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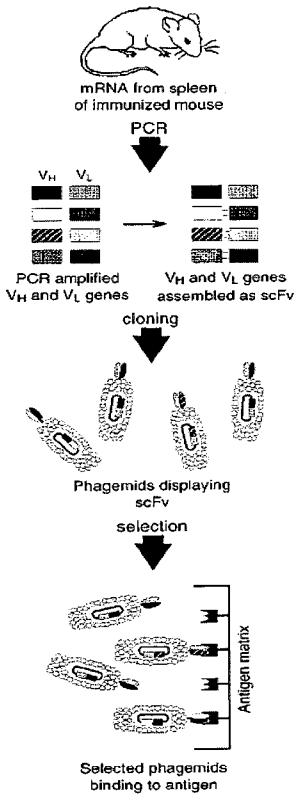

Fig. 1

(57) Abstract: This invention provides antibodies that specifically bind to and typically neutralize botulinum neurotoxins (e.g., BoNT/A, BoNT/B, BoNT/E, etc.) and the epitopes bound by those antibodies. The antibodies and derivatives thereof and/or other antibodies that specifically bind to the neutralizing epitopes provided herein can be used to neutralize botulinum neurotoxin and are therefore also useful in the treatment of botulism.

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THERAPEUTIC MONOCLONAL ANTIBODIES THAT NEUTRALIZE BOTULINUM NEUROTOXINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of and priority to USSN 60/896,332, filed on March 22, 2007, and USSN 60/942,173, filed on June 5, 2007, both of which are incorporated herein by reference in their entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support by Grant No: UO1 AI056493, awarded by the National Institutes of Health, and by Department of Defense Grant DAMD17-98-C-8030. The Government of the United States of America has certain rights in this invention.

FIELD OF THE INVENTION

[0003] This invention relates antibodies that neutralize botulinum neurotoxins (*e.g.*, BoNT/A) and their use in the treatment of botulism.

BACKGROUND OF THE INVENTION

[0004] Botulism is caused by botulinum neurotoxin secreted by members of the genus *Clostridium* and is characterized by flaccid paralysis, which if not immediately fatal requires prolonged hospitalization in an intensive care unit and mechanical ventilation. Naturally occurring botulism is found in infants or adults whose gastrointestinal tracts become colonized by Clostridial bacteria (infant or intestinal botulism), after ingestion of contaminated food products (food botulism), or in anaerobic wound infections (wound botulism) (Center for Disease Control (1998) Botulism in the United States, 1899-1998. Handbook for epidemiologists, clinicians,and laboratory workers. Atlanta, Georgia U.S. Department of Health and Human Services, Public Health Service: downloadable at "www.bt.cdc.gov/agent/botulism/index.asp"). Botulism neurotoxins (BoNTs) are also classified by the Centers for Disease Control (CDC) as one of the six highest-risk threat agents for bioterrorism (the "Category A agents"), due to their extreme potency and lethality, ease of production and transport, and need for prolonged intensive care (Arnon *et*

al. (2001) *JAMA* 285: 1059-1070). Both Iraq and the former Soviet Union produced BoNT for use as weapons (United Nations Security Council (1995) Tenth report of the executive committee of the special commission established by the secretary-general pursuant to paragraph 9(b)(I) of security council resolution 687 (1991), and paragraph 3 of resolution 699 (1991) on the activities of the Special Commision; Bozheyeva *et al.* (1999) Former soviet biological weapons facilities in Kazakhstan: past, present, and future. Center for Nonproliferation Studies, Monterey Institute of International Studies), and the Japanese cult Aum Shinrikyo attempted to use BoNT for bioterrorism (Arnon *et al.* (2001) *supra*). As a result of these threats, specific pharmaceutical agents are needed for prevention and treatment of intoxication.

[0005] No specific small molecule drugs exist for prevention or treatment of botulism, but an investigational pentavalent toxoid vaccine is available from the CDC (Siegel (1988) *J. Clin. Microbiol.* 26: 2351-2356) and a recombinant vaccine is under development (Smith (1998) *Toxicon* 36: 1539-1548). Regardless, mass civilian or military vaccination is unlikely due to the rarity of disease or exposure and the fact that vaccination would prevent subsequent medicinal use of BoNT. Post-exposure vaccination is useless, due to the rapid onset of disease. Toxin neutralizing antibody (Ab) can be used for pre- or post-exposure prophylaxis or for treatment (Franz *et al.* (1993) Pp. 473-476 In B. R. DasGupta (ed.), Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects. Plenum Press, New York). Small quantities of both equine antitoxin and human botulinum immune globulin exist and are currently used to treat adult (Black and Gunn. (1980) *Am. J. Med.,* 69: 567-570; Hibbs *et al.* (1996) *Clin. Infect. Dis.,* 23: 337-340) and infant botulism (Arnon (1993). Clinical trial of human botulism immune globulin., p. 477-482. In B. R. DasGupta (ed.), Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects. Plenum Press, New York) respectively.

[0006] Recombinant monoclonal antibody (mAb) could provide an unlimited supply of antitoxin free of infectious disease risk and not requiring human donors for plasmapheresis. Given the extreme lethality of the BoNTs, mAbs must be of high potency in order to provide an adequate number of doses at reasonable cost. The development of such mAbs has become a high priority research aim of the National Institute of Allergy and Infectious Diseases. While to date no single highly potent mAbs have been described, we recently reported that combining two to three mAbs could yield highly potent BoNT neutralization (Nowakowski *et al.* (2002) *Proc. Natl. Acad. Sci. U S A,* 99: 11346-50).

[0007] The development of mAb therapy for botulism is complicated by the fact that there are at least seven BoNT serotypes (A-G) (Hatheway (1995) *Curr. Top. Microbio. Immunol,* 195: 55-75.) that show little, if any, antibody cross-reactivity. While only four of the BoNT serotypes routinely cause human disease (A, B, E, and F), there has been one reported case of infant botulism caused by BoNT C (Oguma *et al.* (1990) *Lancet* 336: 1449-1450), one outbreak of foodborne botulism linked to BoNT D (Demarchi, *et al.* (1958) *Bull. Acad. Nat. Med.,* 142: 580-582), and several cases of suspicious deaths where BoNT G was isolated (Sonnabend *et al.* (1981) *J. Infect. Dis.,* 143: 22-27). Aerosolized BoNT/C, D, and G have also been shown to produce botulism in primates by the inhalation route (Middlebrook and Franz (1997) Botulinum Toxins, chapter 33. In F.R. Sidell, E.T. Takafuji, D.R. Franz (eds.), Medical Aspects of Chemical and Biological Warfare. TMM publications, Washington, D.C.), and would most likely also affect humans. Thus it is likely that any one of the seven BoNT serotypes can be used as a biothreat agent.

[0008] Variability of the BoNT gene and protein sequence within serotypes has also been reported and there is evidence that such variability can affect the binding of monoclonal antibodies to BoNT/A (Kozaki et al. (1998) *Infect. Immun.,* 66: 4811-4816; Kozaki et al. (1995) *Microbiol. Immunol.*, 39: 767-774).

SUMMARY OF THE INVENTION

[0009] This invention pertains to antibodies that bind to and neutralize botulinum neurotoxin(s). We have discovered that particularly effective neutralization of a Botulism neurotoxin (BoNT) serotype can be achieved by the use of neutralizing antibodies that bind two or more subtypes of the particular neurotoxin serotype with high affinity and/or by combinations of such antibodies. In certain embodiments this invention provides improved antibodies that bind BoNT subtypes BoNT/A, BoNT/B, and BoNT/E. In certain embodiments this invention provides for compositions comprising neutralizing antibodies that bind two or more BoNT subtypes (*e.g.*, BoNT/A1, BoNT/A2, BoNT/A3, *etc.*) with high affinity.

[0010] In certain embodiments this invention provides a neutralizing antibody for Botulinuym neurotoxin (BoNT). The antibody typically comprises at least one VH complementarity determining region (CDR) selected from the group consisting of a 2A10 VH CDR, a 3E1VH CDR, a 3E2VH CDR, a 3E3VH CDR, a 3E4VH CDR, a 3E4.1VH CDR, a 3E5VH CDR, a 3E6VH CDR, a 3E6.1VH CDR, a 4E11VH CDR, a 4E13VH CDR,

a 4E16VH CDR, a 4E16.1VH CDR, a 4E17VH CDR, a 4E17.1VH CDR, an A12 VH CDR, a 6A12 VH CDR, a B1.1 VH CDR, a B6 VH CDR, a B6.1 VH CDR, a B8 VH CDR, a B8.1 VH CDR, a B11 VH CDR, a B11C3 VH CDR, a B11E8 VH CDR, a B12 VH CDR, a B12.1 VH CDR, a B12.2 VH CDR, a 1B18 VH CDR, a 2B18.1 VH CDR, a 4B19 VH CDR, and a 1B22 VH CDR; and/or at least one VL complementarity determining region selected from the group consisting of a 2A10 VL CDR, a 3E1VL CDR, a 3E2VL CDR, a 3E3VL CDR, a 3E4VL CDR, a 3E4.1VL CDR, a 3E5VL CDR, a 3E6VL CDR, a 3E6.1VL CDR, a 4E11VL CDR, a 4E13VL CDR, a 4E16VL CDR, a 4E16.1VL CDR, a 4E17VL CDR, a 4E17.1VL CDR, an A12 VL CDR, a 6A12 VL CDR, a B1.1 VL CDR, a B6 VL CDR, a B6.1 VL CDR, a B8 VL CDR, a B8.1 VL CDR, a B11 VL CDR, a B11C3 VL CDR, a B11E8 VL CDR, a B12 VL CDR, a B12.1. VL CDR, a B12.2 VL CDR, a 1B18 VL CDR, a 2B18.1 VL CDR, a 4B19 VL CDR, and a 1B22 VL CDR. In various embodiments the antibody comprises the VH CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22; and/or the VL CDRs CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22. In various embodiments the antibody comprises the VH and VL CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22. In various embodiments the antibody comprises the VH and VL domains of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22. In certain embodiments the antibody is a single chain Fv (scFv), a FAB , a $(Fab')_2$, an $(ScFv)_2$, and the like. In certain embodiments the antibody is an IgG. In certain

embodiments the antibody is selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22. In various embodiments the antibody is in a pharmaceutically acceptable excipient (*e.g.*, in a unit dosage formulation).

[0011] In various embodiments method of method of inhibiting the activity of Botulinum neurotoxin in a mammal are provided. The methods typically involve administering to a mammal in need thereof a composition comprising at least one neutralizing anti-BoNT antibody as described herein. In certain embodiments the composition comprises at least two different antibodies that each bind different BoNT serotypes. In certain embodiments the composition comprises at least three different antibodies that each bind different BoNT epitopes.

[0012] In certain embodiments compositions are provided that partially or fully neutralize a Botulinum neurotoxin (BoNT). The compositions typically comprise a first antibody that binds a BoNT/B or a BoNT/E serotype, *e.g,.* one or more antibodies as described above, and a second antibody that binds a BoNT serotype selected from the group consisting of BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, and BoNT/F.

[0013] In various embodiments nucleic acids are provided that encode one or more antibodies as described herein. In certain embodiments cells containing such antibodies are also provided herein. Kits are also provided for neutralizing a Botulinum neurotoxin. The kits typically comprise a composition comprising one or more antibodies as described herein. The kits optionally also include instructional materials teaching the use of the composition to neutralize a Botulinum neurotoxin. In certain embodiments the composition is stored in a disposable syringe.

Definitions.

[0014] A "BoNT polypeptide" refers to a Botulinum neurotoxin polypeptide (*e.g.*, a BoNT/A polypeptide, a BoNT/B polypeptide, a BoNT/C polypeptide, and so forth). The BoNT polypeptide can refer to a full-length polypeptide or to a fragment thereof. Thus, for example, the term "BoNT/A polypeptide" refers to either a full-length BoNT/A (a neurotoxin produced by *Clostridium botulinum* of the type A serotype) or a fragment thereof (*e.g.* the Hc fragment). The H_C fragment approximately a 50 Da C-terminal

fragment (residues 873-1296) of BoNT/A (Lacy and Stevens (1999) *J. Mol. Biol.*, 291: 1091-1104).

[0015] A "BoNT" serotype refers one of the standard known BoNT serotypes (*e.g.* BoNT/A, BoNT/C, BoNT/D, BoNT/E, BoNT/F, *etc.*). BoNT serotypes differ from each other by as little as about 35% at the amino acid level (*e.g.*, between BoNT/E and BoNT/F) up to about 66% at the amino acid level, (*e.g.*, for BoNT/A vs BoNT/C or D). Thus, BoNT serotypes differ from each other by about 35-66% at the amino acid level.

[0016] The term "BoNT subtype" (*e.g.*, a BoNT/A1A subtype) refers to botulinum neurotoxin gene sequences of a particular serotype (*e.g.*, A, C, D, F, *etc.*) that differ from each other sufficiently to produce differential antibody binding. In certain embodiments, the subtypes differ from each other by at least 2.5%, preferably by at least 5%, or 10%, more preferably by at least 15% or 20% at the amino acid level. In certain embodiments, the subtypes differ from each other by nor more than 35%, preferably by no more than 31.6%, still more preferably by no more than 30%, or 25%, more preferably by less than about 20% or 16% at the amino acid level.. In certain embodiments, BoNT subtypes differ from each other by at least 2.6%, more preferably by at least 3%, and most preferably by at least 3.6% at the amino acid level. BoNT subtypes typically differ from each other by less than about 31.6%, more preferally by less than about 16%, at the amino acid level.

[0017] An "anti-BoNT antibody" refers to an antibody that binds a BoNT polypeptide, preferably specifically binds a BoNT polypeptide with a KD less than 10^{-7}, preferably less than 10^{-8}, or 10^{-9}, more preferably less than 10^{-10}, 10^{-11}, or 10^{-12}.

[0018] "Neutralization" refers to a measurable decrease in the toxicity of a Botulinum neurotoxin (*e.g.*, BoNT/A).

