(12) PATENT (11) Application No. AU 199954583 B2
(19) AUSTRALIAN PATENT OFFICE (10) Patent No. 759461

(54) Title
Botulinum neurotoxin vaccine

(51)[7] International Patent Classification(s)
C12N 015/31

(21) Application No: **199954583** (22) Application Date: **1999.07.09**

(87) WIPO No: **WO00/02524**

(30) Priority Data

(31) Number	(32) Date	(33) Country
60/092416	**1998.07.10**	**US**
60/133870	**1999.05.12**	**US**

(43) Publication Date : **2000.02.01**
(43) Publication Journal Date : **2000.04.13**
(44) Accepted Journal Date : **2003.04.17**

(71) Applicant(s)
U.S. Medical Research Institute of Infectious Diseases

(72) Inventor(s)
John S. Lee; Peter Pushko; Jonathan F Smith; Michael Parker; Mark T. Dertzbaugh; Leonard Smith

(74) Agent/Attorney
FISHER ADAMS KELLY,GPO Box 1413,BRISBANE QLD 4001

CORRECTED VERSION*

S4583/99

PCT pages 1/5-5/5, drawings, replaced by new pages 1/5-5/5; pages 1-16, sequence listing, replaced by new pages 1-10; due to late transmittal by the receiving Office

INTERNATIONAL ... ERATION TREATY (PCT)

(51) International Patent Classification 7 : ~~A61K~~ C12N 15/31

A2

(11) International Publication Number: WO 00/02524

(43) International Publication Date: 20 January 2000 (20.01.00)

(21) International Application Number: PCT/US99/15570

(22) International Filing Date: 9 July 1999 (09.07.99)

(30) Priority Data:

60/092,416	10 July 1998 (10.07.98)	US
60/133,870	12 May 1999 (12.05.99)	US

(71) Applicant *(for all designated States except US)*: U.S. MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES [US/US]; Department of the Army, 504 Scott Street, Frederick, MD 21702 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LEE, John, S. [US/US]; 1807 Elizabeth Court, Hagerstown, MD 21740 (US). PUSHKO, Peter [US/US]; 917 Seminole Road, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US]; 6936 Eylers Valley Flint Road, Sabillasville, MD 21780 (US). PARKER, Michael [US/US]; Apartment T13, 1001 Carroll Parkway, Frederick, MD 21701 (US). DERTZBAUGH, Mark, T. [US/US]; 335 Fairfield Station Road, Fairfield, PA 17320 (US). SMITH, Leonard [US/US]; 2208 Regina Drive, Clarksburg, MD 20871 (US).

(74) Agents: HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 (US) et al.

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

IP AUSTRALIA
- 1 AUG 2000

(54) Title: BOTULINUM NEUROTOXIN VACCINE

(57) Abstract

Using the nontoxic heavy chain fragment from botulinum neurotoxins A–G, compositions and methods of use in inducing an immune response which is protective against intoxication with botulinum in subjects is described.

*(Referred to in PCT Gazette No. 28/2000 Section II)

TITLE OF THE INVENTION

Botulinum Neurotoxin Vaccine

5

10

FIELD OF THE INVENTION

This invention relates to vaccines for bacterial toxins from *Clostridium botulinum*.

15

INTRODUCTION

Botulism is a disease resulting from the activity of botulinum neurotoxin produced by *Clostridium*
20 *botulinum* on the transmission of neuromuscular stimuli. The blockage of stimuli produces neuromuscular weakness and flaccid paralysis which can lead to respiratory failure and death. Food poisoning, infant botulism, and wound botulism are the three ways
25 in which humans are naturally affected by botulinum neurotoxin (BoNT). Ingestion of improperly prepared or canned foods has resulted in numerous cases of botulism. Seven different serotypes of botulinum neurotoxin have been characterized, types A through G,
30 which are antigenically distinct. BoNT are usually expressed in *Clostridium botulinum* as a single polypeptide chain and then posttranslationally nicked, forming a dichain consisting of a 100-kDa heavy chain and a 50-kDa light chain held together by a single

disulfide bond (DasGupta, B. R. 1989, In L.L. simpson (ed.), Botulinum Neurotoxin and Tetanus Toxin. Academic Press, New York, N.Y.). Topologically, these neurotoxins are composed of three domains, a binding
5 domain, a translocation domain, and a catalytic domain, each of which is believed to play a role in intoxication. The carboxy-terminal portion of the heavy chain is responsible for binding nerve cell receptor(s). After toxin binding, it is thought to be
10 internalized into an endosome through receptor-mediated endocytosis (Byrne, M. P. et al., 1998, supra). The product of a gene encoding only the binding domain of BoNT is nontoxic when administered to an organism since it cannot enter the nerve cell
15 without the translocation domain and it lacks the catalytic domain.

The vaccine currently used against botulism is comprised of the complete toxoid (Byrne, M. P. et al., *Infect. Immun.* 66:4817, 1998). The toxoid vaccine is
20 dangerous and expensive to produce, contains formalin, which is very painful for the recipient, and is incomplete; only five, A-E, of the seven serotypes are represented in the formulation.

Previous work with BoNT serotype A (BoNT/A)
25 demonstrated that the recombinant carboxy terminal of the heavy chain polypeptide (Hc fragment) produced in *Escherichia coli* only partially protected mice challenged with up to 1,200 LD_{50} of BoNT/A (LaPenotiere, H. F. et al., 1995, *Toxicon* 33:1383-
30 1386; Clayton, M.A. et al., 1995, *Infect. Immun.* 63: 2738-2742). This preparation was difficult to produce due to inclusion bodies and the resulting amount of polypeptide was not large enough to justify large scale production. Furthermore, the product contained
35 *E. coli* endotoxin.

Therefore, there is a need for an efficacious vaccine against botulism, useful for protecting humans.

SUMMARY OF THE INVENTION

5 The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against intoxication with botulinum neurtoxin (BoNT) serotypes A (BoNTA), B 10 (BoNTB), C (BoNTC), D (BoNTD), E (BoNTE), and F (BoNTF), and G (BoNTG). The invention relates to the use of a replicon vector which results in production of large amounts of a protein encoded by a sequence cloned into the replicon. The protein product is 15 easily purified, available in large quantities, and devoid of endotoxin. Furthermore, immunization with the replicon encoding the desired antigen has the advantage of expressing genes in lymph nodes for a better immune response, and for stimulating mucosal 20 immune responses (Davis et al., 1996, *J. Virol.* 70, 3781-3787).

The sequences encoding the Hc 50,000 Kd nontoxic fragment of BoNT A-G (see attached sequence and Clayton et al., 1995, *Infection and Immunity* 63, 2738- 25 2742) were inserted into the Venezuelan equine encephalitis (VEE) virus replicon described in U.S. Patent No. 5,792,462 (Hc-replicon). In this vaccine strategy, a gene coding for a protein of interest is cloned in place of the VEE virus structural genes; the 30 result is a self-replicating RNA molecule that encodes its own replicase and transcriptase functions, and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that 35 express the VEE structural proteins (glycoproteins and

nucleocapsid), the replicon RNA is packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided *in trans*. Since the helper RNAs lack packaging signals neccessary for
5 further propagation, the resulting VEE replicon particles (VRPs) which are produced are infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell
10 produces the protein of interest in abundance, is ultimately killed by the infection, but does not produce any viral progeny (Pushko *et al*., 1997, *Virology* 239, 389-401).

Experiments carried out in cell culture using the
15 Hc-replicon demonstrated that the construct could produce high levels of the Hc polypeptides in eukaryotic cells. Inoculation of VRP containing the Hc-replicon into an inbred mouse strain (BALB/c) or into an outbred mouse strain (SWISS) produced high
20 antibody titers and protected the mice from the effects of botulinum neurotoxin.

Therefore, it is one object of the present invention to provide a VEE virus replicon vector comprising a VEE virus replicon and a DNA fragment
25 encoding any of the botulinum neurotoxin heavy chain fragments of serotypes A, B, C, D, E, F, G, alone or in combination, and fragments thereof such as ASubHc1 or ASubHc2, which define domains within serotype A toxin.

30 It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the botulinum neurotoxin fragments described above.

It is another object of the present invention to
35 provide infectious VEE virus replicon particles

produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against botulinum intoxication comprising VEE virus replicon particles containing any of the botulinum neurotoxin fragments described above or a combination of different VEE virus replicons each containing a different botulinum neurotoxin fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Western blot of BHK cell lysates showing expression of Hc from recombinant VEE replicons. a) infected cell lysate; b) commercially available product.

Figure 2. ELISA titers for Balb/c mice immunized with Hc-VRP at different intervals. 1) 0.2 ml human vaccine given at day 0 and 28; 2) 10^7 iu Lassa N-VRP given at day 0 and 28; 3) 10^7 iu Hc-VRP per inoculation; 4) Titers less than 2 logs or greater than 5.61 logs were estimated. Challenge was 31 days after last inoculation with 1000 LD_{50} BoNT/A.

Figure 3. ELISA titers for Balb/c mice immunized with varying amounts of Hc-VRP. 1) 0.2 ml human vaccine given at day 0 and 28; 2) 10^7 iu Lassa N-VRP given at day 0 and 28; 3) Hc-VRP inoculation given at day 0 and 28; 4) Titers less than 2 logs or greater than 5.61 logs were estimated. Challenge was 31 days after last inoculation with 1000 LD_{50} BoNT/A.

Figure 4. ELISA titers for Balb/c and Swiss mice immunized with Hc-VRP. 1) 0.2 ml human vaccine given

at day 0 and 28; 2) 10^7 iu Lassa N-VRP given at day 0 and 28; 3) 10^7 iu Hc-VRP given at day 0 and 28; 4) Titers less than 2 logs or greater than 5.61 logs were estimated. Challenge was 31 days after last
5 inoculation with 1000 LD_{50} BoNT/A.

Figure 5. Schematic of constructs containing BoNT/Hc fragments.

