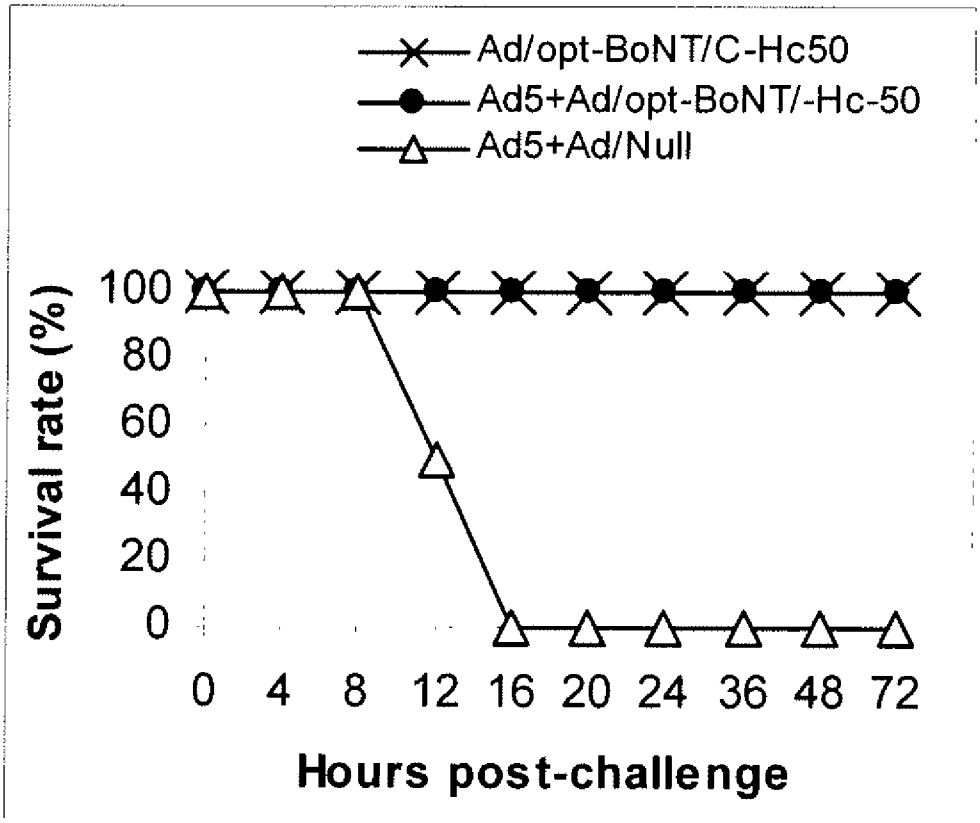

Ad/opt-BoNT/C-Hc50
Ad5+Ad/opt-BoNT/-Hc-50
Ad5+Ad/Null
Survival rate (%)
100
80
60
40
20
0
0 4 8 12 16 20 24 36 48 72
Hours post-challenge

Figure 14