[0019] The term "high affinity" when used with respect to an antibody refers to an antibody that specifically binds to its target(s) with an affinity (K_D) of at least about 10^{-8} M, preferably at least about 10^{-9}M, more preferably at least about 10^{-10}M, and most preferably at last about 10^{-11} M. In certain embodiments "high affinity" antibodies have a K_D that ranges from about 1 nM to about 5 pM.

[0020] The following abbreviations are used herein: AMP, ampicillin; BIG, botulinum immune globulin; BoNT, botulinum neurotoxin; BoNT/A, BoNT type A; CDR, omplementarity determining region; ELISA, enzyme-linked immunosorbent assay; GLU, glucose; HBS, HEPES-buffered saline (10 mM HEPES, 150 mM NaCl [pH 7.4]); H_c, c-

terminal domain of BoNT heavy chain (binding domain); H_N, N-terminal domain of BoNT heavy chain (translocation domain); IgG, immunoglobutin G; IMAC, immobilized-metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; KAN, kanamycin; K_d, equilibrium constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; MPBS, skim milk powder in PBS; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline (25 mM NaH_2PO_4, 125 mM NaCl [pH 7.0]; RU, resonance units; scFv, single-chain Fv antibody fragments; TPBS, 0.05% (vol/vol) Tween 20 in PBS; TMPBS, 0.05% (vol/vol) Tween 20 in MPBS; TU, transducing units; V_H, immunoglobulin heavy-chain variable region; V_K, immunoglobulin kappa light-chain variable region; V_L immunoglobulin light-chain variable region; wt, wild type.

[0021] The terms "polypeptide", "peptide", or "protein" are used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The amino acid residues are preferably in the natural "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. In addition, the amino acids, in addition to the 20 "standard" amino acids, include modified and unusual amino acids, which include, but are not limited to those listed in 37 CFR (1.822(b)(4). Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates either a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to a carboxyl or hydroxyl end group.

[0022] As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0023] A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily

responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0024] Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $(Fab')_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see*, *Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Preferred antibodies include, but are not limited to, Fab'_2, IgG, IgM, IgA, and single chain antibodies, more preferably single chain Fv (scFv) antibodies in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide.

[0025] An "antigen-binding site" or "binding portion" refers to the part of an immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions" or "FRs". Thus, the term "FR" refers to amino acid sequences that are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen binding "surface". This surface mediates recognition and binding of the target antigen. The three hypervariable regions of each of the heavy and light chains are referred to as "complementarity determining regions" or "CDRs" and are characterized, for example by

Kabat *et al. Sequences of proteins of immunological interest*, 4th ed. U.S. Dept. Health and Human Services, Public Health Services, Bethesda, MD (1987).

[0026] An S25 antibody refers to an antibody expressed by clone S25 or to an antibody synthesized in other manners, but having the same CDRs and preferably, but not necessarily, the same framework regions as the antibody expressed by clone s25. Similarly, antibodies C25, 1C6, 3D12, B4, 1F3, HuC25, AR1, AR2, AR3, AR4, WR1(V), WR1(T), 3-1, 3-8, 3-10, ING1, CR1, RAZ1, or ING2 refer to antibodies expressed by the corresponding clone(s) and/or to antibodies synthesized in other manners, but having the same CDRs and preferably, but not necessarily, the same framework regions as the referenced antibodies.

[0027] As used herein, the terms "immunological binding" and "immunological binding properties" refer to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller Kd represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off}/K_{on} enables cancellation of all parameters not related to affinity and is thus equal to the dissociation constant K_d (*see, generally*, Davies *et al.* (1990) *Ann. Rev. Biochem.*, 59: 439-473).

[0028] A "BoNT-neutralizing antibody" refers to an antibody that binds to one or more Botulinum neurotoxin(s) (e.g., BoNT/A1, BoNT/A2, *etc.*) and that by so-binding reduces the toxicity of that BoNT neurotoxin. Thus, for example the term "BoNT/A-neutralizing antibody", as used herein refers to an antibody that specifically binds to a BoNT/A polypeptide (*e.g.* a BoNT/A1 polypeptide), in certain embodiments, to an H_C domain of a BoNT/A polypeptide and that by so-binding reduces the toxicity of the BoNT/A polypeptide. Reduced toxicity can be measured as an increase in the time that paralysis developed and/or as a lethal dosage (*e.g.*, LD_{50}) as described herein. Antibodies

derived from BoNT-neutralizing antibodies include, but are not limited to, the antibodies whose sequence is expressly provided herein.

[0029] Antibodies derived from BoNT-neutralizing antibodies preferably have a binding affinity of about 1.6×10^{-8} or better and can be derived by screening libraries of single chain Fv fragments displayed on phage or yeast constructed from heavy (VH) and light (VL) chain variable region genes obtained from mammals, including mice and humans, immunized with botulinum toxoid, toxin, or BoNT fragments. Antibodies can also be derived by screening phage or yeast display libraries in which a known BoNT-neutralizing variable heavy (V_H) chain is expressed in combination with a multiplicity of variable light (V_L) chains or conversely a known BoNT-neutralizing variable light chain is expressed in combination with a multiplicity of variable heavy (V_H) chains. BoNT-neutralizing antibodies also include those antibodies produced by the introduction of mutations into the variable heavy or variable light complementarity determining regions (CDR1, CDR2 or CDR3) as described herein. Finally BoNT-neutralizing antibodies include those antibodies produced by any combination of these modification methods as applied to the BoNT-neutralizing antibodies described herein and their derivatives.

[0030] A neutralizing epitope refers to the epitope specifically bound by a neutralizing antibody.

[0031] A single chain Fv ("scFv" or "scFv") polypeptide is a covalently linked V_H::V_L heterodimer which may be expressed from a nucleic acid including V_H- and V_L-encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, *et al.* (1988) *Proc. Nat. Acad. Sci. USA*, 85: 5879-5883. A number of structures for converting the naturally aggregated-- but chemically separated light and heavy polypeptide chains from an antibody V region into an scFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. *See, e.g.* U.S. Patent Nos. 5, 091,513 and 5,132,405 and 4,956,778.

[0032] In one class of embodiments, recombinant design methods can be used to develop suitable chemical structures (linkers) for converting two naturally associated--but chemically separate--heavy and light polypeptide chains from an antibody variable region into a scFv molecule which will fold into a three-dimensional structure that is substantially similar to native antibody structure.

[0033] Design criteria include determination of the appropriate length to span the distance between the C-terminal of one chain and the N-terminal of the other, wherein the linker is generally formed from small hydrophilic amino acid residues that do not tend to coil or form secondary structures. Such methods have been described in the art. *See, e.g.*, U.S. Patent Nos. 5,091,513 and 5,132,405 to Huston *et al.*; and U.S. Patent No. 4,946,778 to Ladner *et al.*

[0034] In this regard, the first general step of linker design involves identification of plausible sites to be linked. Appropriate linkage sites on each of the V_H and V_L polypeptide domains include those which will result in the minimum loss of residues from the polypeptide domains, and which will necessitate a linker comprising a minimum number of residues consistent with the need for molecule stability. A pair of sites defines a "gap" to be linked. Linkers connecting the C-terminus of one domain to the N-terminus of the next generally comprise hydrophilic amino acids which assume an unstructured configuration in physiological solutions and preferably are free of residues having large side groups which might interfere with proper folding of the V_H and V_L chains. Thus, suitable linkers under the invention generally comprise polypeptide chains of alternating sets of glycine and serine residues, and may include glutamic acid and lysine residues inserted to enhance solubility. One particular linker under the invention has the amino acid sequence $[(Gly)_4Ser]_3$ (SEQ ID NO:1). Another particularly preferred linker has the amino acid sequence comprising 2 or 3 repeats of $[(Ser)_4Gly]$ (SEQ ID NO:2), such as $[(Ser)_4Gly]_3$ (SEQ ID NO:3), and the like. Nucleotide sequences encoding such linker moieties can be readily provided using various oligonucleotide synthesis techniques known in the art (*see, e.g.,* Sambrook, *supra.*).

[0035] The phrase "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, BoNT/A-neutralizing antibodies can be raised to BoNT/A protein(s) that specifically bind to BoNT/A protein(s), and not to other proteins present in a tissue sample. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select

monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

[0036] The term "conservative substitution" is used in reference to proteins or peptides to reflect amino acid substitutions that do not substantially alter the activity (specificity or binding affinity) of the molecule. Typically conservative amino acid substitutions involve substitution one amino acid for another amino acid with similar chemical properties (*e.g.* charge or hydrophobicity). The following six groups each contain amino acids that are typical conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figure 1 illustrates one strategy for *in vitro antibody* production using phage libraries. mRNA is prepared from splenocytes, first-strand cDNA is prepared, and antibody V_H and V_L genes are amplified by PCR. V_H and V_L genes are spliced together randomly using PCR to create a repertoire of scFv genes. The scFv gene repertoire is cloned into a phagemid vector in frame with a gene (gIII) encoding a phagemid minor coat protein (pIII). Each phage in the resulting phage antibody library expresses and scFv-pIII fusion protein on its surface and contains the gene encoding the scFv inside. Phage antibodies binding a specific antigen can be separated from nonbinding phage antibodies by affinity chromatography on immobilized antigen. A single round of selection increases the number of antigen-binding phage antibodies by a factor ranging from 20 to 10,000 depending on the affinity of the antibody. Eluted phage antibodies are used to infect *E. coli*, which then produce more phage antibodies for the next round of selection. Repeated rounds of selection make it possible to isolate antigen-binding phage antibodies that were originally present at frequencies of less than one in a billion.

[0038] Figure 2 shows deduced protein sequences of heavy (VH) and light (VL) chain variable regions of BoNT B binders. VH domains: A12 (SEQ ID NO:4), 6A12 (SEQ ID NO:5), B1.1 (SEQ ID NO:6), B6 (SEQ ID NO:7), B6.1 (SEQ ID NO:8), B8 (SEQ ID NO:9), B8.1 (SEQ ID NO:10), B11 (SEQ ID NO:11), B11C3 (SEQ ID NO:12), B11E8

(SEQ ID NO:13), B12 (SEQ ID NO:14), B12.1 (SEQ ID NO:15), B12.2 (SEQ ID NO:16), 1B18 (SEQ ID NO:17), 2B18.1 (SEQ ID NO:18), 4B19 (SEQ ID NO:19), 1B22 (SEQ ID NO:20). VL domains: A12 (SEQ ID NO:21), 6A12 (SEQ ID NO:22), B1.1 (SEQ ID NO:23), B6 (SEQ ID NO:24), B6.1 (SEQ ID NO:25), B8 (SEQ ID NO:26), B8.1 (SEQ ID NO:27), B11 (SEQ ID NO:28), B11C3 (SEQ ID NO:29), B11E8 (SEQ ID NO:30), B12 (SEQ ID NO:31), B12.1 (SEQ ID NO:32), B12.2 (SEQ ID NO:33), 1B18 (SEQ ID NO:34), 2B18.1 (SEQ ID NO:35), 4B19 (SEQ ID NO:36) , 1B22 (SEQ ID NO:37). Dashes indicate conserved residues. Letters indicate mutated residues.

[0039] Figure 3 shows deduced protein sequences of heavy and light chain variable regions of BoNT/E binders. VH domains: 2A10 (SEQ ID NO:38), 3E1 (SEQ ID NO:39), 3E2 (SEQ ID NO:40), 3E3 (SEQ ID NO:41), 3E4 (SEQ ID NO:42), 3E4.1 (SEQ ID NO:43), 3E5 (SEQ ID NO:44), 3E6 (SEQ ID NO:45), 3E6.1 (SEQ ID NO:46), 4E11 (SEQ ID NO:47), 4E13 (SEQ ID NO:48), 4E16 (SEQ ID NO:49), 4E16.1 (SEQ ID NO:50), 4E17 (SEQ ID NO:51), 4E17.1 (SEQ ID NO:52); VL domains: 2A10 (SEQ ID NO:53), 3E1 (SEQ ID NO:54), 3E2 (SEQ ID NO:55), 3E3 (SEQ ID NO:56), 3E4 (SEQ ID NO:57), 3E4.1 (SEQ ID NO:58), 3E5 (SEQ ID NO:59), 3E6 (SEQ ID NO:60), 3E6.1 (SEQ ID NO:61), 4E11 (SEQ ID NO:62), 4E13 (SEQ ID NO:63), 4E16 (SEQ ID NO:64), 4E16.1 (SEQ ID NO:65), 4E17 (SEQ ID NO:66), 4E17.1 (SEQ ID NO:67)Dashes indicate conserved residues. Letters indicate mutated residues.

[0040] Figure 4 shows a phylogenetic tree of published botulinum neurotoxin genes. The phylogenetic tree was constructed from the DNA sequences of published Clostridial neurotoxin genes using Vector NTI software.

[0041] Figures 5 shows an analysis of BoNT/A gene sequences. The phylogenetic tree of BoNT/A genes reveals two clusters, A1 and A2.

[0042] Figure 6 shows an analysis of BoNT/B gene sequences. A phylogenetic tree of BoNT/B genes reveals four clusters: BoNT/B1, BoNT/B2, nonproteolytic BoNT/B, and bivalent BoNT/B. Percent differences between clusters range from 3.6 to 7.7%. As with BoNT/A, the greatest differences are seen in the heavy chain.

[0043] Figures 7A and 7B show a scheme used for affinity maturation of HuC25 (Figure 7A) and 3D12 (Figure 7B) scFv using yeast display.

DETAILED DESCRIPTION

[0044] This invention provides novel antibodies that specifically bind to and neutralize botulinum neurotoxin type B and E, and in certain embodiments, other botulinum neurotoxin serotypes (*e.g.*, A, C, D, F, *etc.*, *see, e.g.*, Figures 4-6). Botulinum neurotoxin is produced by the anaerobic bacterium *Clostridium botulinum.* Botulinum neurotoxin poisoning (botulism) arises in a number of contexts including, but not limited to food poisoning (food borne botulism), infected wounds (wound botulism), "infant botulism" from ingestion of spores and production of toxin in the intestine of infants, and as aa chemical/biological warfare agent. Botulism is a paralytic disease that typically begins with cranial nerve involvement and progresses caudally to involve the extremities. In acute cases, botulism can prove fatal.