DETAILED DESCRIPTION

10 In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be
15 given such terms, the following definitions are provided.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site
20 can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell
25 identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural
30 proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA *in trans*. This is typically done with two helpers
35 also called defective helper RNAs. One helper

consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for
5 subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro*
10 and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the
15 cell. The particles can then by inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only
20 the viral nostructural proteins and the product of the heterologous gene cloned in place of the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which
25 the structural proteins genes are replaced with a gene from an immunogen of interest, in this invention, the BoNT Hc proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the
30 glycoprotein and capsid proteins of the VEE virus.

Subject. Includes both human, animal, e.g., horse, cattle, donkey, monkey, pig, dog, guinea pig, mouse, hamster, avian e.g., chicken, pheasant or turkey, fish and other marine animals, and insects
35 such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding BoNT proteins. Both native and synthetic DNA sequences encoding BoNT heavy chain were used in this invention. The synthetic BoNT(Hc) DNA fragments were optimized for codon usage for expression in yeast. Other modifications in codon usage which result in a different nucleotide sequence but still produce an immunologically identifiable heavy chain fragment can also be used. The synthetic Hc fragments used in the examples below are as follows: Hc BoNTA (SEQ ID NO:1), Hc BoNTB (SEQ ID NO:2), Hc BoNTC (SEQ ID NO:3), Hc BoNTE (SEQ ID NO:4), Hc BoNTF (SEQ ID NO:5), Hc BoNTG (SEQ ID NO:6). Native DNA sequences encoding heavy chain region of the neurotoxins as well as the carboxy or amino terminus of the heavy chain region of the neurotoxin were used. The native sequence of heavy chain serotype A is known (Thompson, D. E. et al., 1990, *Eur. J. Biochem.* 189, 73-81) and is identified in SEQ ID NO: 7. The amino terminal region of the native heavy chain (SEQ ID NO: 8) and the carboxy terminal region of the native heavy chain (SEQ ID NO:9) were cloned into a replicon vector and are exemplified below. DNA or polynucleotide sequences to which the invention also relates include sequences of at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, most preferably at least about 15-20 nucleotides corresponding, i.e., homologous to or complementary to, a region of the Hc BoNT nucleotide sequences. Regions from which typical DNA sequences may be derived include but are not limited to, for example, regions encoding specific epitopes, as well as non-transcribed and/or non-translated regions.

DNA sequences also embodied in the present invention include the BoNT sequence encoding a subfragment of the Hc. The exemplified subfragments were chosen based on domains within the protein itself (Lacy et al, 1998, *Science* 5, 898-902). The subfragments exemplified were chosen from serotype A Hc fragment (Clayton et al., 1995, *supra*, GenBank accession number is U22962): AsubHc1 includes amino acid #1 Met to amino acid # 233 Arg (SEQ ID NO:10, nucleotides 9-707 of Genbank U22962) of serotype A Hc fragment, and AsubHc2 includes amino acid # 234 Ser to amino acid # 438 Leu (SEQ ID NO:11, nucleotide 708 to 1325 of Genbank U22962) of serotype A Hc fragment. A methionine was added to AsubHc2 in order to initiate proper translation. Other subfragments of any size can be used for different purposes. For example, a subfragment overlapping the AsubHc1 and AsubHc2 would include epitopes previously broken apart in AsubHc1 and AsubHc2. Methods for manupulating nucleic acid sequences are known in the art, please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: a Laboratory Manual (1982) or DNA Cloning, volumes I and II (D. N. glover ed. 1985) or Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds.) John Wiley & Sons, Inc., for general cloning methods.

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence shown in SEQ ID NO:1-11, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated

sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain
5 reaction assays and for the detection of BoNT sequences. Since the nucleic acid sequence is synthetic, i.e. the codon usage was optimized for expression in a yeast system, it could not be used effectively in hybridization assays without correcting
10 the codon usage for a specific purpose.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and one or more nucleic acid sequences as described above. The vector can take the form of a
15 plasmid, such as pUC19, or any other vector which replicates in any host such as *E. coli*, yeast, insect cells, or mammalian cells. The recombinant DNA molecule can be used to generate more DNA molecules or, when the nucleic acid sequences are inserted into
20 an expression vector, the protein encoded by the nucleic acid sequence can be produced.

When the DNA sequences described above are in a replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in*
25 *vivo*. The DNA sequence for any of the BoNT proteins described above can be cloned into the multiple cloning site of a replicon such that transcription of the RNA from the replicon yields an infectious RNA containing the BoNT sequence encoding a protein or
30 proteins of interest. Use of helper RNA containing sequences necessary for encapsulation of the viral transcript will result in the production of viral particles containing replicon RNA which are able to infect a host and initiate a single round of
35 replication resulting in the expression of the BoNT

proteins. Such replicon constructs include those presented in Table 1.

Table 1

Replicon Plasmid Name	Serotype	expresses
p3014-40A	A	full length synthetic C fragment
p3014-114a1	A	N-terminal sub-synthetic C-fragment
p3014-102a2	A	C-terminal sub-synthetic C-fragment
p3014-73B	B	full length synthetic C fragment
p3014-110C	C	full length synthetic C fragment
p3014-75E	E	full length synthetic C fragment
p3014-77F	F	full length synthetic C fragment
p3014-107G	G	full length synthetic C fragment
pXrep-BoNTA/H	A	heavy chain native sequence
pXrep-BoNTA/H_N	A	amino terminal of native heavy chain sequence
pXrep-BoNTA/H_C	A	carboxy teminal of native heavy chain sequence

The sequences encoding the BoNT proteins were cloned into the replicon vector by methods known in the art and described below in Materials and Methods. Schematic diagrams of the resulting constructs are shown in the Figures. The VEE constructs containing BoNT Hc proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

In another embodiment, the present invention relates to RNA molecules resulting from transcription of the constructs described above. The RNA molecules can be prepared by transcription using methods known in the art and described in the Examples below. Alternatively, the RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for

example, to transfect cells along with RNA from helper plasmids, one of which expresses VEE glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

5 In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, 10 yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to mouse and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are 15 compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors 20 compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance 25 markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general 30 cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of BoNT Hc proteins, such as glutathione S-transferase. The recombinant molecule

can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae, Saccharomyces carlsbergensis,* and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as CHO cells, vero cells, and COS cells to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art.

The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein or polypeptide cloned into the VEE replicon, or a source of RNA transcribed from the replicon as described above, or a source of replicon particles.

In a further embodiment, the present invention relates to a method of producing the recombinant or fusion protein which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and the recombinant or fusion protein encoded by said DNA fragment is produced. The recombinant or fusion protein can then

be isolated using methodology well known in the art. The recombinant or fusion protein can be used as a vaccine for immunizing against intoxication with BoNT or as a diagnostic tool for detection of botulism. 5 The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit toxin effects, such as host proteins or chemically derived agents or other proteins which may interact with the toxin to inibit its function. Increased or decreased 10 botulinum toxicity can be measured using a mouse bioassay. The bioassay is normally used to determine whether serum antibodies, from any animal sources, can protect a naïve mouse from the effects of botulinum neurotoxin. The assay is performed by mixing a serum 15 sample with active toxin which is then injected into a naïve mouse. If the mouse survives, then the serum sample contained protective, neutralizing antibodies. The assay could easliy be modified for testing anti-botulism drugs or agents. The drug or agent could be 20 mixed with active toxin and then injected into a naïve mouse. If the mouse survives, then the drug or agent is effective at preventing botulism.

In another embodiment, the present invention relates to a botulinum neurotoxin vaccine comprising 25 one or more replicon particles derived from one or more replicons encoding one or more BoNT Hc proteins or polypeptides as described above. The present invention also relates to a method for providing immunity against botulism said method comprising 30 administering one or more replicon particles containing any combination of the BoNT proteins to a subject such that a protective immune reaction is generated.

Vaccine formulations of the present invention 35 comprise an immunogenic amount of a replicon particle,

resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^2 to 10^7 per dose is suitable, more or less can be used depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. An "immunogenic amount" is an amount of the

replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to elicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Plasmids. Construction of the VEE replicon, capsid (C-) helper, and glycoprotein (GP-) helper plasmids was previously described (Pushko, 1997, supra). The Hc gene (Clayton, 1995, supra) was cloned into the VEE replicon plasmid as a *Xho*I / *Hind* III fragment utilizing a shuttle vector. The Lassa nucleocapsid replicon (Lassa N-replicon) was constructed as previously described (Pushko 1997, supra) and used as a negative control replicon.

Replicon p3014-40A was cloned as follows: The BoNT/A Hc gene was cut out of pMutAC-1 (obtained from Clayton et al., 1995, supra) with Xho I and Hind III . The gene was ligated into pALTER-1 (Promega, Inc.) at a compatible Sal I and a Hind III site. The BoNT/A Hc gene was cut out of pALTER/Hc with Xba I (the site was located just upstream of the Sal I site) and Hind III. The gene was ligated into the KS2 Shuttle vector, cut out with Apa I and Not I, and ligated into the replicon pVR2 (Drawing sheet 1, U.S. Patent No. 5,792,462 to Johnston et al.).

Replicons p3014-115a1 and -102a2 were cloned as follows: The BoNT/A subHc a1 and a2 genes were PCR amplified from p3014-40A using forward primers containing a 5′ Cla I recognition site, a start codon, and complimentary to the 5′ end of the subgenes, from nucleotide 9 to 28 for a1 and from nucleotide 708 to 731 for a2, and reverse primers containing a 3′ Cla I recognition site, a stop codon, and complimentary to the 3′ end of the subgenes, from nucleotide 686 to 707 for a1 and from nucleotide 1305 to 1325 for a2 (numbering according to Clayton et al., 1995, supra). The PCR products were gel purified and then ligated

into pCR2.1(Invitrogen, Inc.). The genes were cut out of pCR-suba1 or pCR-suba2 with Cla I and ligated into pVR2 replicon.

