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(54) **COMPOSITIONS ET PROCEDES D'APPORT SYSTEMIQUE DE VACCINS ET D'AGENTS THERAPEUTIQUES PAR VOIE ORALE**

(54) **COMPOSITIONS AND METHODS FOR SYSTEMIC DELIVERY OF ORAL VACCINES AND THERAPEUTIC AGENTS**

(57) Cette invention se rapporte à des compositions et à des procédés d'administration par voie orale d'un antigène ou d'un agent thérapeutique dans la circulation générale, au moyen d'une toxine de botulinum modifiée qui est capable de passer de l'intestin dans la circulation générale, et qui est modifiée pour être non toxique.

(57) Compositions and methods of oral delivery of an antigen or therapeutic agent to the general circulation using a modified botulinum toxin which is capable of translocating from the gut to the general circulation but which is altered to be nontoxic are provided.

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(54) Title: COMPOSITIONS AND METHODS FOR SYSTEMIC DELIVERY OF ORAL VACCINES AND THERAPEUTIC AGENTS

(57) Abstract

Compositions and methods of oral delivery of an antigen or therapeutic agent to the general circulation using a modified botulinum toxin which is capable of translocating from the gut to the general circulation but which is altered to be nontoxic are provided.

COMPOSITIONS AND METHODS FOR SYSTEMIC DELIVERY OF ORAL VACCINES AND THERAPEUTIC AGENTS

Introduction

This invention was made in the course of research sponsored by the National Institutes of Health. The U.S. Government may have certain rights in this invention.

Field of the Invention

The present invention relates to compositions and methods for systemic delivery of orally administered vaccines and therapeutic agents via a modified botulinum toxin, wherein said toxin maintains its ability to translocate across the gut wall but has been altered to be non-toxic.

Background of the Invention

Clostridial neurotoxins are the most potent protein toxins known. The neurotoxin produced from *Clostridium tetani* (tetanus toxin) is encountered by humans as a result of open wounds. However, tetanus poisoning at least in industrial countries is no longer a major public health problem due to the availability and widespread use of a safe, effective and inexpensive vaccine. This vaccine is basically a formalin-inactivated culture supernatant from *C. tetani* grown in fermentors.

Botulinum neurotoxin (BoNT), which is produced by the organisms *Clostridium botulinum*, *Clostridium butyricum* and *Clostridium baratii*, is the potent etiologic agent associated with the disease botulism (Simpson, L. *Annu. Rev. Pharmacol. Toxicol.* 1986 26:427-453). Humans are usually exposed to this neurotoxin through food poisoning, although there are rare incidents of wound botulism. A similar vaccine to the tetanus vaccine has been developed to provide protection from botulinum toxin poisoning. However, since there are seven different serotypes of botulinum toxin, complete protection with this inactivated toxin can be afforded only by making seven distinct vaccines and combining them for administration. Presently, only five of the seven serotypes are represented in the botulinum toxin vaccine. Further, some of the serotypes are composed of strains that do not produce high levels of toxin in culture. Thus, growth, purification and inactivation of the toxins for vaccine purposes is time consuming and expensive, owing to the high hazards associated with handling fully active toxin (Clayton et al. *Infection and Immunity* 1995 63(7):2738-2742). At this time this vaccine is only available through the Center of Disease Control for primarily experimental use.

Typically, botulism results from ingestion of food that is tainted with the toxin, or by the ingestion of food contaminated with organisms that can manufacture the toxin in the gut. Regardless of origin, botulinum toxin is synthesized as a relatively nontoxic single chain polypeptide with a molecular weight of approximately 150 kDa. To become fully toxic, it must undergo posttranslational processing, during which the molecule is cleaved by a protease to yield a dichain structure in which a heavy chain (approximately 100,000 daltons) is linked by a disulfide bond to a light chain (approximately 50,000 daltons). The dichain molecule is the holotoxin that accounts for biological activity. BoNT translocates from the gut into the general circulation (lymph and blood) wherein it is then distributed to cholinergic nerve endings which are the target sites of toxin action. The toxin enters these nerves, where it acts as a zinc-dependent endoprotease to cleave polypeptides that are essential for exocytosis (Montecucco, C. and Schiavo, G. *Mol. Microbiol.* 1994 13:1-8). Cleavage of these polypeptides leads to blockade of transmitter release and paralysis.

The heavy chain of the toxin is believed to be essential for binding and translocation of the toxin from the outside to the inside of the cholinergic nerve endings, while the light chain possesses the zinc-dependent endoprotease activity that accounts for the ability of the toxin to poison cholinergic nerve endings (Neimann et al. *Behring Inst. Mitt.* 1991 89:153-162). Accordingly, vaccines against botulism comprising a nontoxic 50 kDa carboxyterminal fragment of *Clostridium botulinum* have been described. LaPenotiere et al. *Toxicon* 1995 33(10):1383-6 and Clayton et al. *Infection and Immunity* 1995 63(7):2738-2742. Further, it has been suggested that this highly selective neurotoxin and tetanus toxin may be converted into nontoxic therapeutic tools that can be applied in delivery of drugs, hormones, enzymes or antiviral substances to the central nervous system.