[0045] Botulism neurotoxins (BoNTs) are classified by the Centers for Disease Control (CDC) as one of the six highest-risk threat agents for bioterrorism (the "Category A agents"), due to their extreme potency and lethality, ease of production and transport, and the need for prolonged intensive care (Arnon *et al.* (2001) *JAMA* 285: 1059-1070). Both Iraq and the former Soviet Union produced BoNT for use as weapons (UN Security Council (1995) *supra*; Bozheyeva (1999) *supra.*) and the Japanese cult Aum Shinrikyo attempted to use BoNT for bioterrorism (Arnon (2001) *supra.*). As a result of these threats, specific pharmaceutical agents are needed for prevention and treatment of intoxication.

[0046] It has recently been discovered that there are multiple subtypes of various BoNT serotypes. Moreover, we have further discovered that many antibodies that bind, for example the BoNT/A1 subtype will not bind the BoNT/A2 subtype, and so forth

[0047] In certain embodiments this inventnion pertains to the discovery that that particularly efficient neutralization of a botulism neurotoxin (BoNT) subtype is achieved by the use of neutralizing antibodies that bind two or more subtypes of the particular BoNT serotype with high affinity. In various embodiments this can be accomplished by using two or more different antibodies directed against each of the subtypes, or alternatively, by the use of antibodies that are cross-reactive for different BoNT subtypes, or by bispecific or polyspecific antibodies with specificities for two or more BoNT epitopes, and/or serotypes, and/or subtypes.

[0048] It was also a surprising discovery that when one starts combining neutralizing antibodies that the potency of the antibody combination increases dramatically. This increase makes it possible to generate a multi-antibody, and/or multi-specific

antibodies of the required potency for therapeutic use. It was also surprising that as one begins combining two and three monoclonal antibodies, the particular BoNT epitope that is recognized becomes less important. Thus, in certain embodiments, this invention contemplates compositions comprising at least two, more preferably at least three high affinity antibodies that bind non-overlapping epitopes on the BoNT.

[0049] Thus, in certain embodiments, this invention contemplates compositions comprising two or more, in certain embodiments preferably three or more different antibodies selected from the antibodies described herein (*see, e.g.,* Figures 2, and 3) and/or antibodies comprising one or more CDRs from these antibodies, and/or one or more antibodies comprising mutants of these antibodies.

[0050] As indicated above, in certain embodiments, the antibodies provided by this invention bind to and neutralize one or more botulinum neurotoxin type B, E, and in certain instances Bont/A subtypes. Neutralization, in this context, refers to a measurable decrease in the toxicity of the target neurotoxin. Such a decrease in toxicity can be measured *in vitro* by a number of methods well known to those of skill in the art. One such assay involves measuring the time to a given percentage (*e.g.,* 50%) twitch tension reduction in a hemidiaphragm preparation. Toxicity can be determined *in vivo, e.g.* as an LD_{50} in a test animal (*e.g.* mouse) botulinum neurotoxin type A in the presence of one or more putative neutralizing antibodies. The neutralizing antibody or antibody combination can be combined with the botulinum neurotoxin prior to administration, or the animal can be administered the antibody prior to, simultaneous with, or after administration of the neurotoxin.

[0051] As the antibodies of this invention act to neutralize botulinum neurotoxins, they are useful in the treatment of pathologies associated with botulinum neurotoxin poisoning. The treatments essentially comprise administering to the poisoned organism (*e.g.* human or non-human mammal) a quantity of one or more neutralizing antibodies sufficient to neutralize (*e.g.* mitigate or eliminate) symptoms of BoNT poisoning.

[0052] Such treatments are most desired and efficacious in acute cases (*e.g.* where vital capacity is less than 30-40 percent of predicted and/or paralysis is progressing rapidly and/or hypoxemia with absolute or relative hypercarbia is present. These antibodies can also be used to treat early cases with symptoms milder than indicated (to prevent prgression) or even prophylactically (a use the military envisions for soldiers going in harms

way). Treatment with the neutralizing antibody can be provided as an adjunct to other therapies (*e.g.* antibiotic treatment).

[0053] The antibodies provided by this invention can also be used for the rapid detection/diagnosis of botulism (type B, E, or A toxin(s)) and thereby supplement and/or replace previous laboratory diagnostics.

[0054] In another embodiment this invention provides the epitopes specifically bound by botulinum neurotoxin antibodies described herein. These epitopes can be used to isolate, and/or identify and/or screen for other antibodies BoNT neutralizing antibodies as described herein.

I. Potency of Botulinum neurotoxin (BoNT)-neutralizing antibodies.

[0055] Without being bound to a particular theory, it is believed that the current antitoxins used to treat botulism (horse and human) have a potency of about 5000 mouse LD50s/mg (human) and 55,000 mouse LD50s mg (horse).

[0056] Based on our calculations, we believe a commercially desirable antitoxin will have a have a potency greater than about 10,000 to 100,000 LD50s/mg. Combinations of the antibodies described herein (*e.g.*, two or three antibodies) can meet this potency. Thus, in certain embodiments, this invention pvoides antibodies and/or antibody combinations that neutralize at least about 10,000 mouse LD50s/mg of antibody, preferably at least about 15,000 mouse LD50s/mg of antibody, more preferably at least about 20,000 mouse LD50s/mg of antibody, and most preferably at least about 25,000 mouse LD50s/mg of antibody.

II. Botulinum neurotoxin (BoNT)-neutralizing antibodies.

[0057] In certain preferred embodiments, BoNT neutralizing antibodies are selected that bind to or more BoNT subtypes. A number of subtypes are known for each BoNT serotype. Thus, for example, BoNT/A subtypes include, but are not limited to, BoNT/A1, BoNT/A2, BoNT/A3, and the like (*see, e.g.,* Figure 4). It is also noted, for example, that the BoNT/A1 subtype includes, but is not limited to 62A, NCTC 2916, ATCC 3502, and Hall hyper (Hall Allergan) and are identical (99.9-100% identity at the amino acid level.) and have been classified as subtype A1 (Figure 5A). The BoNT/A2 sequences (Kyoto-F and FRI-A2H) (Willems, *et al.* (1993) *Res. Microbiol.* 144:547-556) are 100% identical at

the amino acid level. Another BoNT/A subtype, (that we are calling A3) is produced by a strain called Loch Maree that killed a number of people in an outbreak in Scotland.

[0058] Similarly, as shown in Figure 4, a number of subtypes are also known for serotypes B, C, E, and F. Using, the methods described herein, it was discovered that high-affinity antibodies that are cross-reactive with two or more subtypes within a serotype can also be produced (*e.g.*, selected/engineered). Moreover, without being bound to a particular theory, it appears that these cross-reactive antibodies can substantially more efficient in neutralizing Botulinum neurotoxin, particularly when used in combination one or more different neutralizing antibodies.

[0059] The sequences of the variable heavy (VH) and variable light (VL) domains for a number of prototypical BoNT/B and BoNT/E antibodies are illustrated in Tables 1-4, and in Figures 2-3.

[0060] These antibodies can be used individually, and/or in combination with each other, and/or in combination with other known anti-BoNT antibodies (*see, e.g.*, copending application Nos: 11/342,27, filed on 01/26/2006, 09/144,886, filed in 08/31/1998, 10/632,706, filed on 08/01/2003, and PCT application Nos: PCT/US2006/003070 and PCT/US03/24371, which are incorporated herein by reference for all purposes) to form bispecific or polyspecific antibodies

[0061] Table 1. Deduced protein sequences of heavy chain variable regions of BoNT/E binders.

VH Clone/ Gene Family	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
2A10 VH1	QVQLQQS GAEVKKP GSSVKVS CKASGGT FT (SEQ ID NO:68)	RYTIT (SEQ ID NO:69)	WVRQAPG QGLEWM G (SEQ ID NO:70)	GIIPIFDKA NYAQKFQ S (SEQ ID NO:71)	RVTFTAD ASTSTAY MELGSLR PEDTAVY YCAA (SEQ ID NO:72)	YSRGY VHFDY (SEQ ID NO:73)	WGPGTL VTVSS (SEQ ID NO:74)
3E1 VH1	QVQLVES GAEVKKP GSSVKVS CKASGGT FS (SEQ ID NO:75)	NSGFT (SEQ ID NO:76)	WVRQVPG QGLEWM G (SEQ ID NO:77)	GIIPMFGP ANYAQKF QG (SEQ ID NO:78)	RVTITADE STRMVYM ELRSLRSE DTAVYYC AR (SEQ ID NO:79)	DQGEY TVGML LYYAM DV (SEQ ID NO:80)	WGEGTT VTVSS (SEQ ID NO:81)

3E2 VH1	QVQLQES GAEVKKP GSSVKVS CKASGGD LN (SEQ ID NO:82)	KYAIT (SEQ ID NO:83)	WLRQAPG QGFEWMG (SEQ ID NO:84)	GITPIFATT NYAQKFQ G (SEQ ID NO:85)	RVMITAD EVTSTVY MDLSSLG SEDTAIYF CAK (SEQ ID NO:86)	SPRGGI VGTFD T (SEQ ID NO:87)	WGQGTM VTVSS (SEQ ID NO:88)
3E3 VH3	QVQLVES GGGLVKP GESLRLSC AASGFTFS (SEQ ID NO:89)	NYNMN (SEQ ID NO:90)	WVRQAPG KGLEWVS (SEQ ID NO:91)	SISDGGSY RYYAYSV KG (SEQ ID NO:92)	RFTISRDN TKNSLYL QMNSLRA EDTALYY CAR (SEQ ID NO:93)	DEMVH GILVYY GMDV (SEQ ID NO:94)	WGQGTT VTVSS (SEQ ID NO:95)
3E4 VH3	QVQLQES GGGLVQP GGSLRLSC GASGFTFS (SEQ ID NO:96)	SDAMS (SEQ ID NO:97)	WVRQAPG KGLEWVA (SEQ ID NO:98)	AILPSGEA TYYADSV KG (SEQ ID NO:99)	RFTISRHS SKNTLYL QMNSLRA DDTAVYY CAR (SEQ ID NO:100)	DSYHS RLAAF DI (SEQ ID NO:101)	WGQGTM VTVSS (SEQ ID NO:102)
3E4.1 VH3	QVQLQES GGGLVQP GGSLRLSC GASGFTFS (SEQ ID NO:103)	SDAMS (SEQ ID NO:104)	WVRQAPG KGLEWVA (SEQ ID NO:105)	AILPSGEA TYYADSV KG (SEQ ID NO:106)	RFTISRHS SKNTLYL QMNSLRA DDTAVYY CAR (SEQ ID NO:107)	DSYHS RLAAF DI (SEQ ID NO:108)	WGQGTM VTVSS (SEQ ID NO:109)
3E5 VH3	QVQLVQS GGGVVQP GRPLRLSC AASTFNFR (SEQ ID NO:110)	DFYMS (SEQ ID NO:111)	WIRQAPG KGLEWVS (SEQ ID NO:112)	YIGSSGSA LQYADSV KG (SEQ ID NO:113)	RFTISRDN DKNVLYL QMTSLRA EDTAVYY CAR (SEQ ID NO:114)	VASRY HDVLT DGFDI (SEQ ID NO:115)	WGQGTM VTVSS (SEQ ID NO:116)
3E6 VH3	QVQLVQS GGGVVQP GKSLRLSC AASGFTFS (SEQ ID NO:117)	SYAMH (SEQ ID NO:118)	WVRQAPG KGLEWVA (SEQ ID NO:119)	VISYDGN KKYYADS VKG (SEQ ID NO:120)	RFTISRDN SKNTLYL QMNSLRA EDAAVFY CAR (SEQ ID NO:121)	ARLCTS TSCYW TFDP (SEQ ID NO:122)	WGQGTL VTVSS (SEQ ID NO:123)
3E6.1 VH3	QVQLVQS GGGVVQP GKSLRLSC AASGFTFS (SEQ ID NO:124)	SYAMH (SEQ ID NO:125)	WVRQAPG KGLEWVA (SEQ ID NO:126)	VISYDGN KKYYADS VKG (SEQ ID NO:127)	RFTISRDN SKNTLYL QMNSLRA EDAAVFY CAR (SEQ ID NO:128)	ARLCTS TSCYW TFDP (SEQ ID NO:129)	WGQGTL VTVSS (SEQ ID NO:130)
4E11 VH3	QVQLVQS GGGLVQP GGSLRLSC AASGFRFS (SEQ ID NO:131)	GYSFN (SEQ ID NO:132)	WVRQAPG KGLEWVA (SEQ ID NO:133)	YMSSGGSI KNYADSV KG (SEQ ID NO:134)	RFTISRDN AKNSLYL QVNSLRD EDTALYY CAR (SEQ ID NO:135)	GPPGRP NDAFDI (SEQ ID NO:136)	WGQGTM VTVSS (SEQ ID NO:137)
4E13 VH3	EVQLVQS GGGLVQP GGSLRLSC AASGFTFS	SYAMT (SEQ ID NO:139)	WVRQAPG KGLEWVS (SEQ ID NO:140)	SISVSGDS TYYADSV KG (SEQ ID	RFTISRDN SKNTVSL QMNSLRA EDTALYY	GLSKA DLFGM DV (SEQ ID	WGQGTM VTVSS (SEQ ID NO:144)

	(SEQ ID NO:138)			NO:141)	CAK (SEQ ID NO:142)	NO:143)	
4E16 VH4	QVQLQES GPGLVKPS ETLSLTCS VSGVSIS	DYYWS (SEQ ID NO:145)	WIRQPPG KGLEWIG (SEQ ID NO:146)	YIYYSGST NYNPSLKS (SEQ ID NO:147)	RVTISVDT SKNQFSLN LSSVTAA DTAVYYC AR (SEQ ID NO:148)	HTSGW SGGAF DI (SEQ ID NO:149)	WGQGTM VTVSS (SEQ ID NO:150)
4E16.1 VH4	QVQLQES GPGLVKPS ETLSLTCS VSGVSIS (SEQ ID NO:151)	DYYWS (SEQ ID NO:152)	WIRQPPG KGLEWIG (SEQ ID NO:153)	YIYYSGST NYNPSLKS (SEQ ID NO:154)	RVTISVDT SKNQFSLN LSSVTAA DTAVYYC AR (SEQ ID NO:155)	HTSGW SGGAF DI (SEQ ID NO:156)	WGQGTM VTVSS (SEQ ID NO:157)
4E17 VH3	EVQLVQS GGNLVQP GGSLRLSC AATGPIGS (SEQ ID NO:158)	HWMT (SEQ ID NO:159)	WVRQAPG QGLEWVA (SEQ ID NO:160)	NINLDGTE KFYVDSV KG (SEQ ID NO:161)	RFTVSRD NRKSSVFL QMNNLRV DDTAVYY CAR (SEQ ID NO:162)	LQWGG YNGWL SP (SEQ ID NO:163)	WGQGTL VTVSS (SEQ ID NO:164)
4E17.1	EVQLVQS GGNLVQP GGSLRLSC AATGPIGS (SEQ ID NO:165)	HWMT (SEQ ID NO:166)	WVRQAPG QGLEWVA (SEQ ID NO:167)	NINLDGTE KFYVDSV KG (SEQ ID NO:168)	RFTVSRD NRKSSVFL QMNNLRV DDTAVYY CAR (SEQ ID NO:169)	LQWGG YNGWL SP (SEQ ID NO:170)	WGQGTL VTVSS (SEQ ID NO:171)

[0062] Table 2. Deduced protein sequences of light chain variable regions (VL) of BoNT/E binders.