Replicon 3014-73B, -110C, -75E, -77F, and -107G were cloned as follows: An EcoRI digest of plasmids pBoNT/B(Hc), pBoNT/C(Hc), pBoNT/E(Hc), pBoNT/F(Hc), and pBoNT/G(Hc) resulted in an EcoRI DNA fragment containing Hc fragments of serotypes B, C, E, F, and G, respectively. The EcoRI fragments were each ligated into the KS2 Shuttle. The orientation of each gene was determined and then the genes were cut out of the shuttle with Apa I and Not I. The Apa I/Not I genes were ligated into the pVR2 replicon.

Production of VRP. Plasmid templates for the Hc-replicon, C-helper, GP-helper, and the Lassa N-replicon were linearized by digestion with *Not*I at a unique site downstream from the coding sequences, and capped run-off transcripts were prepared *in vitro* using T7 RNA polymerase. Packaging of the replicons into VEE replicon particles (VRPs) was accomplished by electroporating the replicon RNA and the two helper RNAs into BHK cells. VRPs were harvested between 20 and 27 hours after transfection and purified from cell culture supernatants by ultracentrifugation through a discontinuous sucrose gradient (20%). After reconstituting the pelleted VRP in 1/50 volume phosphate buffered saline, the VRPs were stored at -70°C.

Analysis of expression products and titration of VRP. Subconfluent monolayers were infected with Hc-VRP or Lassa N-VRP (m.o.i. = 2) or cell suspensions were electroporated with replicon RNA. Cells were harvested at approximately 20-24 hours and expressed proteins were separated by SDS-PAGE. Visualization of Hc protein (50kDa) was accomplished

using a chemiluminescence western blot assay and antibodies specific for each protein. Titration of VRPs was accomplished by infecting subconfluent monolayers with increasing dilutions of purified VRP. Antigen positive cells were visualized in an indirect immunofluorescence assay using a monoclonal antibody specific for each protein, or in a direct immunofluorescence assay using an FITC-conjugated monkey anti-Lassa serum.

Immunization of mice. Mice were inoculated 1 to 4 times at 7 to 28 day intervals with 10^5 to 10^7 infectious units (iu) of either Hc-VRP or Lassa N-VRP (negative control). Positive control mice for the botulinum study were inoculated subcutaneously with 0.2 ml of human botulinum vaccine at 28 day intervals. Serum for ELISA was obtained 2 days before each inoculation and 3 days before challenge.

For the duration of immunity challenge, the mice were challenged intraperitoneally with 10^3 LD_{50} units of BoNT/A in 0.2% gelatin/PBS 24 weeks after the last inoculation. Swiss mice were challenged intraperitoneally with 10^3 LD50 units of BoNT/B in 0.2% gelatin/PBS 31 days after the last inoculation.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with botulinum neurotoxin (1 ug/ml) in PBS and allowed to absorb overnight at 4°C. Four fold serum dilutions in blocking buffer were applied to the plates and incubated at 37°C for 1 hour. After washing, an anti-mouse secondary antibody (HRP conjugated) was added to the plate and incubated for an additional hour at 37°C. After washing, bound antibody was detected colormetrically using ABTS as a substrate.

Challenge of mice.

Botulinum neurotoxin challenge: Balb/c and Swiss mice were challenged intraperitoneally with 10^2 to 10^5 LD_{50} units of BoNT/A in 0.2% gelatin/PBS 31 days after the last inoculation.

5

Example 1

Packaging and expression of Hc-replicon

The Hc-replicons were efficiently packaged into VRPs using the double helper system. Stock solutions contained about 10^8 iu of purified VRP per milliliter.
10 No replication competent VEE virus was detected in any of the preparations using a standard plaque assay. Cells infected with VRP or transfected with replicons encoding Hc expressed high levels of these proteins as demonstrated by western blot (Figure 1) and by
15 immunofluorescence. VEE replicons expressing the above genes produced proteins that comigrated on gels with authentic proteins and reacted efficiently with antibodies raised to the authentic proteins.

Example 2

20

Protection against challenge with BoNT.

Results from animal studies demonstrated that the VEE replicon expressing the 50-kDa carboxy-terminal fragment of botulinum neurotoxin (Hc) type A or type B polypeptide could immunize and protect mice from a
25 lethal challenge of BoNT/A or BoNT/B, respectively. Balb/c mice inoculation with Hc-VRP (serotype A) at day 0 produced a maximum antibody response around day 19 which remained constant to at least day 26 (Figure 2). Booster inoculations given at day 7, 14, 21, or 28
30 stimulated a good secondary antibody response. If both doses of the Hc-VRP were given on the same day, the primary antibody response was 2.96 logs (serum was obtained 28 days post inoculation) as compared to 1.73 logs for mice that received 2 doses of the Lassa N-VRP

(Lassa N-VRP was given at day 0 and 28; serum was obtained 28 days post-second inoculation). Even though the mice that received 2 doses of Hc-VRP on day 0 were not protected from challenge, the time to death was
5 increased from 6 hours (for mice that received the Lassa N-VRP) to 33 hours. Mice that received a booster inoculation on day 7 produced a secondary antibody response of 3.23 logs with 8 out of 10 surviving challenge with 1000 LD_{50} units of BoNT/A. Mice that
10 received booster inoculations on day 14, 21, or 28 produced higher secondary antibody responses of 3.87, 4.44, and 4.56 logs, respectively, with all mice surviving challenge with 1000 LD_{50} units of BoNT/A. Thus, the most beneficial inoculation schedule was 2
15 doses of Hc-VRP given at least 21 days apart.

Figure 3 shows the ELISA titers and survival for BALB/c mice inoculated with doses of Hc-VRP ranging from 10^5 to 10^7 iu. The dose of Hc-VRP (given twice at an interval of 28 days) required to completely protect
20 BALB/c mice from a lethal challenge of 1000 LD_{50} BoNT/A was between 10^6 and 10^7 iu. The pre-challenge serum ELISA titers from BALB/c mice immunized with 10^5, 10^6 or 10^7 iu of Hc-VRP was 1.27, 3.81, and 4.56 logs, respectively, as compared to 1.73 logs for mice
25 that received the negative control replicon. None of the animals inoculated with 10^5 iu of Hc-VRP or the negative control replicon survived challenge whereas 8 of 10 and 10 of 10 mice that received 10^6 or 10^7 iu of Hc-VRP survived challenge. Table 2 shows a comparison
30 of data obtained from animals challenge with 100 to 100,000 LD_{50} units of BoNT/A. Two inoculations of 10^7 iu of Hc-VRP protected 90% of the BALB/c mice from a lethal challenge of 100,000 LD_{50} units of BoNT/A.

Figure 4 shows ELISA titers and survival for Swiss mice inoculated with Hc-VRP. Two doses of 10^7 iu Hc-VRP protected 100% of the mice from an otherwise lethal challenge of 1000 LD_{50} units of BoNT/A. The
5 protection achieved in the SWISS mice demonstrated the ability of the Hc-replicon to protect outbred animals. The geometric mean titer from SWISS mice immunized with the Hc-VRP was 5.13 logs as compared to 1 log for mice that received the negative control replicon. None
10 of the animals inoculated with the negative control replicon survived challenge.

Table 2. BoNT/A Hc replicon protects Balb/c mice from challenge.

Replicon Dose (ffu)[1]	ELISA GMT[2]	Challenge Dose (LD50)[3]	Survived/Total[4]
10^7	67558	10^2	10/10
10^7	51200	10^3	10/10
10^7	75250	10^4	9/10
10^7	87781	10^5	9/10

15 1. Replicon inoculated at day 0 and 28; ffu, fucus forming units.
2. Prechallenge end point titers.
3. Challenge was 28 days after the last inoculation with the above LD50 BoNT/A.
20 4. Mice that died, titer = 100; 102400

Swiss mice inoculation with different amounts of Hc-VRP (serotype **B**) at day 0 and day 28 were partially to fully protected from challenge. Two doses of 10^6
25 Hc-VRP only protected 3 out of 10 mice whereas two

doses of 10^7 Hc-VRP protected 10 out of 10 mice from a 1000 LD_{50} BoNT/B challenge.

Swiss mice inoculted with Hc-VRP (serotype **E**) were not protected from challenge. Some modifications of the gene, either shortening or adding some sequences (from the N-terminal part of the Heavy Chain) may help increase protection with Hc-VRP (serotype E).

Swiss mice inoculation with different amounts of Hc-VRP (serotype **F**) at day 0 and day 28 were partially protected from challenge. Two doses of 10^6 Hc-VRP only protected 1 out of 10 mice whereas two doses of 10^7 Hc-VRP protected 3 out of 10 mice from a 1000 LD_{50} BoNT/F challenge.

The Hc-replicon's ability to induce long term immunity was investigated by inoculating mice with Hc-VRP (serotype A) and then challenging 6 months post vaccination. Swiss mice were inoculated with either 10^6 or 10^7 Hc-VRP at week 0 and 4 and then challenged during week 28. Mice that received 10^6 Hc-VRP were almost fully protected, 9 out of 10 survived, while mice that received 10^7 Hc-VRP were completely protected, 10 out of 10 survived, from a 1000 LD_{50} BoNT/A challenge.

The ability of Hc-VRP (serotypes E, C and D) to protect animals from challenge with these toxins is being investigated.

Example 3

Immunogenicity and efficacy of replicons, expressing either the native or synthetic gene fragments of the heavy chain of BoNT/A, and the botulinum toxoid vaccine were compared. VEE replicon vector containing native sequences encoding the BoNT/A heavy chain N fragment (BoNT/A HN), the BoNT/A heavy

chain C fragment (BoNT/A HC), the BoNT/A heavy chain (BoNT/A H), and a synthetically derived sequence encoding the BoNT/A heavy chain C fragment (synBoNT/A Hc) (Clayton et al., 1995, supra) were used in this
5 experiment.