Summary of the Invention

An object of the present invention is to provide a modified botulinum toxin which maintains its ability to translocate from the gut into the general circulation but which is nontoxic. The modified botulinum toxin can be used as an oral vaccine for antigenic peptides including botulinum toxin and for the oral delivery of other therapeutic agents to the general circulation.

Brief Description of the Drawings

Figure 1 is diagram of the native botulinum toxin. This figure illustrates the light chain with the zinc binding motif linked by a disulfide bond to the heavy chain of the native toxin.

Figure 2 is a diagram illustrating an example of a modified botulinum toxin of the present invention. This figure illustrates the light chain with a modified zinc binding motif linked to the intact heavy chain of botulinum toxin.

Detailed Description of the Invention

One of the major challenges of modern medicine is the development of drugs that can be administered by the oral route. The development of oral peptide vaccines that evoke systemic immunity has proven to be especially problematic. Difficulties associated with the development of oral peptide vaccines include: degradation upon exposure to conditions of low pH and proteolytic enzymes found in the human gut; the antigenic domain of the agent which produces the illness being too large to allow for significant non-specific diffusion from the lumen of the gut to the general circulation; and an inability to design peptide vaccines that will bind exploitatively to receptors in the gut and undergo active transport to the general circulation. Despite these difficulties, considerable effort is being invested in the search for oral vaccines. For example, the concept of using engineered food such as a potato or a banana as a vector for widescale vaccination has recently been proposed. However, engineering the antigenic peptide into a food which is then ingested does not overcome these difficulties. Accordingly, there is a need for drug delivery vehicles which will reliably and reproducibly translocate an antigenic peptide or other therapeutic agent from the gut to the general circulation.

The present invention provides a modified botulinum toxin which can be used as an oral delivery vehicle for antigenic peptides including, but not limited to, botulinum toxin and other therapeutic agents to the general circulation. It has now been found that botulinum toxin translocates from the gut to the general circulation by binding to serospecific receptors on the mucosal side of polarized gut cells grown in a monolayer. Bound toxin is actively transported across the cells and delivered intact and unmodified on the serosal side of the monolayers. It has been suggested that auxiliary proteins such as hemagglutinin, which is a component of the non-covalent complex of proteins including the botulinum toxin which is released by *Clostridium*, may mediate binding and transport of the toxin across the gut wall. However, experiments performed with a recombinant form of the holotoxin now demonstrate that the botulinum toxin itself possesses the binding domain that recognizes receptors on the surface of gut cells. Further, it has now been demonstrated that modifications can be made to the light chain of the toxin to render it nontoxic without altering the capability of the protein to translocate from the gut to the general circulation. Accordingly, for the purposes of the present invention, by "modified botulinum toxin" is meant a botulinum toxin which maintains its capability of translocating from the gut to the general circulation but which is nontoxic. Alterations which will render the botulinum toxin nontoxic include mutations to the amino acid sequence of the light chain and deletion of the light chain or portions thereof. In a preferred embodiment, mutations are made to the zinc binding motif or the substrate binding motif of the light chain. For the purposes of this invention, by "nontoxic" it is meant that exposure of the cholinergic nerve endings to the modified botulinum toxin does not result in blockade of transmitter release in the nerve endings and paralysis. The effects of alterations rendering the botulinum toxin nontoxic on the ability of the toxin to translocate from the gut to the general circulation can be routinely performed in accordance with the teachings provided herein so that one of skill may identify modified botulinum toxins of the present invention. Included within this definition of modified botulinum toxins are botulinum toxins which further comprise a selected antigen for a protein other than botulinum toxin or a therapeutic agent.

For example, compositions were prepared comprising a botulinum neurotoxin in which the zinc binding motif of the light chain of the holotoxin was inactivated. The modified toxin is nontoxic because the holotoxin does not retain the ability to cause neuromuscular blockade, but the modification to the light chain does not adversely affect the ability of the remainder of the toxin molecule to escape the lumen of the gut into the general circulation. In this preferred embodiment at least three of the amino acids comprising the zinc binding motif of the light chain were modified. Specifically, the amino acids His (at position 229), Glu (at position 230), and His (at position 233) of the native sequence were substituted with amino acids Gly, Thr and Asn, respectively, resulting in SEQ ID NO: 1. The nucleic acid sequence encoding this modified botulinum toxin is depicted as SEQ ID NO: 2. These amino acid substitutions eliminated the ability of the holotoxin to bind catalytic zinc or other divalent cations.

Experiments have also been performed demonstrating that unnicked or single chain botulinum toxin also binds and is transported across the gut wall. Accordingly, modified botulinum toxins of the present invention also include compositions wherein the nicking site has been eliminated.