VL Clone/ Gene Family	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
E1 VK1 (ZA1D VK1)	DIVMTQSP SFLSASVG DRVTITC (SEQ ID NO:172)	WASQG ISSYLA (SEQ ID NO:173)	WYQQKPG KAPKLLIY (SEQ ID NO:174)	AASTLQ S (SEQ ID NO:175)	GVPSRFSGS GSGTEFTLTI SSLQPEDFA TYYC (SEQ ID NO:176)	QQLNSY PLT (SEQ ID NO:177)	FGGGTK VDIKR (SEQ ID NO:178)
3E1 VK1	EIVLTQSP DSLSASVG DRVTITC (SEQ ID NO:179)	RASQGI SGYLA (SEQ ID NO:180)	WYQHKA GKAPKLLI Y (SEQ ID NO:181)	AASSLQ S (SEQ ID NO:182)	GVPSRFSGS GYGTEFTLTI SSLQPDDFA TYYC (SEQ ID NO:183)	QQYNSY PFT (SEQ ID NO:184)	FGGGTK VEIKR (SEQ ID NO:185)
3E2 VK1	EIVLTQSP SFLSAFVG DRVTITC (SEQ ID NO:186)	RTSQSI NNYLN (SEQ ID NO:187)	WYQQKA GKAPKLLI Y (SEQ ID NO:188)	AASTLH T (SEQ ID NO:189)	GVPSRFSGS GSGTEFTLTI SSLQPEDFA TYYC (SEQ ID NO:190)	QQSYSIP LT (SEQ ID NO:191)	FGGGTK VEIKR (SEQ ID NO:192)
3E3	DIVMTQSP	RASQSF	WYQQKPG	AASSRA	GVPTGSVAD	QQSYST	FGGGTK

VK3	DSLSASVG DSVTITC (SEQ ID NO:193)	SSSYLA (SEQ ID NO:194)	QAPRLLIY (SEQ ID NO:195)	A (SEQ ID NO:196)	GSGTDFTLTI SGLQPEDFA AYYC (SEQ ID NO:197)	PYT (SEQ ID NO:198)	VEIKR (SEQ ID NO:199)
3E4 VK1	DIVMTQSP SFLSAFVG DRVTITC (SEQ ID NO:200)	RASQSI SNWLA (SEQ ID NO:201)	WYQQKPG KAPKVLIY (SEQ ID NO:202)	KASSLE N (SEQ ID NO:203)	GVPSRFSGS GSGTDFTLTI TSLQPDDFA TYYC (SEQ ID NO:204)	QQYNA YPLT (SEQ ID NO:205)	FGGGTK VEIKR (SEQ ID NO:206)
3E4.1 VK1	EIVLTQSP STLSASVG DRVAITC (SEQ ID NO:207)	RASQRI GSWLA (SEQ ID NO:208)	WYQQKPG KAPNPLIY (SEQ ID NO:209)	KAFSLE S (SEQ ID NO:210)	GVPSRFSGS RSGTEFTLTI SSLQPDDFA TYFC (SEQ ID NO:211)	QQYDSY PYT (SEQ ID NO:212)	FGQGTKL EIKR (SEQ ID NO:213)
3E5 VK1	DVVMTQS PSSLSASIG DRVTFTC (SEQ ID NO:214)	QASQDI SNRLN (SEQ ID NO:215)	WYQQKPG KVPKLLIS (SEQ ID NO:216)	DASNLE T (SEQ ID NO:217)	GVPSRFSGS GSGTDFTFTI SSLQPEDIAT YYC (SEQ ID NO:218)	QQYDPL LT (SEQ ID NO:219)	FGGGTK VEIKR (SEQ ID NO:220)
3E6 VK1	DIQMTQSP SSVSASVG DTVTISC (SEQ ID NO:221)	RASQGI SSWLA (SEQ ID NO:222)	WYQQKSG QAPTLLIY (SEQ ID NO:223)	AASSLQ S (SEQ ID NO:224)	GVPSRFSGS GSGTDFTLII SSLQPEDFA TYYC (SEQ ID NO:225)	QQAYRT PIT (SEQ ID NO:226)	FGGGTK VEIKR (SEQ ID NO:227)
3E6.1 VK1	DIQMTQSP SSVSASVG DRVSITC (SEQ ID NO:228)	QASQDI SNYLN (SEQ ID NO:229)	WYQQKPG KAPKLLIY (SEQ ID NO:230)	AASSLQ S (SEQ ID NO:231)	GVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:232)	QQSYNT PPT (SEQ ID NO:233)	FGQGTKL EIKR (SEQ ID NO:234)
4E11 VL3	ASVLTQD PAVSVAL GQTVRITC (SEQ ID NO:235)	QGDSL RSYYA S (SEQ ID NO:236)	WYQQKPG QAPVLVIY (SEQ ID NO:237)	GKSNRP S (SEQ ID NO:238)	GIPDRFSGSS SGNTASLTIT GAQAEDEA DYYC (SEQ ID NO:239)	NSRDST GNQL (SEQ ID NO:240)	FGGGTK VTVLG (SEQ ID NO:241)
4E13 VL3	AELTQDP AVSVALG QTVRITC (SEQ ID NO:242)	QGDSL RSYYA S (SEQ ID NO:243)	WYQQKPG QAPVLVIY (SEQ ID NO:244)	GENSRP S (SEQ ID NO:245)	GIPDRFSGSS SGNTASLTI AGAQAEDE ADYYC (SEQ ID NO:246)	NSPDSS GIHLV (SEQ ID NO:247)	FGGGTK VTVLG (SEQ ID NO:248)
4E16 VK4	EIVLTQSP DSLAVSL GERATINC (SEQ ID NO:249)	KSSQSV LYSSN NKNYL A (SEQ ID NO:250)	WYQQKPG QPPKLLFY (SEQ ID NO:251)	WASTRE S (SEQ ID NO:252)	GVPDRFSGS GSGTDFTLTI SSLQAEDVA VYYC (SEQ ID NO:253)	HQYYSS PLT (SEQ ID NO:254)	FGGGTKL EIKR (SEQ ID NO:255)
4E16.1 VK4	EIVLTQSP NSLAVSL GERATIRC (SEQ ID NO:256)	KSSQSV LYSGN NKNYI A (SEQ ID NO:257)	WYQQKPG QPPKLLIY (SEQ ID NO:258)	WASTRE S (SEQ ID NO:259)	GVPDRFSGS GSETDFTLTI SSLRAEDVA LYYC (SEQ ID NO:260)	QQYYSS RWT (SEQ ID NO:261)	FGQGTKL EIKR (SEQ ID NO:262)
4E17 VK1	DIVMTQSP SSVSASVG DRVTITC (SEQ ID NO:263)	RASQSI SSYLN (SEQ ID NO:264)	WYQQKPG KAPKLLIY (SEQ ID NO:265)	GTSNLQ S (SEQ ID NO:266)	GVPSGFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:267)	QETYST PPT (SEQ ID NO:268)	FGGGTKL EIKR (SEQ ID NO:269)
4E17.1 VK1	DIVMTQSP SSLSASVG DRVTISC (SEQ ID	RASQSI RHYVN (SEQ ID NO:271)	WYQQKPG KAPKLLIY (SEQ ID NO:272)	KASSLA S (SEQ ID NO:273)	GAPSRFSGS GSGTDFTLTI SSLQPDDFA TYYC (SEQ	QQSYSIP LT (SEQ ID NO:275)	FGGGTK VEIKR (SEQ ID NO:276)

	NO:270)				ID NO:274)		

[0063] Table 3. Deduced protein sequences of heavy chain variable regions of BoNT/B binders.

VH Clone/ Gene Family	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
A12 VH3	EVQLVES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:277)	SYGMH (SEQ ID NO:278)	WVRQAP GKGLEW VA (SEQ ID NO:279)	VIWYD GSNKY YADSV KG (SEQ ID NO:280)	RFTISRDNSK NTLYLQMN SLRAEDTAV YYCAR (SEQ ID NO:281)	GYSNYD YYYGM DV (SEQ ID NO:282)	WGQGTT VTVSS (SEQ ID NO:283)
6A12 VH3	QVQLVES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:284)	SYGMH (SEQ ID NO:285)	WVRQAP GKGLEW VS (SEQ ID NO:286)	YISSSG STIYYA DSVKG (SEQ ID NO:287)	RFTISRDNA KNSLYLQM NSLRAEDTA VYYCAR (SEQ ID NO:288)	VSIVGG PYGMD V (SEQ ID NO:289)	WGQGTT VTVSS (SEQ ID NO:290)
B1.1 VH1	QVQLVQS GAEVEKP GSSVKVS CKASGGS FS (SEQ ID NO:291)	SYAFT (SEQ ID NO:292)	WVRQAP GQGLEW MG (SEQ ID NO:293)	RIVPFL GVPYY TQKFR G (SEQ ID NO:294)	RVTITADKA TSTVYMELS SLTFDDTAV YYCAR (SEQ ID NO:295)	DKRTYE YNWNSL WF (SEQ ID NO:296)	WGRGTL VTVSS (SEQ ID NO:297)
B6 VH5	QVQLVQS GAEVKKP GESLVISC KASGDKD TFT (SEQ ID NO:298)	SFWIA (SEQ ID NO:299)	WVRQMP GKGLEW MG (SEQ ID NO:300)	IIYAGD SDTRYS PSFQG (SEQ ID NO:301)	HVNISVDRS TNTAYLQW SSLKASDTA MYYCAR (SEQ ID NO:302)	HDSRYK YFYFGM DV (SEQ ID NO:303)	WGQGTT VTVSS (SEQ ID NO:304)
B6.1 VH5	QVQLVQS GAEVKKP GESLVISC KASGDKD TFT (SEQ ID NO:305)	SFWIA (SEQ ID NO:306)	WVRQMP GKGLEW MG (SEQ ID NO:307)	IIYAGD SDTRYS PSFQG (SEQ ID NO:308)	HVNISVDRS TNTAYLQW SSLKASDTA MYYCAR (SEQ ID NO:309)	HDSRYK YFYFGM DV (SEQ ID NO:310)	WGQGTT VTVSS (SEQ ID NO:311)
B8 VH3	QVQLLES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:312)	SYGMH (SEQ ID NO:313)	WVRQAP GKGLEW VA (SEQ ID NO:314)	VIWYD GSNKY YADSV KG (SEQ ID NO:315)	RFTISRDNSK DTLYLQMN SLRAEDTAV YYCAR (SEQ ID NO:316)	GYSNYD YYYGM DV (SEQ ID NO:317)	WGQGTT VTVSS (SEQ ID NO:318)
B8.1 VH3	QVQLLES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:319)	SYGMH (SEQ ID NO:320)	WVRQAP GKGLEW VA (SEQ ID NO:321)	VIWYD GSNKY YADSV KG (SEQ ID NO:322)	RFTISRDNSK NTLYLQMN SLRAEDTAV YYCAR (SEQ ID NO:323)	GYSNYD YYYGM DV (SEQ ID NO:324)	WGQGTT VTVSS (SEQ ID NO:325)
B11 VH3	QVQLLQS AGGVVQP GRSLRLSC AASGFIFR (SEQ ID NO:326)	TYGMH (SEQ ID NO:327)	WVRQAP GKGLEW VA (SEQ ID NO:328)	FVSSDG NNKFY SDSVK G (SEQ ID NO:329)	RFTIPRDNA KNTLYLQM NSLETEDTA VYYCAK (SEQ ID NO:330)	DRYPID CSGGSC FSYGMD V (SEQ ID NO:331)	WGQGTT VTVSS (SEQ ID NO:332)
B11C3	EVQLVES	TYGMH	WVRQAP	FVSSDG	RFTIPRDNA	DRYPID	WGQGTL