After production of VRPs as described above, Swiss mice were inoculated subcutaneously on days 0, 38, and 56 with 10^5, 10^6, or 10^7 VRPs either expressing BoNT/A H_N, BoNT/A H_C, or BoNT/A H; all encoded by
10 their respective native gene sequences. Another group of mice received 10^7 VRPs expressing synBoNT/A Hc. For a negative control, mice were immunized with 10^7 VRPs expressing the Lassa virus nucleocapsid replicon (Lassa N-Rep), and for a positive control, mice were
15 immunized with 0.2 ml of the current human BoNt toxoid vaccine (pentavalent,A-E, formaldehyde-inactivated vaccine with adjuvant). Before each inoculation, the mice were retro-orbitally bled, and sera was isolated on days 23, 51, and 79. Antibody (Ab) titers to
20 BoNT/A were determined on each serum sample using ELISA. Purified BoNT/A was used as ELISA antigen. On day 84, each mouse was challenged intraperitoneally with 1000 LD_{50} of BoNT/A. The mice were then examined daily for 7 days after challenge to determine if
25 protection was conferred.

Mice immunized with either the toxoid vaccine or 10^7 VRPs expressing the synthetic BoNT/A Hc (synBoNT/A Hc) were completely protected from challenge while mice inoculated with 10^7 VRPs expressing the native
30 BoNT/A H_C were almost completely protected (16 of 20 mice survived challenge) (see table 3). In addition, partial protection was observed in mice immunized with 10^6 VRPs expressing the native BoNT/A Hc (9 of 19 mice survived challenge).

Table 3. Survival and ELISA titers of Swiss mice inoculated with different immunogens and challenged with BoNT/A

Immunogen	Gene Sequence	Dose	Survivors /total	Pre-challenge GMT[b] ($\log_{10}$)	MDD[a]
synBoNT/A Hc replicon	Synthetic	107	18/18	5.25	-
Toxoid vaccine	n/a	0.2ml	20/20	6.07	-
Lassa N replicon	n/a	10^7	0/20	1.65	1
BoNT/A Hn replicon	Native	10^5	0/20	1.43	1
BoNT/A Hn replicon	Native	10^6	0/18	2.05	1
BoNT/A Hn replicon	Native	10^7	0/20	2.68	1
BoNT/A Hc replicon	Native	10^5	1/20	1.88	1
BoNT/A Hc replicon	Native	10^6	9/19[a]	3.63	1
BoNT/A Hc replicon	Native	10^7	16/20	5.19	1
BoNT/A H replicon	Native	10^5	0/20	1.66	1
BoNT/A H replicon	Native	10^6	0/20	2.10	1
BoNT/A H replicon	Native	10^7	0/20	2.82	1

Mice were intraperitoneally challenged with 1000 LD50 BoNT/A.
[a] Mice died during retro-orbital bleed;
[b]GMT, geometric mean titer determined on serum obtained 5 days prior to challenge;
5 MDD, mean day to death for those mice that failed to survive challenge

Discussion/Conclusion

Since VEE virus replicates in the cytoplasm of 10 eukaryotic cells, the VEE replicon vaccine vector is a useful tool for the expression of prokaryotic genes in eukaryotic cells. Cytoplasmic expression of genes alleviates the difficulties imposed by splicing and nuclear transport of mRNA. We used the VEE replicon as 15 a way to express the prokaryotic Hc genes in eukaryotic cells and to develop new vaccine candidates against botulinum neurotoxin.

Development of a new candidate vaccine against botulinum neurotoxin would overcome problems 20 associated with the current human vaccine. The current vaccine requires 4 inoculations over 12 months and only protects against 5 of the 7 serotypes. High production costs and high reactogenicity (up to 20% of the recipients developed mild to moderate local 25 reaction after receiving booster inoculations) are just two other problems associated with the current vaccine. We constructed new candidate vaccines, the Hc-replicon vaccines, that may overcome these problems.

30 Hc-replicons produced large amounts of protein *in vitro*, as determined by western blot analysis of cell lysates and by immunofluorescence of fixed cells, and elicited a good immune response when inoculated into mice. We found that two inoculations of Hc-VRP 35 (serotype A) given on day 0 and 21 or 28 produced the strongest secondary antibody response and protected mice from the effects of botulinum neurotoxin serotype

A. Mice inoculated with Hc-VRP (serotype B) were similarly protected from challenge; complete protection was observed using a homologous challenge of BoNT/B. Mice that received 2 inoculation of Hc-VRP (serotype A) were also protected against 100,000 LD_{50} units of BoNT/A, an extremely high challenge dose. The Hc-replicon was also able to induce long term protection in mice; with protection lasting for at least 6 months. The Hc-replicon vaccines, using the synthetic or native sequence of the heavy chain, were effective in blocking the effects of botulinum neurotoxin and may alleviate most of the problems associated with the current vaccine.

EDITORIAL NOTE - NO 54583/99

The following Sequence listing is part of the description.
The claims follow on page 28 to 31.

SEQUENCE LISTING

<110> U.S. Army Medical Reseach Institute for Infectious
Diseases
John S. Lee
Peter Pushko
Michael D. Parker
Jonathan F. Smith
Mark T. Dertzbaugh
Leonard Smith

<120> Botulinum Neurotoxin Vaccine

<130> 003/124/SAP RIID 98-21

<140> PCT/US99/15570

<141> 1999-07-09

<150> US 60/092,416

<151> 1998-07-10

<160> 11

<170> Apple Macintosh Microsoft Word 6.0

<210> 1

<211> 1338

<212> DNA

<213> Artificial

<220>

<223>/note="synthetic sequence encoding botulinum neurotoxin"

<400> 1

```
gtcgagccat ggctcgtctg ctgtctacct tcactgaata catcaagaac
atcatcaata cctccatcct gaacctgcgc tacgaatcca atcacctgat
cgacctgtct cgctacgctt ccaaaatcaa catcggttct aaagttaact
tcgatccgat cgacaagaat cagatccagc tgttcaatct ggaatcttcc
aaaatcgaag ttatcctgaa gaatgctatc gtatacaact ctatgtacga
aaacttctcc acctccttct ggatccgtat cccgaaatac ttcaactcca
tctctctgaa caatgaatac accatcatca actgcatgga aaacaattct
ggttggaaag tatctctgaa ctacggtgaa atcatctgga ctctgcagga
```

cactcaggaa atcaaacagc gtgttgtatt caaatactct cagatgatca
acatctctga ctacatcaat cgctggatct tcgttaccat caccaacaat
cgtctgaata actccaaaat ctacatcaac ggccgtctga tcgaccagaa
accgatctcc aatctgggta acatccacgc ttctaataac atcatgttca
aactggacgg ttgtcgtgac actcaccgct acatctggat caaatacttc
aatctgttcg acaaagaact gaacgaaaaa gaaatcaaag acctgtacga
caaccagtcc aattctggta tcctgaaaga cttctggggt gactacctgc
agtacgacaa accgtactac atgctgaatc tgtacgatcc gaacaaatac
gttgacgtca acaatgtagg tatccgcggt tacatgtacc tgaaaggtcc
gcgtggttct gttatgacta ccaacatcta cctgaactct tccctgtacc
gtggtaccaa attcatcatc aagaaatacg cgtctggtaa caaggacaat
atcgttcgca acaatgatcg tgtatacatc aatgttgtag ttaagaacaa
agaataccgt ctggctacca atgcttctca ggctggtgta gaaaagatct
tgtctgctct ggaaatcccg gacgttggta atctgtctca ggtagttgta
atgaaatcca agaacgacca gggtatcact aacaaatgca aaatgaatct
gcaggacaac aatggtaacg atatcggttt catcggtttc caccagttca
acaatatcgc taaactggtt gcttccaact ggtacaatcg tcagatcgaa
cgttcctctc gcactctggg ttgctcttgg gagttcatcc cggttgatga
cggttggggt gaacgtccgc tgtaacccgg gaaagctt

<210> 2

<211> 1341

<212> DNA

<213> Artificial

<220>

<223>/note="synthetic sequence encoding botulinum neurotoxin"

<400> 2

gaattcacga tggccaacaa atacaattcc gaaatcctga acaatatcat
cctgaacctg cgttacaaag acaacaatct gatcgatctg tctggttacg
gtgctaaagt tgaagtatac gacgctgttg aactgaatga caagaaccag
ttcaaactga cctcttccgc taactctaag atccgtgtta ctcagaatca
gaacatcatc ttcaactccg tattcctgga cttctctgtt tccttctgga
ttcgtatccc gaaatacaag aacgacggta tccagaatta catccacaat
gaatacacca tcatcaactg catgaagaat aactctggtt ggaagatctc
catccgcggt aaccgtatca tctggactct gatcgatatc aacggtaaga
ccaaatctgt attcttcgaa tacaacatcc gtgaagacat ctctgaatac
atcaatcgct ggttcttcgt taccatcacc aataacctga acaatgctaa
aatctacatc aacggtaaac tggaatctaa taccgacatc aaagacatcc
gtgaagttat cgctaacggt gaaatcatct tcaaactgga cggtgacatc
gatcgtaccc agttcatctg gatgaaatac ttctccatct tcaacaccga
actgtctcag tccaatatcg aagaacggta caagatccag tcttactccg
aatacctgaa agacttctgg ggtaatccgc tgatgtacaa caaagaatac
tatatgttca atgctggtaa caagaactct tacatcaaac tgaagaaaga
ctctccggtt ggtgaaatcc tgactcgttc caaatacaac cagaactcta
aatacatcaa ctaccgcgac ctgtacatcg gtgaaaagtt catcatccgt
cgcaaatcta actctcagtc catcaatgat gacatcgtac gtaaagaaga
ctacatctac ctggacttct tcaacctgaa tcaggaatgg cgtgtataca

cctacaagta cttcaagaaa gaagaagaaa agcttttcct ggctccgatc
tctgattccg acgaactcta caacaccatc cagatcaaag aatacgacga
acagccgacc tactcttgcc agctgctgtt caagaaagat gaagaatcta
ctgacgaaat cggtctgatc ggtatccacc gtttctacga atctggtatc
gtattcgaag aatacaaaga ctacttctgc atctccaaat ggtacctgaa
ggaagttaaa cgcaaaccgt acaacctgaa actgggttgc aattggcagt
tcatcccgaa agacgaaggt tggaccgaat agtaagaatt c