The biological activity of a modified botulinum toxin of the present invention was determined via an *in vivo* toxicity test, *in vitro* activity on the mouse phrenic nerve-hemidiaphragm preparation, and enzymatic activity in crude synaptosome preparations. For these experiments, the modified botulinum toxin, referred to herein as modified recombinant or modified rBoNT/C, was generated from botulinum toxin serotype C using site-directed mutagenesis to inactivate the zinc binding motif from the light chain of the holotoxin that is essential for endoprotease activity. However, other methods of peptide synthesis including, but not limited to, biochemical techniques, such as enzymatically cutting a peptide and cross linking the resulting fragments which are performed routinely by those of skill in the art can also be used. Further, given the structural and functional similarities of the botulism serotypes, one of skill could routinely prepare modified botulinum toxins from serotypes other than botulinum serotype C. For example, all serotypes of botulinum toxins are synthesized as relatively inactive precursors with molecular weights of approximately 150,000. In each case, the precursors must be "nicked" by a protease to generate a dichain molecule having a heavy chain (100,000 kDa) linked by a disulfide bond to a light chain (50,000 kDa). Every serotype of botulinum toxin acts preferentially on cholinergic nerve endings to block transmitter release, with the heavy chain acting principally as a tissue-targeting domain to direct the toxin to cholinergic nerve endings, and the light chain acting inside the nerve ending to block transmitter release. It is the light chain of every serotype that acts as a zinc-dependent metalloendoprotease to cleave one or more members of a family of polypeptides that is essential for transmitter release. In every serotype, there is a zinc binding motif, His-Glu-X-X-His (SEQ ID NO: 3) that is essential for enzymatic activity. Modification of the binding motif invariably causes loss of enzymatic activity. Further, alignment of the nucleic acid and amino acid sequences for a portion of each serotype encompassing the region of the zinc binding motif demonstrates a high degree of sequence identity in the regions adjacent to and comprising the zinc binding motif. Thus, examples using botulinum serotype C are representative of the entire class.

In vivo toxicity testing of modified rBoNT/C holotoxin demonstrated that the modified botulinum toxin with mutations in the zinc binding motif produced no acute toxicity in mice during a 16 week monitoring period following administration, even at high doses (10 μg per animal, i.p.). No apparent neurotoxicity or other obvious harmful effects were observed in any of the animals. In contrast, mice injected with 100 ng i.p. native BoNT/C died within 2 to 2.5 hours of injection.

The *in vitro* toxicity of modified BoNT/C holotoxin was also compared with that of native BoNT/C in mouse phrenic nerve-hemidiaphragm preparations. It was found that the addition of the modified botulinum toxin to phrenic nerve-hemidiaphragm preparations did not produce neuromuscular blockade (1×10^{-10} M; n=4). By contrast, addition of native BoNT/C (1×10^{-12} M; n=8) invariably produced paralysis of transmission (mean ± S.E.M. = 152 ± 17 min).

The ability of this modified botulinum toxin to evoke an immune response was also tested after oral (p.o.) administration and subcutaneous (s.c.) injection. As determined by immunoblot analysis, both p.o. and s.c. administration of modified rBoNT/C holotoxin evoked systemic antibody production. Accordingly, the modified botulinum toxin of the present invention maintained its ability survive transit through the gut and to undergo active translocation out of the gut. This is further evidenced by the finding that s.c. administration of a non-homogeneous preparation of the modified botulinum toxin, which contained small amounts of unrelated proteins, is able to evoke an immune response against these unrelated proteins, while p.o. administration evoked antibody only against the modified botulinum toxin.

The protective effect of the antibodies elicited by p.o. and s.c. administration of the modified botulinum toxin was then demonstrated in both serum neutralization and *in vivo* toxicity tests. Regardless of the route of administration, serum from animals immunized with modified botulinum toxin inactivated a large dose (~10,000 LD_{50}) of native BoNT/C. Similarly, in *in vivo* toxicity tests, immunization with the modified botulinum toxin by either the p.o. or s.c. route produced a dramatic reduction in the potency of a subsequent injection of native toxin. Animals given the modified botulinum toxin by the oral route of administration had detectable antibodies in serum for at least three months. Further, animals that received the modified botulinum toxin either p.o. or s.c. were protected against native BoNT/C challenge three months after the third booster.

Accordingly, results from these experiments demonstrate that a modified botulinum toxin can be constructed in accordance with the teachings provided herein that is nontoxic but which retains the ability to translocate from the gut to the general circulation and to evoke protective antibodies. Further, compositions comprising a modified botulinum toxin of the present invention are clearly effective as oral vaccines against botulism in animals.