VH3	GGGVVQP GRSLRLSC ATSGFILR (SEQ ID NO:333)	(SEQ ID NO:334)	GKGLEW VA(SEQ ID NO:335)	NNKFY SDSVK G (SEQ ID NO:336)	KNTLYLQM NSLETEDTA VYYCAK (SEQ ID NO:337)	CSGGSC FSYGMD V(SEQ ID NO:338)	VTVSS (SEQ ID NO:339)
B11E8 VH3	EVQLVQS GGGVVQP GRSLRLSC AASGFIFR (SEQ ID NO:340)	TYGMH (SEQ ID NO:341)	WVRQAP GKGLEW VA (SEQ ID NO:342)	FVSSDG NNKFY SDSVK G (SEQ ID NO:343)	RFTISRDNA KNTLYLQM NSLETEDTA MYYCAK (SEQ ID NO:344)	DRYPID CSGGSC FSYGMD V (SEQ ID NO:345)	WGQGTT VTVSS (SEQ ID NO:346)
B12 VH3	QVNLRES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:347)	SYALH (SEQ ID NO:348)	WVRQTP GKGLEW VA (SEQ ID NO:349)	LISYDG SNKYY ADSVK G (SEQ ID NO:350)	RFTISRDNSK NMLYLQMN SLRAEDTAV YYCAK (SEQ ID NO:351)	DRSHYG DYVGYL DY (SEQ ID NO:352)	WGQGTL VTVSS (SEQ ID NO:353)
B12.1 VH3	QVNLRES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:354)	SYALH (SEQ ID NO:355)	WVRQTP GKGLEW VA (SEQ ID NO:356)	LISYDG SNKYY ADSVK G (SEQ ID NO:357)	RFTISRDNSK NMLYLQMN SLRAEDTAV YYCAK (SEQ ID NO:358)	DRSHYG DYVGYL DY (SEQ ID NO:359)	WGQGTL VTVSS (SEQ ID NO:360)
B12.2 VH3	QVNLRES GGGVVQP GRSLRLSC AASGFTFS (SEQ ID NO:361)	SYALH (SEQ ID NO:362)	WVRQTP GKGLEW VA (SEQ ID NO:363)	LISYDG SNKYY ADSVK G (SEQ ID NO:364)	RFTISRDNSK NMLYLQMN SLRAEDTAV YYCAK (SEQ ID NO:365)	DRSHYG DYVGYL DY (SEQ ID NO:366)	WGQGTL VTVSS (SEQ ID NO:367)
1B18 VH3	EVQLVQS GGGLVQP GGSRRLSC AASGFYF N (SEQ ID NO:368)	AYWM T (SEQ ID NO:369)	WVRQAP GKGLEW VA (SEQ ID NO:370)	NINLDG TEIYYL DSVKG (SEQ ID NO:371)	RFTVSRDNV KNSVFLQMS SLRVEDTAV YFCAR (SEQ ID NO:372)	LEWGGR NGWVSP (SEQ ID NO:373)	WGQGTL VTVSS (SEQ ID NO:374)
2B18.1 VH3	QVQLVQS GGGLVQP GGSRRLSC AASGFYF N (SEQ ID NO:375)	AYWM T (SEQ ID NO:376)	WVRQAP GKGLEW VA (SEQ ID NO:377)	NINLDG TEIYYL DSVKG (SEQ ID NO:378)	RFTVSRDNV KNSVFLQMS SLRVEDTAV YFCAR (SEQ ID NO:379)	LEWGGR NGWVSP (SEQ ID NO:380)	WGQGTL VTVSS (SEQ ID NO:381)
4B19 VH1	QVQLVQS GAEVKKP GASVNVS CKASGYT FT (SEQ ID NO:382)	GYYIY (SEQ ID NO:383)	WVRQAP GQGLEW MG (SEQ ID NO:384)	WINPNS GVTKY AQKFQ G (SEQ ID NO:385)	RVTMTIDTS TNTAYMEL NRLRADDT AVYYCAR (SEQ ID NO:386)	EWTQL WSPYDY (SEQ ID NO:387)	WGQGTT VTVSS (SEQ ID NO:388)
1B22 VH4	QVQLQES GSRLVKPS QTLSLTCG VSGGSISS S (SEQ ID NO:389)	SYSWS (SEQ ID NO:390)	WIRQTPG KGLEWIG (SEQ ID NO:391)	YIYHSG STYYN PSLKS (SEQ ID NO:392)	RVTMSVDK SRNQFSLNM SSVTAADTA VYYCAR (SEQ ID NO:393)	TAFYYE NTGPIRC YLDF (SEQ ID NO:394)	WGQGTL VTVSS (SEQ ID NO:395)

[0064] Table 4. Deduced protein sequences of light chain variable regions (VL) of BoNT/B binders.

VL/Clone/ Gene Family	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
A12 VK1	DIQMTQSP SSLSASVG DRVTITC (SEQ ID NO:396)	RASQRI SNYLN (SEQ ID NO:397)	WYQQKP GKAPKLL IY (SEQ ID NO:398)	AASSL QS (SEQ ID NO:399)	EVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:400)	QQSYRP PLT (SEQ ID NO:401)	FGGGTK VEIKR (SEQ ID NO:402)
6A12 VK2	DIQMTQSP SSVSASVG NRVTITC (SEQ ID NO:403)	RASQGI SSWLA (SEQ ID NO:404)	WYQQKP GKAPKLL IY (SEQ ID NO:405)	AASSL QS (SEQ ID NO:406)	GVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:407)	QKANSF PLT (SEQ ID NO:408)	FGGGTK VEIKR (SEQ ID NO:409)
B1.1 VK1	DVVMTQS PSSLSASV GDRVTITC (SEQ ID NO:410)	RASQSI SSYLN (SEQ ID NO:411)	WYQQKP GKAPKLL IY (SEQ ID NO:412)	AASSL QS (SEQ ID NO:413)	GVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:414)	QQSYST PLT (SEQ ID NO:415)	FGQGTKL EIKR (SEQ ID NO:416)
B6 VK1	DVVMTQS PSSLSASV GDRITITC (SEQ ID NO:417)	QAGQD ISNFLN (SEQ ID NO:418)	WYQQKP GKAPKLL IR (SEQ ID NO:419)	DASNL ET (SEQ ID NO:420)	GVPSRFSGG GSGTHFTFTI SSLHPEDIAT YFC (SEQ ID NO:421)	QQYDNL PYT (SEQ ID NO:422)	FGQGTKL EIKR (SEQ ID NO:423)
B6.1 VK1	DIQMTQSP SSLSASVG DRVTITC (SEQ ID NO:424)	RASQSI SSYLN (SEQ ID NO:425)	WYQQEP GKAPKLL IY (SEQ ID NO:426)	SASSLQ S (SEQ ID NO:427)	GVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:428)	QQSYST LPYT (SEQ ID NO:429)	FGQGTKP EIKR (SEQ ID NO:430)
B8 VK1	DIQMTQSP SSLSASVG DRVTITC (SEQ ID NO:431)	RASQRI SNYLN (SEQ ID NO:432)	WYQQKP GKAPKLL IY (SEQ ID NO:433)	AASSL QS (SEQ ID NO:434)	EVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:435)	QQSYRP PLT (SEQ ID NO:436)	FGGGTK VDIKR (SEQ ID NO:437)
B8.1 VK1	DIQMTQSP SSLSASVG DRVTITC (SEQ ID NO:438)	RASQRI SNYLN (SEQ ID NO:439)	WYQQKP GKAPKLL IY (SEQ ID NO:440)	AASSL QS (SEQ ID NO:441)	EVPSRFSGS GYGTDFTLT ISSLQPEDFA TYYC (SEQ ID NO:442)	QQSYRP PLT (SEQ ID NO:443)	FGGGTK VDIKR (SEQ ID NO:444)
B11 VK1	DIVMTQSP STLSASVG DRVTVTC (SEQ ID NO:445)	RASQSI NSWLA (SEQ ID NO:446)	WYQQKP GKAPKLL IY (SEQ ID NO:447)	EASSLE S(SEQ ID NO:448)	GVPSRFSGS GSGTEFTLTI SSLQPDDFA TYYC(SEQ ID NO:449)	QQYDSY WLT(SE Q ID NO:450)	FGGGTK VEIKR(SE Q ID NO:451)
B11C3 VK1	DIQMTQSP SSVSASVG DRVTITC (SEQ ID NO:452)	RASQG VSRWL A (SEQ ID NO:453)	WYQQRP EKAPKLL IY (SEQ ID NO:454)	GASSL QS (SEQ ID NO:455)	GVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:456)	QQYDSF PLT (SEQ ID NO:457)	FGGGTK VEIKR (SEQ ID NO:458)
B11E8 VK1	EIVLTQSP ATLSVSPG ERATLSC (SEQ ID NO:459)	RASQS VSKFL A (SEQ ID NO:460)	WYQQKR GQAPRLL IY (SEQ ID NO:461)	GASTR AT (SEQ ID NO:462)	GIPARFSGSG SGTEFALTIS SLQSEDFAD YYC (SEQ ID NO:463)	QQYDN WPIT (SEQ ID NO:464)	FGQGTRL EIKR (SEQ ID NO:465)
B12 VK1	DIVMTQSP STLSASVG DRVTITC (SEQ ID NO:466)	RASQGI SSWLA (SEQ ID NO:467)	WYQQKP GKAPKLL IY (SEQ ID NO:468)	KASSLE S (SEQ ID NO:469)	GVPSRFSGS GSGTEFTLTI SSLQPEDFA TYYC (SEQ ID NO:470)	LQHNSY PRA (SEQ ID NO:471)	FGQGTKL EIKR (SEQ ID NO:472)
B12.1 VL3	AYVLTQP PSVSVAPG	EGNNV GNKNV	WYQQRP GQAPVL	DDSDR PS (SEQ	GIPERFSGSN SGNTATLTI	QVWDSS SAQWV	FGGGTKL TVLG

	KTAAITC (SEQ ID NO:473)	H (SEQ ID NO:474)	VVH (SEQ ID NO:475)	ID NO:476)	NRVEAGDE ADYYC (SEQ ID NO:477)	(SEQ ID NO:478)	(SEQ ID NO:479)
B12.2 VL1	ESVLTQPP LVSAAPG QKVTISC (SEQ ID NO:480)	SGSSSN IGNNY VS (SEQ ID NO:481)	WYQQLP GTAPKLL IY (SEQ ID NO:482)	ENSKRS S (SEQ ID NO:483)	GIPDRFSGSK SGTSATLGIT GLQTGDEA DYYC (SEQ ID NO:484)	GTWDSS LSAVV (SEQ ID NO:485)	FGGGTKL TVLG (SEQ ID NO:486)
1B18 VK1	DVVMTQS PSSVSASV GDRVTITC (SEQ ID NO:487)	RASQSI SSYLN (SEQ ID NO:488)	WYQQRP GKAPKLL IF (SEQ ID NO:489)	AASSL QS (SEQ ID NO:490)	AVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:491)	QQSYST PPT (SEQ ID NO:492)	FGQGTK VEIKR (SEQ ID NO:493)
2B18.1 VK1	DIVMTQSP SSLSASVG DRVSISC (SEQ ID NO:494)	RASQSI SSYLN (SEQ ID NO:495)	WYQQKP GKAPKLL IY (SEQ ID NO:496)	KTSSLE S (SEQ ID NO:497)	GVPSRFSGR GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:498)	QQSYST PLT (SEQ ID NO:499)	FGGGTK VEIKR (SEQ ID NO:500)
1B22 VK1	DIQMTQSP STLSASIG DRVTISC (SEQ ID NO:501)	RASQSI QSWLA (SEQ ID NO:502)	WYQQRP GEAPKLL IY (SEQ ID NO:503)	SASTLQ T (SEQ ID NO:504)	GVPSRFSGS GSGTDFTLTI SSLQPEDFA TYYC (SEQ ID NO:505)	QQYNSY PLT (SEQ ID NO:506)	FGQGTKL EIKR (SEQ ID NO:507)
4B19 VK1	DIVLTQSP STLSASVG DRVTISC (SEQ ID NO:508)	RASRSI GWYLN (SEQ ID NO:509)	WYQQRP GKAPKLL IY (SEQ ID NO:510)	AASSL HN (SEQ ID NO:511)	GVPSRFSGS GSGTEFTLTI SSLQPDDFA TYYC (SEQ ID NO:512)	QQAFGF PRT (SEQ ID NO:513)	FGQGTK VEIKR (SEQ ID NO:514)

[0065] Table 5. Unique BoNT/A light chain antibodies. Shown are the clone name, VH CDR3, VL CDR3, KD for BoNT/A light chain, and epitope recognized. Epitopes are assigned sequential numbers, if the epitope does not overlap with other light chain antibodies. Affinities are for BoNT/A1 as determined using yeast displayed scFv and soluble BoNT/A1.

Clone	VH CDR3	VL CDR3	KD (nM)	Epitope
ING2	DPYYYSYMDV (SEQ ID NO:515)	QQYYSTPFT (SEQ ID NO:516)	0.25	1
5A20	EASFGWSYLGHDDAFDI (SEQ ID NO:517)	QQYGSSLWT (SEQ ID NO:518)	0.34	2
CON1 (4A1.1)	DPGWIYSDTSAAGWFDP (SEQ ID NO:519)	QQSYDTPRT (SEQ ID NO:520)	10	3

[0066] Using the teachings and the sequence information provided herein, the variable light and variable heavy chains can be joined directly or through a linker (*e.g.*, $(Gly_4Ser)_3$, SEQ ID NO:521) to form a single-chain Fv antibody. The various CDRs and/or framework regions can be used to form full human antibodies, chimeric antibodies, antibody fragments, polyvalent antibodies, and the like.

[0067] In certain embodiments, the anti-BoNT antibodies of this invention have a binding affinity (K_D) for a BoNT protein of at least 10^{-8}, preferably at least 10^{-9}, more preferably at least 10^{-10}, and most preferably at least 10^{-11}, or 10^{-12}.

III. Preparation of BoNT neutralizing antibodies.

A) Recombinant expression of BoNT-neutralizing antibodies.

[0068] Using the information provided herein, the botulinum neurotoxin - neutralizing antibodies of this invention are prepared using standard techniques well known to those of skill in the art.

[0069] For example, the polypeptide sequences provided herein (*see, e.g.,* Tables 1-5, and/or Figures 2-3) can be used to determine appropriate nucleic acid sequences encoding the BoNT-neutralizing antibodies and the nucleic acids sequences then used to express one or more BoNT-neutralizing antibodies. The nucleic acid sequence(s) can be optimized to reflect particular codon "preferences" for various expression systems according to standard methods well known to those of skill in the art.