<210> 3

<211> 1371

<212> DNA

<213> Artificial

<220>

<223>/note="synthetic sequence encoding botulinum neurotoxin"

<400> 3

gaattcacga tcaccatccc attcaacatc ttctcctaca ccaacaactc
cctgttgaag gacatcatca acgagtactt caacaacatc aacgactcca
agatcctgtc cctgcagaac cgtaagaaca ccttggtcga cacctccggt
tacaacgccg aggtctccga ggagggtgac gtccagctga acccaatctt
cccattcgac ttcaagctgg gttcctccgg tgaggacaga ggtaaggtca
tcgtcaccca gaacgagaac atcgtctaca actccatgta cgagtccttc
tccatctcct tctggatcag aatcaacaag tgggtctcca acttgccagg
ttacaccatc atcgactccg tcaagaacaa ctccggttgg tccatcggta
tcatctccac cttcctggtc ttcaccctga agcagaacga ggactccgag
cagtccatca acttctccta cgacatctcc aacaacgctc ctggttacaa
caagtggttc ttcgtcaccg tcaccaacaa catgatgggt aacatgaaga
tctacatcaa cggtaagctg atcgacacca tcaaggtcaa ggagttgacc
ggtatcaact tctccaagac catcaccttc gagatcaaca agatcccaga
caccggtctg atcacctccg actccgacaa tatcaacatg tggatccgtg
acttctacat cttcgccaag gagttggacg gtaaggacat caacatcctg
ttcaactcct tgcagtacac caacgtcgtc aaggactact ggggtaacga
cctgagatac aacaaggagt actacatggt caacatcgac tacttgaaca
gatacatgta cgccaactcc agacagatcg tcttcaacac cagacgtaac
aacaacgact tcaacgaggg ttacaagatc atcatcaagc gtatcagagg
taacaccaac gacaccagag tcagaggtgg tgacatcctg tacttcgaca
tgactatcaa caacaaggcc tacaacctgt tcatgaagaa cgagaccatg
tacgccgaca accactccac cgaggacatc tacgccatcg gtctgcgtga
gcagaccaag gacatcaacg acaacatcat cttccagatc cagccaatga
acaacactta ctactacgct tcccagatct tcaagtccaa cttcaacggt
gagaacatct ccggtatctg ttccatcggt acctacagat tccgtctggg
tggtgactgg tacagacaca actacttggt tccaactgtc aagcagggta
actacgcctc cttgctggag tccacttcca cccactgggg attcgtccca
gtctccgagt aataggaatt c

<210> 4

<211> 1400

<212> DNA

<213> Artificial

<220>

<223>/note="synthetic sequence encoding botulinum neurotoxin"

<400> 4

```
gaattcacca tgggagagag tcagcaagaa ctaaattcta tggtaactga
taccctaaat aatagtattc cttttaagct ttcttcttat acagatgata
aaattttaat ttcctacttc aacaagttct tcaagagaat taagtcttct
tccgttttaa acatgagata caagaatgat aaatacgtcg acacttccgg
ttacgactcc aatatcaaca ttaacggtga cgtgtacaag tacccaacta
acaaaaacca attcggtatc tacaacgaca agcttactga gctgaacatc
tctcaaaacg actacattat ctacgacaac aagtacaaga acttctctat
ttctttctgg gtcaggattc ctaactacga caacaagatc gtcaacgtta
acaacgagta cactatcatc aactgtatga gagacaacaa ctccggttgg
aaggtctctc ttaaccacaa cgagatcatt tggaccttgc aagacaacgc
aggtattaac caaaagttag cattcaacta cggtaacgca aacggtattt
ctgactacat caacaagtgg attttcgtca ctatcactaa cgacagatta
ggtgactcta agctttacat taacggtaac ttaatcgacc aaaagtccat
tttaaactta ggtaacattc acgtttctga caacatctta ttcaagatcg
ttaactgcag ttacaccaga tacattggca ttagatactt caacattttc
gacaaggagt tagacgagac cgagattcaa actttataca gcaacgaacc
taacaccaat atttgaagg acttctgggg taactacttg ctttacgaca
aggaatacta cttattaaac gtgttaaagc caaacaactt cattgatagg
agaaaggatt ctactttaag cattaacaac atcagaagca ctattctttt
agctaacaga ttatactctg gtatcaaggt taagatccaa agagttaaca
actcttctac taacgataac cttgttagaa agaacgatca ggtctatatt
aacttcgtcg ctagcaagac tcacttattc ccattatatg ctgataccgc
taccaccaac aaggagaaga ccatcaagat ctcctcctct ggcaacagat
ttaaccaatg cgtcgttatg aactccgtcg gtaacaactg taccatgaac
tttaaaaata ataatggaaa taatattggg ttgttaggtt tcaaggcaga
tactgtagtt gctagtactt ggtattatac ccacatgaga gatcacacca
acagcaatgg atgtttttgg aactttattt ctgaagaaca tggatggcaa
gaaaaataat agggatccgc ggccgcacgc gtcccgggac tagtgaattc
```

<210> 5

<211> 1317

<212> DNA

<213> Artificial

<220>

<223>/note="synthetic sequence encoding botulinum neurotoxin"

<400> 5

gaattcacga tgtcctacac caacgacaag atcctgatct tgtacttcaa
caagctgtac aagaagatca aggacaactc catcttggac atgagatacg
aaaacaataa gttcatcgac atctccggtt acggttccaa catctccatc
aacggtgacg tctacatcta ctccaccaat agaaaccagt tcggaatcta
ctcctccaag ccttccgagg tcaacatcgc tcagaacaac gacatcatct
acaacggaag ataccagaac ttctccatgt ccttctgggt ccgtatccca
aagtacttca acaaggtcaa cctgaataac gagtacacca tcatcgactg
catccgtaac aataactccg gatggaagat ctccctgaac tacaacaaga
tcatctggac cctgcaggac accgccggta acaatcagaa gttggtcttc
aactacaccc agatgatctc catctccgac tacatcaaca agtggatctt
cgtcaccatc accaataacc gtttgggaaa ctccagaatc tacatcaacg
gtaacttgat cgacgagaag tccatctcca acttgggtga catccacgtc
tccgacaaca ttttgttcaa gatcgtcggt tgtaacgaca cccgttacgt
cgggatccgt tacttcaaag tcttcgacac tgagttgggt aagaccgaga
tcgagacctt gtactcccac gagcctgacc catccatcct gaaggacttc
tggggtaact acctgctgta caacaaacgt tactacttgc tgaacctgtt
gcgtaccgac aagtccatca cccagaactc caacttcttg aacatcaacc
agcagagagg tgtctaccag aagccaaaca tcttctccaa caccagattg
tacaccggag tcgagttcat tatcagaaag aacggatcta ctgatatttc
caacaccgat aacttcgtca gaaagaacga tctggcttac atcaacgttc
tcgacagaga tgtcgaatac cgtctgaacg ccgatatctc tatcgccaaa
cctgaaaaga tcatcaagct gatccgtacc tctaactcta acaactctct
gggacaaatc atcgtcatgg actccatcgg taataactgt accatgaact
tccagaacaa caacggtgga aacatcggtt tgttgggttt ccactccaac
aacttggtcg cttcctcctg gtactacaac aacatccgta agaacacctc
ctccaacggt tgcttctggt ccttcatctc caaggagcac ggttggcagg
agaactaata ggaattc

<210> 6

<211> 1347

<212> DNA

<213> Artificial

<220>

<223>/note="synthetic sequence encoding botulinum neurotoxin"

<400> 6

atgaaggaca ccatcctgat ccaggtcttc aacaactaca tctccaacat
ctcctccaac gccatcctgt ccctgtccta ccgtggtggt cgtctgatcg
actcctccgg ttacggagcc accatcaacg tcggttccga cgtcatcttc
aacgacatcg gtaacggtca gttcaagctg aacaactccg agaactccaa
catcaccgcc caccagtcca agttcgtcgt ctacgactcc atgttcgaca
acttctccat caacttctgg gtccgtaccc caaagtacaa caacaacgac
atccagacct acctgcagaa cgagtacacc atcatctcct gtatcaagaa
cgactccggt tggaaggtct ccatcaaggg aaaccgtatc atctggaccc
tgatcgacgt caacgccaag tccaagtcca tcttcttcga gtactccatc

aaggacaaca tctccgacta catcaacaag tggttctcca tcaccatcac
caacgaccgt ctgggtaacg ccaacatcta catcaacggt tccctgaaga
agtccgagaa gatcctgaac ctggaccgta tcaactcctc caacgacatc
gacttcaagc tgatgaactg taccgacacc accaagttcg tctggatcaa
ggacttcaac atcttcggtc gtgagctgaa cgccaccgag gtctcctccc
tgtactggat ccagtcctcc accaacaccc tgaaggactt ctggggaaac
ccactgcgtt acgacaccca gtactacctg ttcaaccagg gtatgcagaa
catctacatc aagtacttct ccaaggcctc catgggtgag accgcccctc
gtaccaactt caacaacgcc gccatcaact accagaacct gtacctgggt
ctgcgtttca tcatcaagaa ggcctccaac tcccgtaaca tcaacaacga
caacatcgtc cgtgagggtg actacatcta cctgaacatc gacaacatct
ccgacgagtc ctaccgtgtc tacgtcctgg tcaactccaa ggagatccag
acccagctgt tcctggcccc aatcaacgac gaccctacct tctacgacgt
cctgcagatc aagaagtact acgagaagac cacctacaac tgtcagatcc
tgtgcgagaa ggacaccaag accttcggac tgttcggtat cggtaagttc
gtcaaggact acggttacgt ctgggacacc tacgacaact acttctgtat
ctcccagtgg tacctgcgtc gtatctccga gaacatcaac aagctgcgtc
tgggatgtaa ctggcagttc atcccagtcg acgagggttg gaccgag