In addition, because the modified botulinum toxins of the present invention retain their ability to translocate from the gut and to be delivered intact to the general circulation, these modified botulinum toxins can be used as delivery vehicles for oral administration of antigens to proteins other than botulinum toxin and therapeutic agents to the general circulation. There are various ways in which the modified botulinum toxin could be used as a carrier for oral vaccines. For example, because the inactivation of the zinc binding motif of the light chain does not adversely affect the toxin's ability to translocate out of the gut, the zinc binding motif of the native botulinum toxin can be replaced with a selected antigen for a different protein, i.e. a protein other than botulism, to produce an oral vaccine against this different protein. Alternatively, well known techniques of protein chemistry and molecular biology can be used to attach the selected antigen or a portion thereof to a modified botulinum toxin. The resulting modified botulinum toxin would not only be nontoxic, but also retain its ability to translocate from the gut to the general circulation so that the selected antigen, when administered orally, would reach the general circulation to evoke a systemic immune response against the protein. Examples of vaccines which could be administered orally with the modified botulinum toxin include, but are not limited to, vaccines for *Bacille Calmette-Guerin*, cholera, diphtheria, hepatitis B, measles, meningitis, mumps, pertussis, plague, polio, rabies, rubella, tetanus, typhoid, and yellow fever. The oral vaccine can be administered individually or in combination, such as for DTP (diphtheria, tetanus, pertussis). The ability to deliver an oral vaccine is especially important for areas in which medical personnel are not readily available. Moreover, an oral vaccine of the present invention would represent an important economic advantage in addition to diminishing the need for skilled personnel as it would eliminate costs associated with syringes used for injection and/or for the disposal of used syringes.

Formulations of oral vaccines of the present invention preferably comprise the modified botulinum toxin in a pharmacologically acceptable carrier, such as sterile physiological saline, sterile saline with 0.1% gelatin, or sterile saline with 1.0 mg/ml bovine serum albumin. Alternatively, the modified botulinum toxin of the present invention can be genetically engineered into a plant so that food produced by the plant such as a potato or a banana can serve as a vector for widescale vaccination. Methods of genetically engineering plants to express a foreign peptide are well known in the art as exemplified by PCT/US96/09558, filed June 6, 1996.

The modified botulinum toxins of the present invention are also useful in the construction of chimeric oral therapeutics. In this embodiment, a therapeutic agent can be linked to modified botulinum toxin to yield two broad groups of orally administered molecules: (1) new drugs with biologically stable linkages, and (2) conjugate prodrugs having biologically or chemically unstable linkages, which dissociate from the carrier upon reaching the blood. Examples of chimeric therapeutic techniques are described generally by Lautenslager, G.T. and Simpson, L.L., "Chimeric Molecules

Constructed with Endogenous Substances," *Advances in Molecular and Cell Biology*, Vol. 9, pp. 233-262, JAI Press, Inc. (1994). For example, a therapeutic peptide could be attached to a modified botulinum toxin, thus creating an agent which possesses the characteristics of the substituent yet is capable of being administered orally. One example would be the creation of an orally administered thrombolytic agent. A fusion protein constructed by combining P-selectin and tissue plasminogen activator (TPA) is a promising chimera which expresses thrombolytic activity and targets to the thrombi. This chimera must be introduced into the blood stream. However, using either molecular biology or protein chemistry, this 'first order' chimeric molecule could be attached to a modified botulinum toxin of the present invention to create a higher order chimera which possesses the added advantage of being delivered to the general circulation by oral administration. Another example is in the design of an orally administered anti-neoplastic drug. Various antineoplastic drugs which exploit the cytotoxic properties of one molecule, fused to a portion of another which functions to specifically target the toxin have been disclosed. A more recent example employs the amino-terminus of *Pseudomonas exotoxin* (PE) fused to epidermal growth factor (EGF), resulting in chimera EGF-PE which can be used as a cytotoxic agent towards EGF-receptor-bearing cancer cells. Linkage of this chimera to a modified botulinum toxin of the present invention would result in creation of a higher order chimera which can be administered orally.

The general concepts for use of a modified botulinum toxin as a carrier for vaccines or other therapeutic agents are the same for human and for non-human animals, with one exception. All serotypes of botulinum toxin are not likely to be equally efficacious as carriers for drugs in all species. Clinical evidence suggests that humans are especially sensitive to the effects of serotypes A, B, and E.

This may relate to the efficiency with which these three serotypes are absorbed from the gastrointestinal system. Thus, serotypes A, B, and E would be preferred carriers of therapeutic agents for humans.

On the contrary, most non-human animals are particularly sensitive to serotype C. This suggests that as to veterinary medicine, the preferred carrier of therapeutic agents for non-human animal use would be serotype C. Examples of animal vaccines which could be administered orally with the modified botulinum toxin include, but are not limited to, ones for adenovirus type 2, *Bordetella bronchispetica*, botulism, calicivirus, *Chlamydia psittaci*, clostridial diseases, such as *Clostridium Perfringens* type C, coronaviruses, distemper, equine encephalomyelitis, *Escherichia coli*, feline infectious peritonitis, feline leukemia virus, feline panleukopenia, hepatitis, leptospirosis, parainfluenza virus, parvoviruses, rabies, rhinotracheitis virus, and tetanus.

The following examples are provided for illustrative purposes only and are not intended to limit the invention.

EXAMPLES

Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). The expression vector pQE-30 and nickel-nitrilotriacetic acid (Ni-NTA) Agarose were purchased from QIAGEN (Chatsworth, CA). Monoclonal antibodies (mAb) specific for the 6xHis affinity tag were purchased from QIAGEN. Anti-syntaxin mAbs (S-0664; anti-HPC-1) were purchased from SIGMA (St. Louis, MO), and horse anti-BoNT/C antibodies was obtained from the Centers for Disease Control (CDC, Atlanta, GA). Plasmids pCL8 and pCH3 carrying *Eco*RI fragments of BoNT/C DNA have been described previously by Kimura et al. *BBRC* 1990 171:1304-1311.