[0070] Using the sequence information provided, the nucleic acids may be synthesized according to a number of standard methods known to those of skill in the art. Oligonucleotide synthesis, is preferably carried out on commercially available solid phase oligonucleotide synthesis machines (Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.* 12:6159-6168) or manually synthesized using, for example, the solid phase phosphoramidite triester method described by Beaucage *et. al.* (1981) *Tetrahedron Letts.* 22(20): 1859-1862.

[0071] Once a nucleic acid encoding an anti-BoNT antibody is synthesized it can be amplified and/or cloned according to standard methods. Molecular cloning techniques to achieve these ends are known in the art. A wide variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant nucleic acids are known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds.,

Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Methods of producing recombinant immunoglobulins are also known in the art. *See*, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

[0072] Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; and Barringer *et al.* (1990) *Gene* 89, 117. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039.

[0073] Once the nucleic acid for an anti-BoNT antibody is isolated and cloned, one can express the gene in a variety of recombinantly engineered cells known to those of skill in the art. Examples of such cells include bacteria, yeast, filamentous fungi, insect (especially employing baculoviral vectors), and mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of antibodies.

[0074] In brief summary, the expression of natural or synthetic nucleic acids encoding anti-BoNT antibodies will typically be achieved by operably linking a nucleic acid encoding the antibody to a promoter (which is either constitutive or inducible), and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration in prokaryotes, eukaryotes, or both. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the nucleic acid encoding the anti-BoNT antibody. The vectors optionally comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the cassette in both eukaryotes and prokaryotes, *i.e.*, shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems. *See* Sambrook.

[0075] To obtain high levels of expression of a cloned nucleic acid it is common to construct expression plasmids which typically contain a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. Examples of regulatory regions suitable for this purpose in *E. coli* are the promoter and operator region of the *E. coli* tryptophan biosynthetic pathway as described by Yanofsky (1984) *J. Bacteriol.*, 158:1018-1024 and the leftward promoter of phage lambda (P_L) as described by Herskowitz and Hagen (1980) *Ann. Rev. Genet.*, 14:399-445. The inclusion of selection markers in DNA vectors transformed in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol. *See* Sambrook for details concerning selection markers, *e.g.*, for use in *E. coli*.

[0076] Expression systems for expressing anti-BoNT antibodies are available using, for example, *E. coli*, *Bacillus sp.* (*see, e.g.*, Palva, *et al.* (1983) *Gene* 22:229-235; Mosbach *et al.* (1983) *Nature*, 302: 543-545), and *Salmonella*. In certain embodiments, *E. coli* systems are preferred.

[0077] The anti-BoNT antibodies produced by prokaryotic cells may require exposure to chaotropic agents for proper folding. During purification from, *e.g.*, *E. coli*, the expressed protein is optionally denatured and then renatured. This can be accomplished, *e.g.*, by solubilizing the bacterially produced antibodies in a chaotropic agent such as guanidine HCl. The antibody is then renatured, either by slow dialysis or by gel filtration (*see, e.g.*, U.S. Patent No. 4,511,503).

[0078] Methods of transfecting and expressing genes in mammalian cells are known in the art. Transducing cells with nucleic acids can involve, for example, incubating viral vectors containing anti-BoNT nucleic acids with cells within the host range of the vector (*see, e.g.*, Goeddel (1990) *Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA or Krieger (1990) *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, New York, N.Y. and the references cited therein).

[0079] The culture of cells used in the present invention, including cell lines and cultured cells from tissue or blood samples is well known in the art (*see, e.g.*, Freshney (1994) *Culture of Animal Cells, a Manual of Basic Technique, third edition,* Wiley-Liss, N. Y. and the references cited therein).

[0080] Techniques for using and manipulating antibodies are found in Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497.

[0081] In one preferred embodiment the BoNT/A-neutralizing antibody gene (*e.g.* BoNT/A-neutralizing scFv gene) is subcloned into the expression vector pUC119mycHis (Tomlinson *et al.* (1996) *J. Mol. Biol.*, 256: 813-817) or pSYN3, resulting in the addition of a hexahistidine tag at the C-terminal end of the scFv to facilitate purification. Detailed protocols for the cloning and purification of certain BoNT-neutralizing antibodies are found, for example, in Amersdorfer *et al.* (1997) *Infect. Immunity,* 65(9): 3743-3752, and the like.

B) Preparation of whole polyclonal or monoclonal antibodies.

[0082] The anti-BoNT antibodies of this invention include individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. In certain embodiments, preferred antibodies are selected to bind one or more epitopes bound by the antibodies described herein (*e.g.*, 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22). The antibodies can be raised in their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies that specifically bind to a particular epitope are known to persons of skill. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

1) Polyclonal antibody production.

[0083] Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (*e.g.*, BoNT/A, BoNT/B, BoNT/E, *etc.*) subsequences including, but not limited to subsequences comprising epitopes specifically bound by antibodies expressed by clones clones 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1,

4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22 disclosed herein, preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.*, GST, keyhole limpet hemanocyanin, *etc.*), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture (*see, e.g.*, Figure 1). The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the BoNT/A polypeptide is performed where desired (*see, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

[0084] Antibodies that specifically bind to the neutralizing epitopes described herein can be selected from polyclonal sera using the selection techniques described herein.

2) Monoclonal antibody production.

[0085] In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Descriptions of techniques for preparing such monoclonal antibodies are found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497.

[0086] Summarized briefly, monoclonal antibody production proceeds by injecting an animal with an (*e.g.*, BoNT/A, BoNT/B, BoNT/E, *etc.*) subsequences including, but not limited to subsequences comprising epitopes specifically bound by antibodies expressed by clones clones 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22 disclosed herein,. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single

antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

[0087] Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the BoNT antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate (preferably mammalian) host. The antibodies of the present invention are used with or without modification, and include chimeric antibodies such as humanized murine antibodies.

IV. Modification of BoNT neutralizing antibodies.

A) Phage display can be used to increase antibody affinity.

[0088] To create higher affinity antibodies, mutant scFv gene repertories, based on the sequence of a binding scFv (*see, e.g.,* Tables 1-5, and/or Figures 2, and/or 3), can be created and expressed on the surface of phage. Display of antibody fragments on the surface of viruses which infect bacteria (bacteriophage or phage) makes it possible to produce human or other mammalian antibodies (*e.g.,* scFvs) with a wide range of affinities and kinetic characteristics. To display antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (*e.g.,* pIII) and the antibody fragment-pIII fusion protein is expressed on the phage surface (McCafferty *et al.* (1990) *Nature,* 348: 552-554; Hoogenboom *et al.* (1991) *Nucleic Acids Res.,* 19: 4133-4137).

[0089] Since the antibody fragments on the surface of the phage are functional, those phage bearing antigen binding antibody fragments can be separated from non-binding or lower affinity phage by antigen affinity chromatography (McCafferty *et al.* (1990) *Nature,* 348: 552-554). Mixtures of phage are allowed to bind to the affinity matrix, non-binding or lower affinity phage are removed by washing, and bound phage are eluted by treatment with acid or alkali. Depending on the affinity of the antibody fragment, enrichment factors of 20 fold-1,000,000 fold are obtained by single round of affinity selection.

[0090] By infecting bacteria with the eluted phage or modified variants of the eluted phage as described below, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round becomes 1,000,000 fold in two rounds of selection (*see, e.g.,* McCafferty *et al.* (1990) *Nature,* 348: 552-554). Thus, even when enrichments in each round are low, multiple rounds of affinity selection leads to the isolation of rare phage and the genetic material contained within which encodes the sequence of the binding antibody (*see, e.g.,* Marks *et al.* (1991) *J. Mol. Biol.,* 222: 581-597). The physical link between genotype and phenotype provided by phage display makes it possible to test every member of an antibody fragment library for binding to antigen, even with libraries as large as 100,000,000 clones. For example, after multiple rounds of selection on antigen, a binding scFv that occurred with a frequency of only 1/30,000,000 clones was recovered (*Id.*).

1) Chain shuffling.

[0091] One approach for creating mutant scFv gene repertoires involves replacing either the V_H or V_L gene from a binding scFv with a repertoire of V_H or V_L genes (chain shuffling) (*see, e.g.,* Clackson *et al.* (1991) *Nature,* 352: 624-628). Such gene repertoires contain numerous variable genes derived from the same germline gene as the binding scFv, but with point mutations (*see, e.g.,* Marks *et al.* (1992) *Bio/Technology,* 10: 779-783). Using light or heavy chain shuffling and phage display, the binding avidities of, *e.g.,* BoNT/E or BoNT/B -neutralizing antibody fragment can be dramatically increased (*see, e.g.,* Marks *et al.* (1992) *Bio/Technology,* 10: 779-785 in which the affinity of a human scFv antibody fragment which bound the hapten phenyloxazolone (phox) was increased from 300 nM to 15 nM (20 fold)).

[0092] Thus, to alter the affinity of BoNT-neutralizing antibody a mutant scFv gene repertoire is created containing the V_H gene of a known BoNT-neutralizing antibody (*e.g.,* 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22) and a V_L gene repertoire (light chain shuffling). Alternatively, an scFv gene repertoire is created containing the V_L gene of a known BoNT-neutralizing antibody (*e.g*2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22) and a V_H gene repertoire (heavy chain shuffling). The scFv gene repertoire is cloned into a phage display vector (*e.g.,* pHEN-1,

Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137) and after transformation a library of transformants is obtained. Phage are prepared and concentrated and selections are performed. In addition to chain shuffling, it is also possible to shuffle individual complementarity determining regions (CDRs).

[0093] In certain embodiments, the antigen concentration is decreased in each round of selection, reaching a concentration less than the desired K_d by the final rounds of selection. This results in the selection of phage on the basis of affinity (Hawkins *et al.* (1992) *J. Mol. Biol. 226:* 889-896).

2) Increasing the affinity of anti-BoNT antibodies by site directed mutagenesis.

[0094] The majority of antigen contacting amino acid side chains are located in the complementarity determining regions (CDRs), three in the V_H (CDRl, CDR2, and CDR3) and three in the V_L (CDRl, CDR2, and CDR3) (*see, e.g.,* Chothia *et al.* (1987) *J. Mol. Biol.*,196: 901-917; Chothia *et al.* (1986) *Science,* 233: 755-8; Nhan *et al.* (1991) *J. Mol. Biol.*, 217: 133-151). Without being bound to a theory, it is believed that these residues contribute the majority of binding energetics responsible for antibody affinity for antigen. In other molecules, mutating amino acids that contact ligand has been shown to be an effective means of increasing the affinity of one protein molecule for its binding partner (Lowman *et al.* (1993) *J. Mol. Biol.*, 234: 564-578; Wells (1990) *Biochemistry,* 29: 8509-8516). Thus mutation (randomization) of the CDRs and screening against , for example, BoNT/A, BoNT/E, BoNT/B, or the epiotpes thereof, can be used to generate anti-BoNT antibodies having improved binding affinity.

[0095] In certain embodiments, each CDR is randomized in a separate library, using, for example, A12 as a template. To simplify affinity measurement, A12, or other lower affinity anti-BoNT antibodies, are used as a template, rather than a higher affinity scFv. The CDR sequences of the highest affinity mutants from each CDR library are combined to obtain an additive increase in affinity. A similar approach has been used to increase the affinity of human growth hormone (hGH) for the growth hormone receptor over 1500 fold from 3.4×10^{-10} to 9.0×10^{-13} M (*see, e.g.,* Lowman *et al.* (1993) *J. Mol. Biol.*, 234: 564-578).

[0096] To increase the affinity of BoNT-neutralizing antibodies, amino acid residues located in one or more CDRs (*e.g.*, 9 amino acid residues located in V_L CDR3) are

partially randomized by synthesizing a "doped" oligonucleotide in which the wild type nucleotide occurred with a frequency of, *e.g.* 49%. The oligonucleotide is used to amplify the remainder of the BoNT-neutralizing scFv gene(s) using PCR.

[0097] For example in one embodiment, to create a library in which V_H CDR3 is randomized an oligonucleotide is synthesized which anneals to the BoNT-neutralizing antibody V_H framework 3 and encodes V_H CDR3 and a portion of framework 4. At the four positions to be randomized, the sequence NNS can be used, where N is any of the 4 nucleotides, and S is "C" or "T". The oligonucleotide is used to amplify the BoNT/A-neutralizing antibody V_H gene using PCR, creating a mutant BoNT-neutralizing antibody V_H gene repertoire. PCR is used to splice the V_H gene repertoire with the BoNT-neutralizing antibody light chain gene, and the resulting scFv gene repertoire cloned into a phage display vector (*e.g.*, pHEN-1 or pCANTAB5E). Ligated vector DNA is used to transform electrocompetent *E. coli* to produce a phage antibody library.

[0098] To select higher affinity mutant scFv, each round of selection of the phage antibody libraries is conducted on decreasing amounts of one or more BoNT subtypes.. Clones from the third and fourth round of selection can screened for binding to the desired antigen(s) (*e.g.*, BoNT/B BoNT/E, *etc.*) by ELISA on 96 well plates. scFv from, *e.g.*, twenty to forty ELISA positive clones can be expressed, *e.g.* in 10 ml cultures, the periplasm harvested, and the scFv k_{off} determined by BIAcore. Clones with the slowest k_{off} are sequenced, and each unique scFv subcloned into an appropriate vector (*e.g.*, pUC119 mycHis). The scFv are expressed in culture, and purified. Affinities of purified scFv can be determined by BIAcore.

[0099] By way of illustration, Figure 7 show a scheme used for affinity maturation of HuC25 (Figure 7A) and 3D12 (Figure 7B) scFv using yeast display (*see, e.g.*, : 11/342,27, filed on 01/26/2006, 09/144,886, filed in 08/31/1998, 10/632,706, filed on 08/01/2003, and PCT application Nos: PCT/US2006/003070 and PCT/US03/24371, which are incorporated herein by reference for all purposes).

3) Creation of anti-BoNT (scFv')2 homodimers.