<210> 7

<211> 2452

<212> DNA

<213> *Clostridium botulinum*

<220>

<400> 7

atgat caaagttaat aattgggact tgttttttag tccttcagaa
gataatttta ctaatgatct aaataaagga gaagaaatta catctgatac
taatatagaa gcagcagaag aaaatattag tttagattta atacaacaat
attatttaac ctttaatttt gataatgaac ctgaaaatat ttcaatagaa
aatctttcaa gtgacattat aggccaatta gaacttatgc ctaatataga
aagatttcct aatggaaaaa agtatgagtt agataaatat actatgttcc
attatcttcg tgctcaagaa tttgaacatg gtaaatctag gattgcttta
acaaattctg ttaacgaagc attattaaat cctagtcgtg tttatacatt
tttttcttca gactatgtaa agaaagttaa taaagctacg gaggcagcta
tgtttttagg ctgggtagaa caattagtat atgattttac cgatgaaact
agcgaagtaa gtactacgga taaaattgcg gatataacta taattattcc
tttaatattt tcaggagctg ttattctgtt agaatttata ccagagattg
caatacctgt attaggtact tttgcacttg tatcatatat tgcgaataag
gttctaaccg ttcaaacaat agataatgct ttaagtaaaa gaaatgaaaa
atgggatgag gtctataaat atatagtaac aaattggtta gcaaaggtta
atacacagat tgatctaata agaaaaaaaa tgaaagaagc tttagaaaat
caagcagaag caacaaaggc tataataaac tatcagtata atcaatatac
tgaggaagag aaaaataata ttaattttaa tattgatgat ttaagttcga
aacttaatga gtctataaat aaagctatga ttaatataaa taaatttttg
aatcaatgct ctgtttcata tttaatgaat tctatgatcc cttatggtgt
taaacggtta gaagattttg atgctagtct taaagatgca ttattaaagt
atatatatga actttaattg gtcaagtaga tagattaaaa gataaagtta
ataatacact tagtacagat atacctttc agctttccaa atacgtagat

aatcaaagat tattatctac atttactgaa tatattaaga atattattaa
tacttctata ttgaatttaa gatatgaaag taatcattta atagacttat
ctaggtatgc atcaaaaata aatattggta gtaaagtaaa ttttgatcca
atagataaaa atcaaattca attatttaat ttagaaagta gtaaaattga
ggtaatttta aaaaatgcta ttgtatataa tagtatgtat gaaaatttta
gtactagctt ttggataaga attcctaagt attttaacag tataagtcta
aataatgaat atacaataat aaattgtatg gaaaataatt caggatggaa
agtatcactt aattatggtg aaataatctg gactttacag gatactcagg
aaataaaaca aagagtagtt tttaaataca gtcaaatgat taatatatca
acagatggat ttttgtaact atcactaata atagattaaa taactctaaa
atttatataa atggaagatt aatagatcaa aaaccaattt caaatttagg
taatattcat gctagtaata atataatgtt taaattagat ggttgtagag
atacacatag atatatttgg ataaaatatt ttaatctttt tgataaggaa
ttaaatgaaa aagaaatcaa agatttatat gataatcaat caaattcagg
tattttaaaa gacttttggg gtgattattt acaatatgat aaaccatact
atatgttaaa tttatatgat ccaaataaat atgtcgatgt aaataatgta
ggtattagag gttatatgta tcttaaaggg cctagaggta gcgtaatgac
tacaaacatt tatttaaatt caagtttgta taggggggaca aaatttatta
taaaaaaata tgcttctgga aataaagata atattgttag aaataatgat
cgtgtatata ttaatgtagt agttaaaaat aaagaatata ggttagctac
taatgcatca caggcaggcg tagaaaaaat actaagtgca ttagaaatac
ctgatgtagg aaatctaagt caagtagtag taatgaagtc aaaaaatgat
caaggaataa caaataaatg caaaatgaat ttacaagata ataatgggaa
tgatataggc tttataggat ttcatcagtt taataatata gctaaactag
tagcaagtaa ttggtataat agacaaatag aaagatctag taggactttg
ggttgctcat gggaatttat tcctgtagat gatggatggg gagaaaggcc
actgtaa

<210> 8

<211> 1987

<212> DNA

<213> *Clostridium botulinum*

<220>

<400> 8

atgat caaagttaat aattgggact tgttttttag tccttcagaa
gataatttta ctaatgatct aaataaagga gaagaaatta catctgatac
taatatagaa gcagcagaag aaaatattag tttagattta atacaacaat
attatttaac ctttaatttt gataatgaac ctgaaaatat ttcaatagaa
aatctttcaa gtgacattat aggccaatta gaacttatgc ctaatataga
aagatttcct aatggaaaaa agtatgagtt agataaatat actatgttcc
attatcttcg tgctcaagaa tttgaacatg gtaaatctag gattgcttta
acaaattctg ttaacgaagc tgctcaagaa tttgaacatg gtaaatctag
gattgcttta acaaattctg ttaacgaagc attattaaat cctagtcgtg
tttatacatt tttttcttca gactatgtaa agaaagttaa taaagctacg
gaggcagcta tgtttttagg ctgggtagaa caattagtat atgattttac
cgatgaaact agcgaagtaa gtactacgga taaaattgcg gatataacta

taattattcc atatatagga cctgctttaa atataggtaa tatgttatat
aaagatgatt ttgtaggtgc tttaatattt tcaggagctg ttattctgtt
agaatttata ccagagattg caatacctgt attaggtact tttgcacttg
tatcatatat tgcgaataag gttctaaccg ttcaaacaat agataatgct
ttaagtaaaa gaaatgaaaa atgggatgag gtctataaat atatagtaac
aaattggtta gcaaaggtta atacacagat tgatctaata agaaaaaaaa
tgaaagaagc tttagaaaat caagcagaag caacaaaggc tataataaac
tatcagtata atcaatatac tgaggaagag aaaaataata ttaattttaa
tattgatgat ttaagttcga aacttaatga gtctataaat aaagctatga
ttaatataaa taaatttttg aatcaatgct ctgtttcata tttaatgaat
tctatgatcc cttatggtgt taaacggtta gaagattttg atgctagtct
taaagatgca ttattaaagt atatatatga taatagagga actttaattg
gtcaagtaga tagattaaaa gataaagtta ataatacact tagtacagat
atacctttttc agctttccaa atacgtagat aatcaaagat tattatctac
atttactgaa tatattaaga atattattaa tacttctata ttgaatttaa
gatatgaaag taatcattta atagacttat ctaggtatgc atcaaaaata
aatattggta gtaaagtaaa ttttgatcca atagataaaa atcaaattca
attatttaat ttagaaagta gtaaaattga ggtaatttta aaaaatgcta
ttgtatataa tagtatgtat gaaaatttta gtactagctt ttggataaga
attcctaagt attttaacag tataagtcta aataatgaat atacaataat
aaattgtatg gaaaataatt caggatggaa agtatcactt aattatggtg
aaataatctg gactttacag gatactcagg aaataaaaca aagagtagtt
tttaaataca gtcaaatgat taatatatca gattatataa acagatggat
ttttgtaact atcactaata atagattaaa taactctaaa taactctaaa
atttatataa atggaagatt aatagatcaa aaaccaattt caaatttagg
taatattcat gctagtaata atataatgtt taaattagat ggttgtagag
atacacatag atatatttgg ataaaatatt ttaatctttt tgataaggaa
ttaaatgaaa aagaaatcaa agatttatat gataatcaat aa

<210> 9

<211> 1327

<212> DNA

<213> *Clostridium botulinum*

<220

<400> 9

atgcaaagat tattatctac atttactgaa tatattaaga atattattaa
tacttctata ttgaatttaa gatatgaaag taatcattta atagacttat
ctaggtatgc atcaaaaata aatattggta gtaaagtaaa ttttgatcca
atagataaaa atcaaattca attatttaat ttagaaagta gtaaaattga
ggtaatttta aaaaatgcta ttgtatataa tagtatgtat gaaaatttta
gtactagctt ttggataaga attcctaagt attttaacag tataagtcta
aataatgaat atacaataat aaattgtatg gaaaataatt caggatggaa
agtatcactt aattatggtg aaataatctg gactttacag gatactcagg
aaataaaaca aagagtagtt tttaaataca gtcaaatgat taatatatca
gattatataa acagatggat ttttgtaact atcactaata atagattaaa
taactctaaa atttatataa atggaagatt aatagatcaa aaaccaattt
caaatttagg taatattcat gctagtaata atataatgtt taaattagat

ggttgtagag atacacatag atatatttgg ataaaatatt ttaatctttt
tgataaggaa ttaaatgaaa aagaaatcaa agatttatat gataatcaat
caaattcagg tattttaaaa gacttttggg gtgattattt acaatatgat
aaaccatact atatgttaaa tttatatgat ccaaataaat atgtcgatgt
aaataatgta ggtattagag gttatatgta tcttaaaggg cctagaggta
gcgtaatgac tacaaatgac tacaaacatt tatttaaatt caagtttgta
taggggaca aaatttatta taaaaaaata tgcttctgga aataaagata
atattgttag aaataatgat cgtgtatata ttaatgtagt agttaaaaat
aaagaatata ggttagctac taatgcatca caggcaggcg tagaaaaaat
actaagtgca ttagaaatac ctgatgtagg aaatctaagt caagtagtag
taatgaagtc aaaaaatgat caaggaataa caaataaatg caaaatgaat
ttacaagata ataatgggaa tgatataggc tttataggat ttcatcagtt
taataatata gctaaactag tagcaagtaa ttggtataat agacaaatag
aaagatctag taggactttg ggttgctcat gggaatttat tcctgtagat
gatggatggg gagaaaggcc actgtaa