Example 1: Construction of expression vectors for synthesis rBoNT/C holotoxin

A schematic representation of the native botulinum toxin is depicted in Figure 1. A schematic representation of a modified botulinum toxin, rBoNT/C is depicted in Figure 2. The nucleic acid and protein sequences for the modified botulinum toxin, rBoNT/C are depicted in SEQ ID NO: 2 and SEQ ID NO: 1, respectively.

Techniques for DNA fragment isolation, repair of overhanging ends with the Klenow fragment of DNA polymerase I, and ligation with T4 ligase are known to those skilled in the art and have been described, for example, by Sambrook et al., 1989 *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. All cloning steps and expression were performed in *Escherichia coli* strain M-15 (QIAGEN) containing the pREP4 repressor plasmid.

The gene encoding a recombinant modified botulinum toxin was assembled from three separate toxin fragments (fragments I, II and III) generated using PCR and ligated into vector pQE-30 resulting in plasmid pQE-TC1. Initially, a DNA fragment coding for the amino-terminal portion of BoNT/C (fragments I and II) was amplified from plasmid pCL8 in two sequential steps to generate pBot C2. DNA fragment I (nt 4-689) was amplified using the following pair of oligonucleotide primers:

(forward) 5'-CCCAATAACAATTAACAACTTTAAT-3' (SEQ ID NO: 4)

*Kpn*I

(reverse) 5'-TTTGGTACCCATTAAAATTAGTATTGGATCCAT-3' (SEQ ID NO: 5)

One cytosine was added to the 5'-end of the forward primer to provide for reconstruction of the BamHI restriction site, as well as to clone rBoNT/C DNA in frame with the pQE-30 initiation of translation methionine.

A *Kpn*I restriction site was included in the reverse primer to generate amino acid mutations His^{229}→Gly and Glu^{230}→Thr at the 3' end of fragment I. Amplified fragment I was treated with T4 polymerase, cut with *Kpn*I and inserted between the Klenow filled-in *Bam*HI and *Kpn*I sites of the expression vector pQE-30, resulting in plasmid pBot C1. DNA fragment II (nt 689-1633) was then amplified using oligonucleotide primers:

*Kpn*I
(forward) 5'-TTTGGTACCCTTAATAATGCAATGCATAATTTATATGGA-3' (SEQ ID NO: 6)

*Eco*RI
(reverse) 5'-GAATTCAAATAATCAACATTTTGAG-3' (SEQ ID NO: 7)

In the forward primer nucleotide changes were introduced to create a *Kpn*I site and generate amino acid mutations His^{229}→Gly, Glu^{230}→Thr, and His^{233}→Asn at the 5' end of fragment II. The reverse primer was complementary to the BoNT/C sequence and contained an internal *Eco*RI site at nucleotide position 1633. Amplified fragment II was treated with T4 polymerase, cut with *Kpn*I and inserted between the *Kpn*I and Klenow filled-in *Sal*I sites of pBot C1. The resulting plasmid pBot C2 contained the 5'-terminal fragment of BoNT/C (nt 4-1633) in frame with the ATG codon and 6xHis affinity sequence of pQE-30.

DNA fragment III (nt 1633-3873) coding for the carboxy-terminal domain of BoNT/C was amplified from plasmid pCH3 using oligonucleotide primers

*Eco*RI
forward 5'-TTTGAATTCTTATTATTACCTAGAATC-3' (SEQ ID NO: 8)

*Sac*I
reverse 5'-TTTGAGCTCTTATTCACTTACAGGTACAAAAC-3' (SEQ ID NO: 9)

The forward primer was complementary to the BoNT/C sequence and contained an internal *Eco*RI site at position

1632. In the reverse primer, a *Sac*I restriction site was introduced immediately downstream of the stop codon. Amplified fragment III was digested with *Eco*RI and *Sac*I and cloned separately into *Eco*RI and *Sac*I digested plasmid pQE-30 generating plasmid pBot C3. Finally, the DNA encoding a full-size, modified botulinum toxin was reconstructed by introducing the *Eco*RI-*Eco*RI fragment (nt -88 to +1632) from plasmid pBot C2 into *Eco*RI digested, calf intestine alkaline phosphatase dephosphorylated plasmid pBot C3 to give plasmid pQE-TC1. All PCR fragments were reanalyzed by DNA sequencing.

The oligonucleotide primers were designed to engineer a *KpnI* restriction site in the segment of DNA encoding the zinc-binding motif. The creation of the *Kpn*I restriction site in this DNA segment enabled the mutation of three amino acids ($His^{229} \rightarrow Gly$; $Glu^{230} \rightarrow Thr$ and $His^{233} \rightarrow Asn$) that are essential for zinc binding, and provided for the reconstruction of a DNA encoding a modified botulinum toxin without preliminary cloning of wild type BoNT/C DNA. The recombinant modified botulinum toxin, synthesized from plasmid pQE-TC1, contained eleven additional amino acids, Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser (SEQ ID NO: 10), at the amino-terminus.