[0100] To create anti-BoNT (*e.g.*, BoNT-neutralizing) $(scFv')_2$ antibodies, two ANTI-BoNT scFvs are joined, either through a linker (*e.g.*, a carbon linker, a peptide, *etc.*) or through a disulfide bond between, for example, two cysteins. Thus, for example, to create disulfide linked scFv, a cysteine residue can be introduced by site directed

mutagenesis between a myc tag and a hexahistidine tag at the carboxy-terminus of an anti-BoNT/A. Introduction of the correct sequence can be verified by DNA sequencing. In certain embodiments, the construct is in pUC119, so that the pelB leader directs expressed scFv to the periplasm and cloning sites (NcoI and NotI) exist to introduce anti-BoNT mutant scFv. Expressed scFv has the myc tag at the C-terminus, followed by two glycines, a cysteine, and then 6 histidines to facilitate purification by IMAC. After disulfide bond formation between the two cysteine residues, the two scFv can be separated from each other by 26 amino acids (two 11 amino acid myc tags and 4 glycines). An scFv was expressed from this construct, purified by IMAC may predominantly comprise monomeric scFv. To produce $(scFv')_2$ dimers, the cysteine can be reduced by incubation with 1 MM beta-mercaptoethanol, and half of the scFv blocked by the addition of DTNB. Blocked and unblocked scFvs can be incubated together to form $(scFv')_2$ and the resulting material can optionally be analyzed by gel filtration. The affinity of the anti-BoNT scFv' monomer and $(scFv')_2$ dimer can optionally be determined by BIAcore.

[0101] In certain embodiments, the $(scFv')_2$ dimer is created by joining the scFv fragments through a linker, more preferably through a peptide linker. This can be accomplished by a wide variety of means well known to those of skill in the art. For example, one preferred approach is described by Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA,* 90: 6444-6448 (*see also* WO 94/13804).

[0102] Typically, linkers are introduced by PCR cloning. For example, synthetic oligonucleotides encoding the 5 amino acid linker (Gly_4Ser, SEQ ID NO:522) can be used to PCR amplify the BoNT/A-neutralizing antibody V_H and V_L genes which are then spliced together to create the BoNT/A-neutralizing diabody gene. The gene can then be cloned into an appropriate vector, expressed, and purified according to standard methods well known to those of skill in the art.

4) Preparation of anti-BoNT $(scFv)_2$, Fab, and $(Fab')_2$ molecules.

[0103] Anti-BoNTantibodies such as anti-BoNT/E or anti-BoN/B scFv, or variant(s) with higher affinity, are suitable templates for creating size and valency variants. For example, an anti-BoNT $(scFv')_2$ can be created from the parent scFv (*e.g.*, 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19,

1B22, *etc.*) as described above. An scFv gene can be excised using appropriate restriction enzymes and cloned into another vector as described herein.

[0104] In one embodiment, expressed scFv has a myc tag at the C-terminus, followed by two glycines, a cysteine, and six histidines to facilitate purification. In certain embodiments, after disulfide bond formation between the two cystine residues, the two scFv are separated from each other by 26 amino acids (*e.g.*, two eleven amino acid myc tags and four glycines). Single-chain Fv (scFv) can be expressed from this construct and purified.

[0105] To produce $(scFv')_2$ dimers, the cysteine is reduced by incubation with 1 mM β-mercaptoethanol, and half of the scFv blocked by the addition of DTNB. Blocked and unblocked scFv are incubated together to form $(scFv')_2$, which is purified. As higher affinity scFv are isolated, their genes are similarly used to construct $(scFv')_2$.

[0106] In certain embodiments, anti-BoNT Fab are expressed in *E. coli* using an expression vector similar to the one described by Better *et. al.* (1988) *Science,* 240: 1041-1043. For example, to create a BoNT/B or BoNT/E-neutralizing Fab, the V_H and V_L genes are amplified from the scFv using PCR. The V_H gene is cloned into an expression vector (*e.g.*, a PUC119 based bacterial expression vector) that provides an IgG C_H1 domain downstream from, and in frame with, the V_H gene. The vector also contains the lac promoter, a pelb leader sequence to direct expressed V_H-C_H1 domain into the periplasm, a gene 3 leader sequence to direct expressed light chain into the periplasm, and cloning sites for the light chain gene. Clones containing the correct VH gene are identified, *e.g.*, by PCR fingerprinting. The V_L gene is spliced to the C_L gene using PCR and cloned into the vector containing the V_H C_H1 gene.

B) Selection of neutralizing antibodies.

[0107] In certain embodiments, selection of anti-BoNT antibodies (whether produced by phage display, yeast display, immunization methods, hybridoma technology, *etc.*) involves screening the resulting antibodies for specific binding to an appropriate antigen(s). In the instant case, suitable antigens can include, but are not limited to BoNT/E, BoNT/B, BoNT/A1, BoNT/A2, BoNT/A3 H_C, a C-terminal domain of BoNT heavy chain (binding domain), BoNT/A3 holotoxins, r recombinant BoNT domains such as HC (binding domain), HN (translocation domain), or LC (light chain), and the like. In certain embodiments the neutralizing antibodies are selected for specific binding of an epitope recognized by one or more of the antibodies described herein.

[0108] Selection can be by any of a number of methods well known to those of skill in the art. In an illustrative embodiment, selection is by immunochromatography (*e.g.*, using immunotubes, Maxisorp, Nunc) against the desired target, *e.g.*, BoNT/E, BoNT/B, *etc.*. In another embodiment, selection is against a BoNT protein in a surface plasmon resonance system (*e.g.*, BIAcore, Pharmacia) either alone or in combination with an antibody that binds to an epitope specifically bound by one or more of the antibodies described herein. Selection can also be done using flow cytometry for yeast display libraries. In one embodiment, yeast display libraries are sequentially selected, first on BoNT/A1, then on BoNT/A2 to obtain antibodies that bind with high affinity to both subtypes of BoNT/A. This can be repeated for other subtypes.

[0109] For phage display, analysis of binding can be simplified by including an amber codon between the antibody fragment gene and gene III. This makes it possible to easily switch between displayed and soluble antibody fragments simply by changing the host bacterial strain. When phage are grown in a supE suppresser strain of *E. coli,* the amber stop codon between the antibody gene and gene III is read as glutamine and the antibody fragment is displayed on the surface of the phage. When eluted phage are used to infect a non-suppressor strain, the amber codon is read as a stop codon and soluble antibody is secreted from the bacteria into the periplasm and culture media (Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137). Binding of soluble scFv to antigen can be detected, *e.g.*, by ELISA using a murine IgG monoclonal antibody (*e.g.*, 9E10) which recognizes a C-terminal *myc* peptide tag on the scFv (Evan *et al.* (1985) *Mol. Cell Biol.*, 5: 3610-3616; Munro *et al.* (1986) *Cell,* 46: 291-300), *e.g.*, followed by incubation with polyclonal anti-mouse Fc conjugated to a detectable label (*e.g.*, horseradish peroxidase).

[0110] As indicated above, purification of the anti-BoNT antibody can be facilitated by cloning of the scFv gene into an expression vector (*e.g.*, expression vector pUC119mycHIS) that results in the addition of the myc peptide tag followed by a hexa-histidine tag at the C-terminal end of the scFv. The vector also preferably encodes the pectate lyase leader sequence that directs expression of the scFv into the bacterial periplasm where the leader sequence is cleaved. This makes it possible to harvest native properly folded scFv directly from the bacterial periplasm. The BoNT-neutralizing antibody is then expressed and purified from the bacterial supernatant using immobilized metal affinity chromatography.

C) Measurement of anti-BoNT antibody affinity for one or more BoNT subtypes.

[0111] As explained above, selection for increased avidity involves measuring the affinity of an anti-BoNT (*e.g.*, a BoNT-neutralizing) antibody (or a modified BoNT-neutralizing antibody) for one or more targets of interest (*e.g.* BoNT/E subtype(s) or domains thereof. For example, the K_d of a BoNT/E-neutralizing antibody and the kinetics of binding to BoNT/E are determined in a BIAcore, a biosensor based on surface plasmon resonance. For this technique, antigen is coupled to a derivatized sensor chip capable of detecting changes in mass.. When antibody is passed over the sensor chip, antibody binds to the antigen resulting in an increase in mass that is quantifiable. Measurement of the rate of association as a function of antibody concentration can be used to calculate the association rate constant (k_{on}). After the association phase, buffer is passed over the chip and the rate of dissociation of antibody (k_{off}) determined. K_{on} is typically measured in the range 1.0×10^2 to 5.0×10^6 and k_{off} in the range 1.0×10^{-1} to 1.0×10^{-6}. The equilibrium constant K_d is then calculated as k_{off}/k_{on} and thus is typically measured in the range 10^{-5} to 10^{-12}. Affinities measured in this manner correlate well with affinities measured in solution by fluorescence quench titration.

[0112] Phage display and selection generally results in the selection of higher affinity mutant scFvs (Marks *et al.* (1992) *Bio/Technology,* 10: 779-783; Hawkins *et al.* (1992) *J. Mol. Biol. 226:* 889-896; Riechmann *et al.* (1993) *Biochemistry,* 32: 8848-8855; Clackson *et al.* (1991) *Nature,* 352: 624-628), but probably does not result in the separation of mutants with less than a 6 fold difference in affinity (Riechmann *et al.* (1993) *Biochemistry,* 32: 8848-8855). Thus a rapid method is needed to estimate the relative affinities of mutant scFvs isolated after selection. Since increased affinity results primarily from a reduction in the k_{off}, measurement of k_{off} should identify higher affinity scFv. k_{off} can be measured in the BIAcore on unpurified scFv in bacterial periplasm, since expression levels are high enough to give an adequate binding signal and k_{off} is independent of concentration. The value of k_{off} for periplasmic and purified scFv is typically in close agreement.

V. Human or humanized (chimeric) antibody production.

[0113] As indicated above, the anti-BoNT antibodies of this invention can be administered to an organism (*e.g.*, a human patient) for therapeutic purposes (*e.g.*, the

treatment of botulism). Antibodies administered to an organism other than the species in which they are raised can be immunogenic. Thus, for example, murine antibodies repeatedly administered to a human often induce an immunologic response against the antibody (e.g., the human anti-mouse antibody (HAMA) response). While this is typically not a problem for the use of non-human antibodies of this invention as they are typically not utilized repeatedly, the immunogenic properties of the antibody are reduced by altering portions, or all, of the antibody into characteristically human sequences thereby producing chimeric or human antibodies, respectively.

A) Chimeric antibodies.

[0114] Chimeric) antibodies are immunoglobulin molecules comprising a human and non-human portion. More specifically, the antigen combining region (or variable region) of a chimeric antibody is derived from a non-human source (*e.g.*, murine) and the constant region of the chimeric antibody (which confers biological effector function to the immunoglobulin) is derived from a human source. The chimeric antibody should have the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule. A large number of methods of generating chimeric antibodies are well known to those of skill in the art (*see, e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 5,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369).

[0115] In general, the procedures used to produce chimeric antibodies consist of the following steps (the order of some steps may be interchanged): (a) identifying and cloning the correct gene segment encoding the antigen binding portion of the antibody molecule; this gene segment (known as the VDJ, variable, diversity and joining regions for heavy chains or VJ, variable, joining regions for light chains (or simply as the V or variable region) may be in either the cDNA or genomic form; (b) cloning the gene segments encoding the constant region or desired part thereof; (c) ligating the variable region to the constant region so that the complete chimeric antibody is encoded in a transcribable and translatable form; (d) ligating this construct into a vector containing a selectable marker and gene control regions such as promoters, enhancers and poly(A) addition signals; (e) amplifying this construct in a host cell (*e.g.*, bacteria); (f) introducing the DNA into eukaryotic cells (transfection) most often mammalian lymphocytes; and culturing the host cell under conditions suitable for expression of the chimeric antibody.

[0116] Antibodies of several distinct antigen binding specificities have been manipulated by these protocols to produce chimeric proteins (*e.g.*, anti-TNP: Boulianne *et al.* (1984) *Nature,* 312: 643; and anti-tumor antigens: Sahagan *et al.* (1986) *J. Immunol.,* 137: 1066). Likewise several different effector functions have been achieved by linking new sequences to those encoding the antigen binding region. Some of these include enzymes (Neuberger *et al.* (1984) *Nature* 312: 604), immunoglobulin constant regions from another species and constant regions of another immunoglobulin chain (Sharon *et al.* (1984) *Nature* 309: 364; Tan *et al.*, (1985) *J. Immunol.* 135: 3565-3567).

[0117] In one preferred embodiment, a recombinant DNA vector is used to transfect a cell line that produces an anti-BoNT antibody. The novel recombinant DNA vector contains a "replacement gene" to replace all or a portion of the gene encoding the immunoglobulin constant region in the cell line (*e.g.*, a replacement gene may encode all or a portion of a constant region of a human immunoglobulin, a specific immunoglobulin class, or an enzyme, a toxin, a biologically active peptide, a growth factor, inhibitor, or a linker peptide to facilitate conjugation to a drug, toxin, or other molecule, *etc.*), and a "target sequence" which allows for targeted homologous recombination with immunoglobulin sequences within the antibody producing cell.

[0118] In another embodiment, a recombinant DNA vector is used to transfect a cell line that produces an antibody having a desired effector function, (*e.g.*, a constant region of a human immunoglobulin) in which case, the replacement gene contained in the recombinant vector may encode all or a portion of a region of an BoNT/A-neutralizing antibody and the target sequence contained in the recombinant vector allows for homologous recombination and targeted gene modification within the antibody producing cell. In either embodiment, when only a portion of the variable or constant region is replaced, the resulting chimeric antibody may define the same antigen and/or have the same effector function yet be altered or improved so that the chimeric antibody may demonstrate a greater antigen specificity, greater affinity binding constant, increased effector function, or increased secretion and production by the transfected antibody producing cell line, *etc.*

[0119] Regardless of the embodiment practiced, the processes of selection for integrated DNA (via a selectable marker), screening for chimeric antibody production, and cell cloning, can be used to obtain a clone of cells producing the chimeric antibody.