<210> 10

<211> 702

<212> DNA

<213> *Clostridium botulinum*

<220>

<400> 10

at ggctcgtctg ctgtctacct tcactgaata catcaagaac
atcatcaata cctccatcct gaacctgcgc tacgaatcca atcacctgat
cgacctgtct cgctacgctt ccaaaatcaa catcggttct aaagttaact
tcgatccgat cgacaagaat cagatccagc tgttcaatct ggaatcttcc
aaaatcgaag ttatcctgaa gaatgctatc gtatacaact ctatgtacga
aaacttctcc acctccttct ggatccgtat cccgaaatac ttcaactcca
tctctctgaa caatgaatac accatcatca actgcatgga aaacaattct
ggttggaaag tatctctgaa ctacggtgaa atcatctgga ctctgcagga
cactcaggaa atcaaacagc gtgttgtatt caaatactct cagatgatca
acatctctga ctacatcaat cgctggatct tcgttaccat caccaacaat
cgtctgaata actccaaaat ctacatcaac ggccgtctga tcgaccagaa
accgatctcc aatctgggta acatccacgc ttctaataac atcatgttca
aactggacgg ttgtcgtgac actcaccgct acatctggat caaatacttc
aatctgttcg acaaagaact gaacgaaaaa gaaatcaaag acctgtacga
caaccagtaa

<210> 11

<211> 621

<212> DNA

<213> *Clostridium botulinum*

<220>

<400> 11

atgtcc aattctggta tcctgaaaga cttctggggt gactacctgc
agtacgacaa accgtactac atgctgaatc tgtacgatcc gaacaaatac
gttgacgtca acaatgtagg tatccgcggt tacatgtacc tgaaaggtcc
gcgtggttct gttatgacta ccaacatcta cctgaactct tccctgtacc
gtggtaccaa attcatcatc aagaaatacg cgtctggtaa caaggacaat
atcgttcgca acaatgatcg tgtatacatc aatgttgtag ttaagaacaa
agaataccgt ctggctacca atgcttctca ggctggtgta gaaaagatct
tgtctgctct ggaaatcccg gacgttggta atctgtctca ggtagttgta
atgaaatcca agaacgacca gggtatcact aacaaatgca aaatgaatct
gcaggacaac aatggtaacg atatcggttt catcggtttc caccagttca
acaatatcgc taaactggtt gcttccaact ggtacaatcg tcagatcgaa
cgttcctctc gcactctggg ttgctcttgg gagttcatcc cggttgatga
cggttggggt gaacgtccgc tgtaa

What is claimed is:

1. A recombinant DNA construct comprising:

(i) a VEE virus replicon vector, and

(ii) at least one nucleic acid comprising a nucleotide sequence encoding a carboxy terminal heavy chain or fragment thereof, selected from the group consisting of: BoNTA, BoNTB, BoNTC, BoNTD, BoNTE, BoNTF, and BoNTG.

2. A recombinant DNA construct according to claim 1 wherein said vector is an expression vector.

3. A recombinant DNA construct according to claim 1 wherein said vector is a eukaryotic vector.

4. The recombinant DNA construct according to claim 1 wherein said construct is p3014-40A.

5. The recombinant DNA construct according to claim 1 wherein said construct is p3014-114a1.

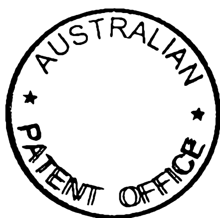
6. The recombinant DNA construct according to claim 1 wherein said construct is p3014-102a2.

7. The recombinant DNA construct according to claim 1 wherein said construct is p3014-73B.

8. The recombinant DNA construct according to claim 1 wherein said construct is p3014-110C.

9. The recombinant DNA construct according to claim 1 wherein said construct is p3014-75E.

10. The recombinant DNA construct according to claim 1 wherein said construct is p3014-77F.



11. The recombinant DNA construct according to claim 1 wherein said construct is p3014-107G.

12. Self replicating RNA produced from any of the constructs selected from the group consisting of: p3014-
5 40a, p3014-114a1, p3014-102a2, p3014-73B, p3014-110C, p3014-75E, p3014-77F and p3014-107G.

13. Infectious alphavirus particles produced from packaging the self replicating RNA of claim 12.

14. A pharmaceutical composition comprising infectious
10 alphavirus particles according to claim 13 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

15. A host cell transformed with a recombinant DNA construct according to claim 1.

15 16. A host cell according to claim 15 wherein said host cell is eukaryotic.

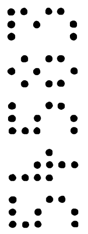
17. A method for producing BoNT Hc protein comprising culturing the cells according to claim 16 under conditions such that said DNA fragment is expressed and
20 said protein is produced.

18. A vaccine for BoNTA comprising viral particles containing one or more replicon RNA encoding one or more BoNTA proteins and, optionally one or more antigens selected from the group consisting of:
25 HcBoNTB, HcBoNTC, HcBoNTD, HcBoNTE, HcBoNTF and HcBoNTG.

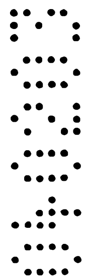
19. A vaccine for BoNTB comprising viral particles

containing one or more replicon RNA encoding one or more BoNTB proteins and, optionally, one or more antigens selected from the group consisting of:
HcBoNTA, HcBoNTC, HcBoNTD, HcBoNTE, HcBoNTF and HcBoNTG.

5 20. A vaccine for BoNTC comprising viral particles containing one or more replicon RNA encoding one or more BoNTC proteins and optionally, one or more antigens selected from the group consisting of:
HcBoNTA, HcBoNTB, HcBoNTD, HcBoNTE, HcBoNTF and HcBoNTG.



10 21. A vaccine for BoNTD comprising viral particles containing one or more replicon RNA encoding one or more BoNTD proteins and, optionally, one or more antigens selected from the group consisting of:
HcBoNTA, HcBoNTB, HcBoNTC, HcBoNTE, HcBoNTF and HcBoNTG.



15 22. A vaccine for BoNTE comprising viral particles containing one or more replicon RNA encoding one or more BoNTE proteins and, optionally, one or more antigens selected from the group consisting of:
HcBoNTA, HcBoNTB, HcBoNTC, HcBoNTD, HcBoNTF and HcBoNTG.

20 23. A vaccine for BoNTF comprising viral particles containing one or more replicon RNA encoding one or more BoNTF proteins and, optionally, one or more antigens selected from the group consisting of:
HcBoNTA, HcBoNTB, HcBoNTC, HcBoNTD, HcBoNTE and HcBoNTG.

25 24. A vaccine for BoNTG comprising viral particles containing one or more replicon RNA encoding one or more

BoNTG proteins and, optionally, one or more antigens selected from the group consisting of:
HcBoNTA, HcBoNTB, HcBoNTC, HcBoNTD, HcBoNTE and HcBoNTF.

25. A pharmaceutical composition comprising the self replication RNA of claim 12 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

26. A pharmaceutical composition comprising one or more recombinant DNA constructs selected from the group consisting of: p3014-40a, p3014-114a1, p3014-102a2, p3014-73B, p3014-110C, p3014-75E, p3014-77F and p3014-107G in a pharmaceutically acceptable amount, in a pharmaceutically acceptable carrier/and or adjuvant.

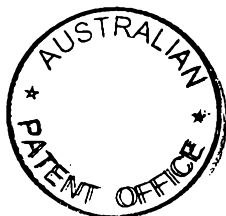
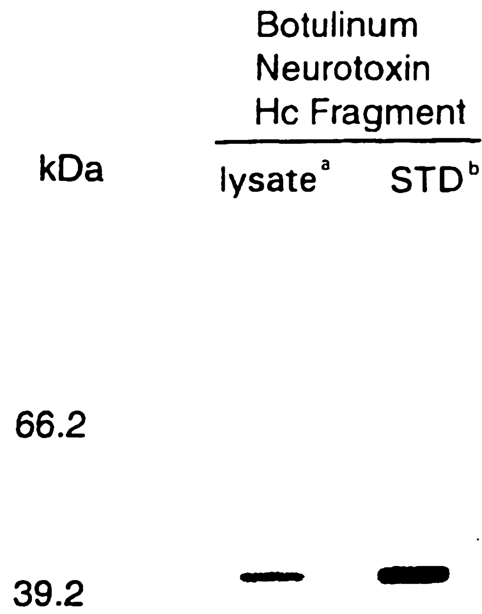