Example 2 Optimization of neurotoxin expression

PCR was used to modify a sequence of the pQE-30 vector preceding the structural gene encoding modified rBoNT/C. A new forward primer, 5'-CGGTACCATGCCAATAACAATTAACAACTTT-3' (SEQ ID NO: 11), containing ten additional nucleotides on the 5'-end and a new reverse primer,

*Bgl*II

5'-AGCTATAGATCTATAATAATCCAA-3'

(SEQ ID NO: 12) covering the *Bgl*II restriction site at position 892 of the BoNT/C sequence (Kimura et al. *Biochem. Biophys. Res. Comm.* 1990 171:1304-1311) were used to reamplify a DNA fragment coding for the amino-terminal portion of the rBoNT/C. The amplified fragment was treated with T4 polymerase, cut with *Bgl*II and inserted between the Klenow filled in *Bam*HI and *Bgl*II sites of pQE-TC1 to give plasmid pQE-TC2.

Example 3 Expression and purification of modified rBoNT/C holotoxin

Cultures were grown in Lennox L broth at 37°C, with shaking, to an A_{600} of 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside was added to 1.0 mM final concentration, and incubation continued for an additional 5 hours. Bacteria from 1 liter of induced culture were harvested by centrifugation at 4°C and resuspended in 20 ml of 50 mM sodium phosphate buffer, pH 7.4, with 300 mM NaCl. The cell suspension was lysed, on ice, by sonication, with 2 pulses of 1 minute duration each at 75% power, using a Model 60 Sonic Dismembrator (Fisher Scientific, Malvern, PA). Lysates were centrifuged at 20,000 x g for 30 minutes at 4°C. The clarified supernatants were mixed with 1 ml of packed Ni-NTA resin, incubated for 1 hour at 4°C on a rotator and finally poured into a 25 ml column. The column was washed with 30 volumes of washing buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 25 mM imidazole). Bound proteins were eluted with elution buffer (50 mM sodium phosphate, pH 4.5, 300 mM NaCl). Purified proteins were analyzed on sodium dodecylsulfate polyacrylamide gels (SDS-PAGE).

Example 4 Immunoblot Analysis

The ability of *E.coli* to drive expression of a recombinant modified botulinum toxin from plasmid pQE-TC1 was examined by immunoblot analysis of cell extract. Proteins for analysis by Western blotting were separated on 10% polyacrylamide gels according to the method of Laemmli, U.K. *Nature* 1970 22:680-685, transferred to nitrocellulose, and processed for detection of immunoreactive proteins containing the 6xHis affinity tag. Incubations with primary antibodies were performed for 1 hour at 37°C with a 1:2000 dilution of the anti-6xHis affinity tag mAb, or with anti-BoNT/C antibodies. Membranes were developed using enhanced chemiluminescence according to manufacturers instructions (ECL; Amersham Corp., Arlington Heights, IL). The synthesis of recombinant proteins was induced with IPTG and aliquots of solubilized cells were run on SDS-PAGE.

Western blot analysis with anti-6xHis tag or anti-BoNT/C antibodies revealed an extremely low level of expression. Accordingly, a new plasmid was constructed which did not contain the stretch of four cytosine nucleotides which originated from cloning of neurotoxin DNA into the *Bam*HI site of pQE-30 vector and designated pQE-TC2. Western blot analysis with anti-6xHis tag antibody revealed that pQE-TC2 was more efficient at driving the synthesis of modified rBoNT/C holotoxin. Indeed, 1-2 mg of modified rBoNT/C holotoxin could be purified from 1L of Lennox broth.

Modified rBoNT/C holotoxin was synthesized in soluble form, without visible degradation, but unlike *Clostridium botulinum* the *E. coli* did not provide for efficient nicking of modified rBoNT/C holotoxin. Only trace amounts of L-chain were detectable in modified rBoNT/C holotoxin by Coomassie staining or Western blotting. However, modified rBoNT/C holotoxin was efficiently nicked with immobilized TPCK-trypsin (Pierce, Rockford, IL) and produced heavy and light chains of the correct molecular weight. Modified rBoNT/C holotoxin synthesized from pQE-TC2 contained fourteen additional amino acids (Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-Gly-Thr (SEQ ID NO: 13)) at the amino terminus. The 6xHis sequence within this fourteen amino acid segment was used for purification and subsequent detection of synthesized protein. The recombinant protein produced in this manner was purified by affinity chromatography on Ni-NTA resin using the 6xHis affinity tag. Specifically bound protein was eluted with low pH (elution buffer pH 4.5) and analyzed on SDS-PAGE. Analysis of protein eluted from the affinity resin revealed that toxin could be purified to a homogeneity of 80% - 90%. The purified modified recombinant BoNT/C or modified rBoNT/C was used for all studies presented herein.

Example 5 Bioassay of recombinant proteins

As described in the examples that follow, the purified recombinant proteins was assayed for biological activity using an *in vivo* toxicity test, *in vitro* activity on the mouse phrenic nerve-hemidiaphragm preparation, and enzymatic activity in crude synaptosome preparations.

A. *In vivo* toxicity testing

The toxicity of modified rBoNT/C holotoxin was tested. Modified rBoNT/C holotoxin purified by elution from the histidine affinity resin was diluted in PBS including 1 mg/ml BSA and injected intraperitoneally (i.p.) into mice. The rBoNT/C holotoxin was administered in a 100 μl aliquot of PBS-BSA at a concentration of 10 μg per animal having an average weight of 25 g. The animals were monitored for a total of 16 weeks to rule out any non-specific toxicity.

B. *In vitro* toxicity testing

Toxicity was bioassayed on the mouse phrenic nerve-hemidiaphragm preparations using the method of Simpson et al. *J. Pharmacol. Exp. Ther.* 1990 254:98-103. Tissues were excised and suspended in physiological buffer that was aerated with 95% O_2, 5% CO_2 and maintained at 35°C. The physiological solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; NaH_2PO_4, 1.0; D-glucose, 11; and gelatin, 0.01%. Phrenic nerves were stimulated continuously (1.0 Hz; 0.1-0.3 millisecond duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a 50% reduction in muscle twitch response to neurogenic stimulation.

C. Cleavage of substrate

Synaptosomes (1 mg/ml) were prepared according to the method of Rosahl et al. Cell 1993 75:661-670. The synaptosomes were incubated in the presence of modified rBoNT/C holotoxin (100 nM) for 90 minutes at 37°C in Tris-buffered saline (TBS) or in TBS containing 10 mM dithiothreitol. In parallel experiments, synaptosomal membranes were incubated in the presence and absence of native BoNT/C. The proteins were separated on 15% SDS-PAGE, transferred to nitrocellulase, and processed for detection of immunoreactive proteins with anti-syntaxin mAb.

Example 6 Serum antibody response in mice immunized with modified rBoNT/C holotoxin

Swiss-Webster female mice weighing approximately 25 grams (Ace Animals, Boyertown, PA) were immunized in parallel experiments either, s.c. or p.o., with rBoNt/C holotoxin or TBS, to assess the ability of this peptide to evoke a serum immune response.

A. Immunization and sample collection

For s.c. injection each animal received 2 μg protein in 0.1 ml of elution buffer. For the oral administration route, each animal was fed 4 μg of protein in 0.2 ml elution buffer administered through an intragastric feeding needle. Mice were immunized on day zero, and boosters were given on days 14, 28, and 42. Samples of serum from identically immunized mice were collected and pooled on days 21, 35, and 49 after immunization. For collection of serum, mice were bled with heparinized capillary tubes at the retro-orbital plexus while under isoflurane anesthesia.

B. Assay of serum for antibody production

Sera from immunized or control mice were assayed for antibodies using immunoblot analysis for immunoreactivity to unnicked modified botulinum toxin. Recombinant antigen (modified botulinum toxin; 0.1 μg/lane) was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat powdered milk in TBS, cut into strips and processed for detection of immunoreactive proteins using various serum samples. Primary incubations were performed overnight (18 hours) at room temperature with a 1:1000 diluted serum. A secondary horseradish peroxidase-labeled anti-mouse IgG was used at a 1:10,000 dilution for 1 hour at room temperature. After extensive washing, membranes were developed using ECL (Amersham).

Example 7 Neutralizing activity of serum from immunized mice

Experiments were performed to assess the ability of various serum samples to neutralize native BoNT/C. Three different sources of serum were tested, as follows: 1) non-immune serum, 2) serum from animals that had received modified rBoNT/C holotoxin p.o., and 3) serum from animals that had received modified rBoNT/C holotoxin s.c. Native BoNT/C (10 μl, 100 ng) was incubated with 10 μl of pre-immune or immune serum at 37°C for 1 hour, or with PBS-BSA. Subsequently, the incubation mixture was diluted with 80 μl PBS including 1 mg/ml BSA and injected i.p. The mice were monitored for 48 hours to assess any residual toxicity of the various mixtures.

Example 8 Protection of mice against challenge with native BoNT/C

Three months after administration of the third booster, mice immunized with rBoNT/C were challenged with an i.p. dose of 100 ng native BoNT/C per animal. The survival of challenged animals was monitored for 5 days.

The disclosures of each and every, patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While the invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

SEQUENCE LISTING

<110> SIMPSON, LANCE
KIYATKIN, NIKITA
MAKSYMOWYCH, ANDREW

<120> COMPOSITIONS AND METHODS FOR SYSTEMIC DELIVERY OF ORAL VACCINES AND THERAPEUTIC AGENTS

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What is claimed is:

1. A modified botulinum toxin comprising a botulinum toxin capable of translocating from the gut to the general circulation which is altered to be nontoxic.

2. The modified botulinum toxin of claim 1 further comprising a selected antigen.

3. The modified botulinum toxin of claim 1 further comprising a therapeutic agent.

4. An oral vaccine against botulism comprising the modified botulinum toxin of claim 1 and a pharmaceutically acceptable vehicle.