FIG. 1



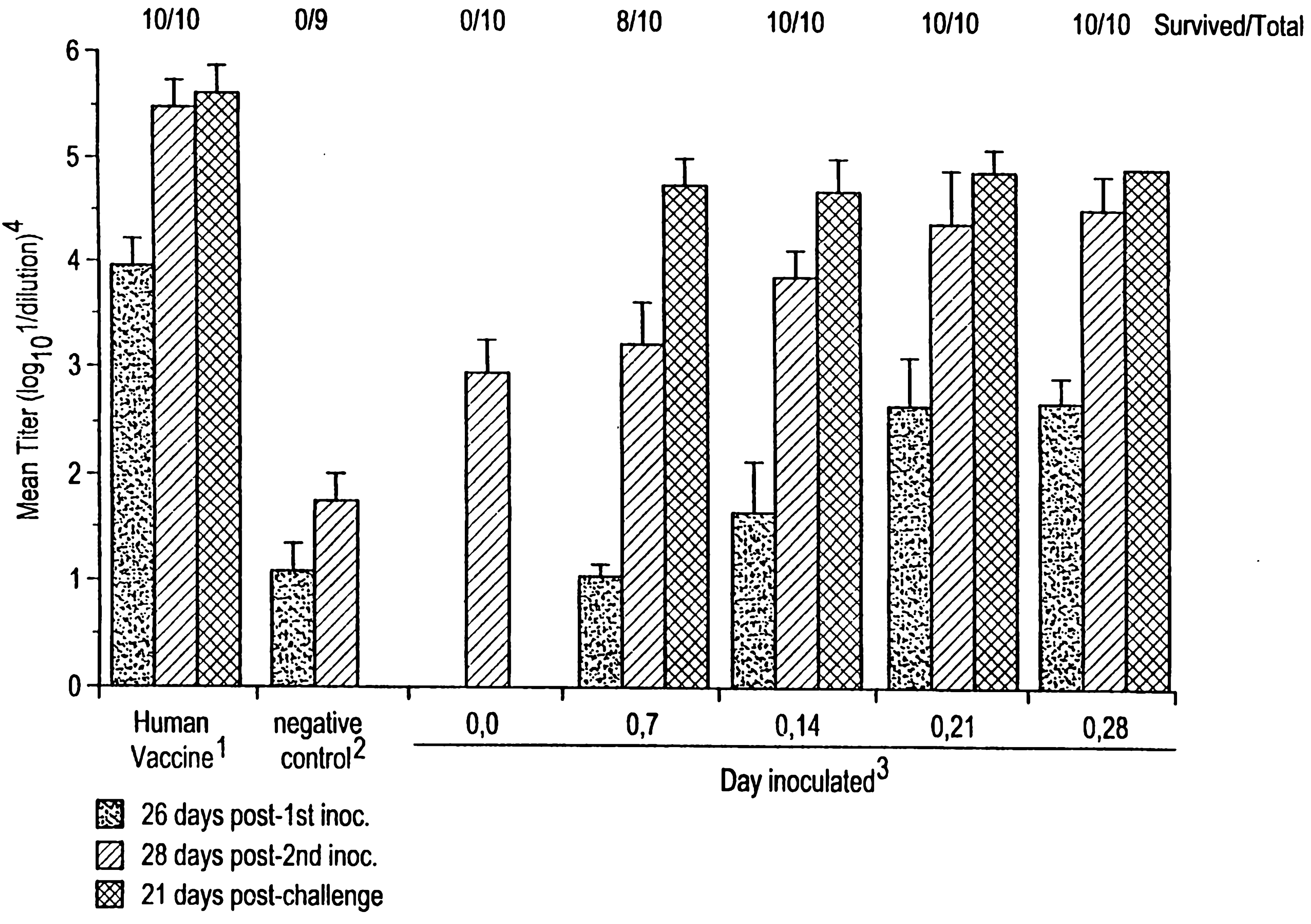


FIG. 2
10/10
0/9
0/10
8/10
10/10
10/10
10/10 Survived/Total
Mean Titer (log10 1/dilution)4
0
1
2
3
4
5
6
Human Vaccine1
negative control2
0,0
0,7
0,14
0,21
0,28
Day inoculated3
26 days post-1st inoc.
28 days post-2nd inoc.
21 days post-challenge

FIG. 3

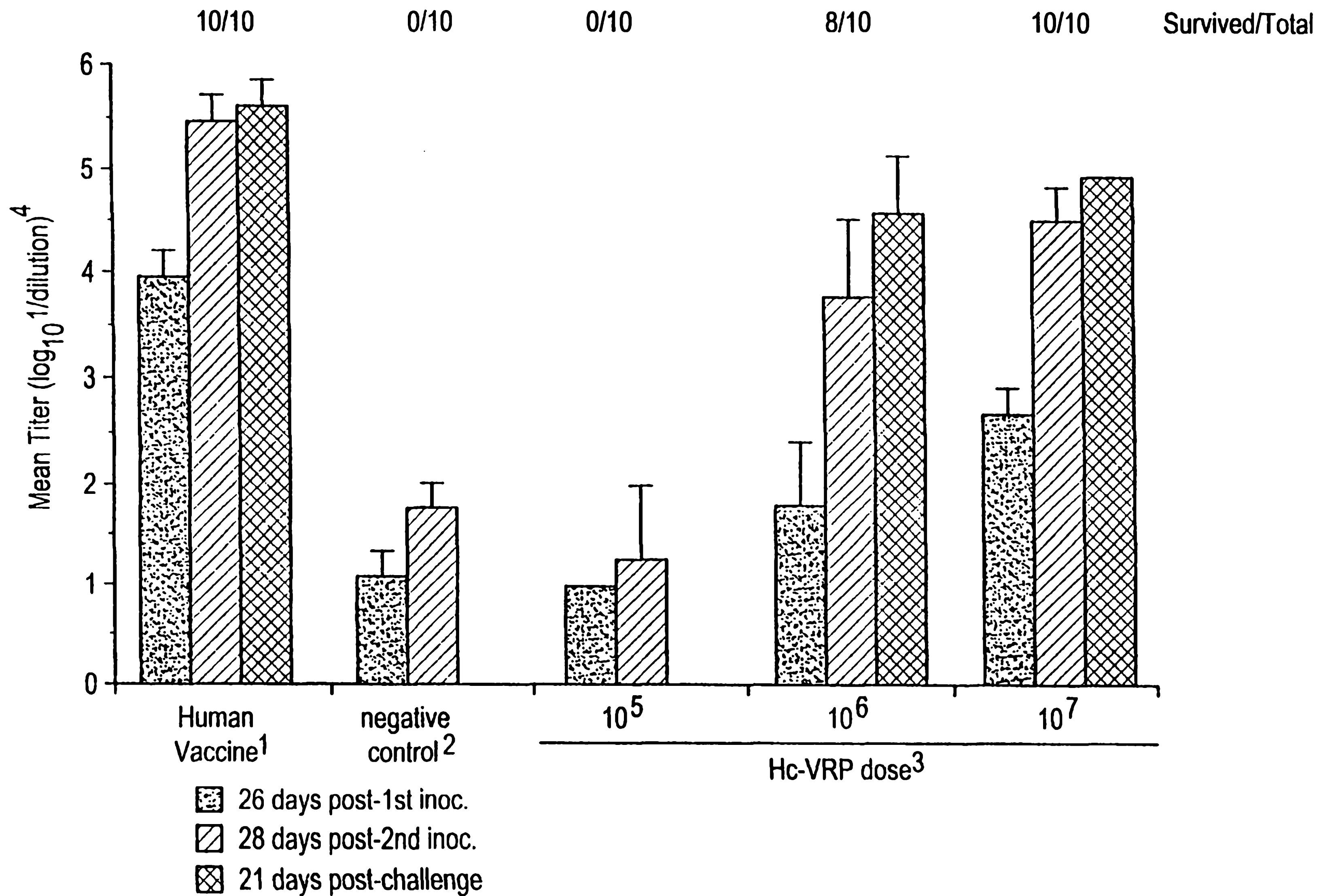
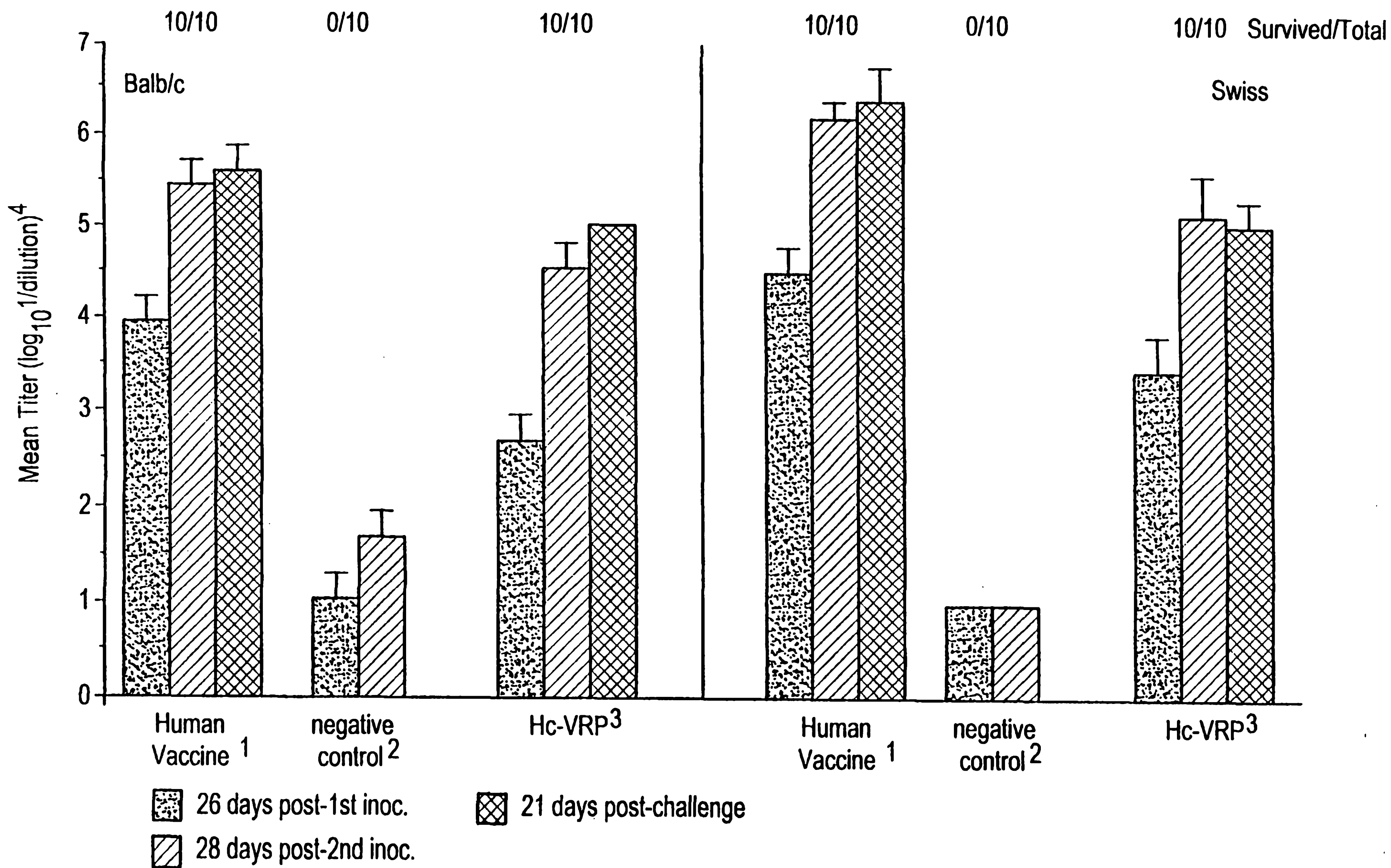


FIG. 4



SUBSTITUTE SHEET (RULE 26)

FIG. 5