5. An oral vaccine against a selected antigen comprising the modified botulinum toxin of claim 2 and a pharmaceutically acceptable vehicle.

6. A method of orally delivering a therapeutic agent to an animal comprising administering to the animal a modified botulinum toxin of claim 3.

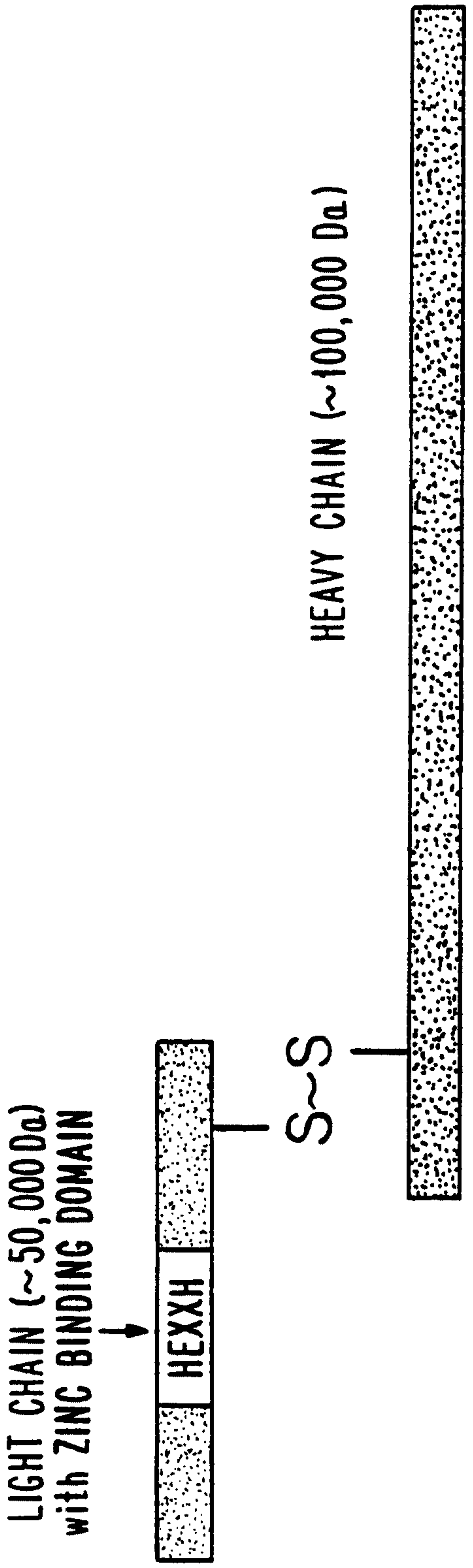
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Fig.1

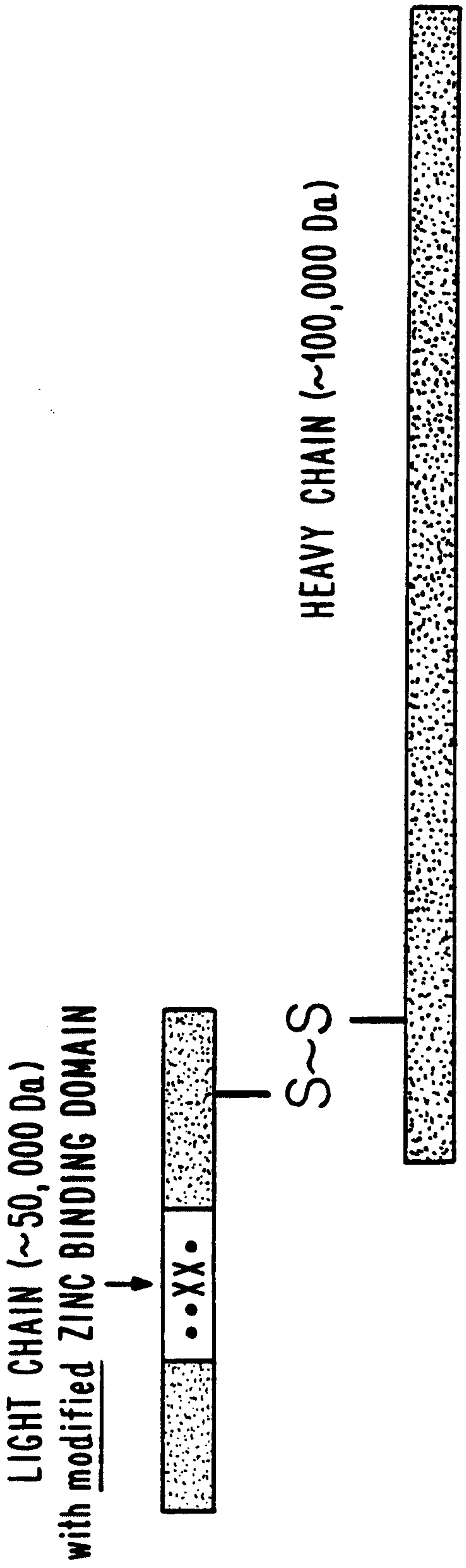
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Fig.2