[0120] Thus, a piece of DNA which encodes a modification for a monoclonal antibody can be targeted directly to the site of the expressed immunoglobulin gene within a

B-cell or hybridoma cell line. DNA constructs for any particular modification may be used to alter the protein product of any monoclonal cell line or hybridoma. Such a procedure circumvents the costly and time consuming task of cloning both heavy and light chain variable region genes from each B-cell clone expressing a useful antigen specificity. In addition to circumventing the process of cloning variable region genes, the level of expression of chimeric antibody should be higher when the gene is at its natural chromosomal location rather than at a random position. Detailed methods for preparation of chimeric (humanized) antibodies can be found in U.S. Patent 5,482,856.

B) Human and humanized antibodies.

[0121] In another embodiment, this invention provides for humanized or fully human anti-BoNT-neutralizing antibodies (*e.g.*, 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, *etc.*). Human antibodies consist entirely of characteristically human polypeptide sequences. The human BoNT-neutralizing antibodies of this invention can be produced in using a wide variety of methods (*see, e.g.*, Larrick *et al.*, U.S. Pat. No. 5,001,065, for review).

[0122] In certain preferred embodiments, fully human scFv antibodies of this invention are obtained by modification and screening of fully human single-chain (*e.g.* scFv) libraries. Thus, in certain embodiments, fully human antibodies are produced using phage and/or yeast display methods as described herein. Methods of producing fully human gene libraries are well known to those of skill in the art (*see, e.g.*, Vaughn *et al.* (1996) *Nature Biotechnology,* 14(3): 309-314, Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581-597, and PCT/US96/10287).

[0123] In another embodiment, human BoNT-neutralizing antibodies of the present invention are can be produced in trioma cells. Genes encoding the antibodies are then cloned and expressed in other cells, particularly, nonhuman mammalian cells.

[0124] The general approach for producing human antibodies by trioma technology has been described by Ostberg *et al.* (1983) *Hybridoma* 2: 361-367, Ostberg, U.S. Pat.No. 4,634,664, and Engelman *et al.*, U.S. Pat. No. 4,634,666. The antibody-producing cell lines obtained by this method are called triomas because they are descended from three cells; two human and one mouse. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

[0125] Preparation of trioma cells requires an initial fusion of a mouse myeloma cell line with unimmunized human peripheral B lymphocytes. This fusion generates a xenogenic hybrid cell containing both human and mouse chromosomes (*see,* Engelman, *supra.*). Xenogenic cells that have lost the capacity to secrete antibodies are selected. Preferably, a xenogenic cell is selected that is resistant to 8-azaguanine. Such cells are unable to propagate on hypoxanthine-aminopterin-thymidine (HAT) or azaserine-hypoxanthine (AH) media.

[0126] The capacity to secrete antibodies is conferred by a further fusion between the xenogenic cell and B-lymphocytes immunized against a BoNT polypeptide (*e.g.,* BoNT/A, BoNT/A H_c, BoNT/A subsequences including, but not limited to subsequences comprising epitopes specifically bound by the antibodies described herein, *etc.*). The B-lymphocytes are obtained from the spleen, blood or lymph nodes of human donor. If antibodies against a specific antigen or epitope are desired, it is preferable to use that antigen or epitope thereof as the immunogen rather than the entire polypeptide. Alternatively, B-lymphocytes are obtained from an unimmunized individual and stimulated with a BoNT polypeptide, or a epitope thereof, *in vitro*. In a further variation, B-lymphocytes are obtained from an infected, or otherwise immunized individual, and then hyperimmunized by exposure to a BoNT polypeptide for about seven to fourteen days, *in vitro.*

[0127] The immunized B-lymphocytes prepared by one of the above procedures are fused with a xenogenic hybrid cell by well known methods. For example, the cells are treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37°C for about 5-10 min. Cells are separated from the fusion mixture and propagated in media selective for the desired hybrids. When the xenogenic hybrid cell is resistant to 8-azaguanine, immortalized trioma cells are conveniently selected by successive passage of cells on HAT or AH medium. Other selective procedures are, of course, possible depending on the nature of the cells used in fusion. Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to the BoNT polypeptide or an epitope thereof. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown *in vitro* in culture medium, or are injected into selected host animals and grown *in vivo.*

[0128] The trioma cell lines obtained are then tested for the ability to bind a BoNT polypeptide or an epitope thereof. Antibodies are separated from the resulting culture

medium or body fluids by conventional antibody-fractionation procedures, such as ammonium sulfate precipitation, DEAE cellulose chromatography and affinity chromatography.

[0129] Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into a cell line such as the cell lines typically used for expression of recombinant or humanized immunoglobulins. As well as increasing yield of antibody, this strategy offers the additional advantage that immunoglobulins are obtained from a cell line that does not have a human component, and does not therefore need to be subjected to the especially extensive viral screening required for human cell lines.

[0130] The genes encoding the heavy and light chains of immunoglobulins secreted by trioma cell lines are cloned according to methods, including but not limited to, the polymerase chain reaction (PCR), known in the art (*see, e.g.,* Sambrook *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, N.Y., 1989; Berger & Kimmel, *Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques,* Academic Press, Inc., San Diego, Calif., 1987; Co *et al.* (1992) *J. Immunol.,* 148: 1149). For example, genes encoding heavy and light chains are cloned from a trioma's genomic DNA or cDNA produced by reverse transcription of the trioma's RNA. Cloning is accomplished by conventional techniques including the use of PCR primers that hybridize to the sequences flanking or overlapping the genes, or segments of genes, to be cloned.

[0131] Typically, recombinant constructs comprise DNA segments encoding a complete human immunoglobulin heavy chain and/or a complete human immunoglobulin light chain of an immunoglobulin expressed by a trioma cell line. Alternatively, DNA segments encoding only a portion of the primary antibody genes are produced, which portions possess binding and/or effector activities. Other recombinant constructs contain segments of trioma cell line immunoglobulin genes fused to segments of other immunoglobulin genes, particularly segments of other human constant region sequences (heavy and/or light chain). Human constant region sequences can be selected from various reference sources, including but not limited to those listed in Kabat *et al.* (1987) *Sequences of Proteins of Immunological Interest,* U.S. Department of Health and Human Services.

[0132] In addition to the DNA segments encoding anti-BoNT immunoglobulins or fragments thereof, other substantially homologous modified immunoglobulins can be

readily designed and manufactured utilizing various recombinant DNA techniques known to those skilled in the art such as site-directed mutagenesis (*see* Gillman & Smith (1979) *Gene,* 8: 81-97; Roberts *et al.* (1987) *Nature* 328: 731-734). Such modified segments will usually retain antigen binding capacity and/or effector function. Moreover, the modified segments are usually not so far changed from the original trioma genomic sequences to prevent hybridization to these sequences under stringent conditions. Because, like many genes, immunoglobulin genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes to produce fusion proteins (*e.g.,* immunotoxins) having novel properties or novel combinations of properties.

[0133] The genomic sequences can be cloned and expressed according to standard methods as described herein.

[0134] Other approaches to antibody production include *in vitro* immunization of human blood. In this approach, human blood lymphocytes capable of producing human antibodies are produced. Human peripheral blood is collected from the patient and is treated to recover mononuclear cells. The suppressor T-cells then are removed and remaining cells are suspended in a tissue culture medium to which is added the antigen and autologous serum and, preferably, a nonspecific lymphocyte activator. The cells then are incubated for a period of time so that they produce the specific antibody desired. The cells then can be fused to human myeloma cells to immortalize the cell line, thereby to permit continuous production of antibody (*see* U.S. Patent 4,716,111).

[0135] In another approach, mouse-human hybridomas which produce human BoNT-neutralizing antibodies are prepared (*see, e.g.,* U.S. Patent 5,506,132). Other approaches include immunization of murines transformed to express human immunoglobulin genes, and phage display screening (Vaughan *et al. supra.*).

VI. Assaying for cross-reactivity at a neutralizing epitope.

[0136] In a preferred embodiment, the antibodies of this invention specifically bind to one or more epitopes recognized by antibodies described herein (*e.g.,* 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, *etc.*). In other words, particularly preferred antibodies are cross-reactive with

one of more of these antibodies. Means of assaying for cross-reactivity are well known to those of skill in the art (*see, e.g.,* Dowbenko *et al.* (1988) *J. Virol.* 62: 4703-4711).

[0137] This can be ascertained by providing one or more isolated target BoNT polypeptide(s) (*e.g.* BoNT/A1 and/or BoNT/A2, or recombinant domains of said toxin, such as H_c) attached to a solid support and assaying the ability of a test antibody to compete with, *e.g.,* 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, *etc* for binding to the target BoNT peptide. Thus, immunoassays in a competitive binding format are preferably used for crossreactivity determinations. For example, in one embodiment, a BoNT/E and/or BoNT/B polypeptide is immobilized to a solid support. Antibodies to be tested (*e.g.* generated by selection from a phage-display library) added to the assay compete with2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, *etc* antibodies binding to the immobilized BoNT polypeptide(s). The ability of test antibodies to compete with the binding of the 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, *etc* antibodies to the immobilized protein(s) are compared. The percent crossreactivity above proteins is then calculated, using standard calculations.

[0138] If the test antibody competes with one or more of the 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, *etc* antibodies and has a binding affinity comparable to or greater than about 1 x 10^{-8} M with the same target then the test antibody is expected to be a BoNT-neutralizing antibody.

[0139] In a particularly preferred embodiment, cross-reactivity is performed by using surface plasmon resonance in a BIAcore. In a BlAcore flow cell, the BoNT polypeptide(s) (*e.g.,* BoNT/B and/or BoNT/E) are coupled to a sensor chip (*e.g.* CM5) as described in the examples. With a flow rate of 5 µl/min, a titration of 100 nM to 1 µM antibody is injected over the flow cell surface for about 5 minutes to determine an antibody concentration that results in near saturation of the surface. Epitope mapping or cross-reactivity is then evaluated using pairs of antibodies at concentrations resulting in near

saturation and at least 100 RU of antibody bound. The amount of antibody bound is determined for each member of a pair, and then the two antibodies are mixed together to give a final concentration equal to the concentration used for measurements of the individual antibodies. Antibodies recognizing different epitopes show an essentially additive increase in the RU bound when injected together, while antibodies recognizing identical epitopes show only a minimal increase in RU (*see the examples*). In a particularly preferred embodiment, antibodies are said to be cross-reactive if, when "injected" together they show an essentially additive increase (preferably an increase by at least a factor of about 1.4, more preferably an increase by at least a factor of about 1.6, and most preferably an increase by at least a factor of about 1.8 or 2.

[0140] Cross-reactivity at the desired epitopes can ascertained by a number of other standard techniques (*see, e.g.*, Geysen *et al* (1987) *J. Immunol. Meth.* 102, 259-274). This technique involves the synthesis of large numbers of overlapping BoNT peptides. The synthesized peptides are then screened against one or more of the prototypical antibodies (*e.g.*, CR1, RAZ1, ING1, ING2, *etc.*) and the characteristic epitopes specifically bound by these antibodies can be identified by binding specificity and affinity. The epitopes thus identified can be conveniently used for competitive assays as described herein to identify cross-reacting antibodies.

[0141] The peptides for epitope mapping can be conveniently prepared using "Multipin" peptide synthesis techniques (*see, e.g.*, Geysen *et al* (1987) *Science,* 235: 1184-1190). Using the known sequence of one or more BoNT subtypes (*see, e.g.*, Atassi *et al.* (1996) *J. Prot. Chem.,* 7: 691-700 and references cited therein), overlapping BoNT polypeptide sequences can be synthesized individually in a sequential manner on plastic pins in an array of one or more 96-well microtest plate(s).

[0142] The procedure for epitope mapping using this multipin peptide system is described in U.S. Patent 5,739,306. Briefly, the pins are first treated with a pre-coat buffer containing 2% bovine serum albumin and 0.1% Tween 20 in PBS for 1 hour at room temperature. Then the pins are then inserted into the individual wells of 96-well microtest plate containing the antibodies in the pre-coat buffer, *e.g.* at 2 μg/ml. The incubation is preferably for about 1 hour at room temperature. The pins are washed in PBST (*e.g.*, 3 rinses for every 10 minutes), and then incubated in the wells of a 96-well microtest plate containing 100 mu l of HRP-conjugated goat anti-mouse IgG (Fc) (Jackson ImmunoResearch Laboratories) at a 1:4,000 dilution for 1 hour at room temperature. After

the pins are washed as before, the pins are put into wells containing peroxidase substrate solution of diammonium 2,2'-azino-bis [3-ethylbenzthiazoline-b-sulfonate] (ABTS) and H_2O_2 (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) for 30 minutes at room temperature for color reaction. The plate is read at 405 nm by a plate reader (*e.g.*, BioTek ELISA plate reader) against a background absorption wavelength of 492 nm. Wells showing color development indicated reactivity of the BoNT/A H_C peptides in such wells with S25, C25, C39, 1C6, or 1F3 antibodies.

VII. Assaying for neutralizing activity of anti-BoNT antibodies.

[0143] Preferred antibodies of this invention act, individually or in combination, to neutralize (reduce or eliminate) the toxicity of botulinum neurotoxin type. Neutralization can be evaluated *in vivo* or *in vitro*. In vivo neutralization measurements simply involve measuring changes in the lethality (*e.g.*, LD_{50} or other standard metric) due to a BoNT neurotoxin administration due to the presence of one or more antibodies being tested for neutralizing activity. The neurotoxin can be directly administered to the test organism (*e.g.* mouse) or the organism can harbor a botulism infection (*e.g.*, be infected with *Clostridium botulinum*). The antibody can be administered before, during, or after the injection of BoNT neurotoxin or infection of the test animal. A decrease in the rate of progression, or mortality rate indicates that the antibody(s) have neutralizing activity.

[0144] One suitable *in vitro* assay for neutralizing activity uses a hemidiaphragm preparation (Deshpande *et al.* (1995) *Toxicon*, 33: 551-557). Briefly, left and right phrenic nerve hemidiaphragm preparations are suspended in physiological solution and maintained at a constant temperature (*e.g.* 36°C). The phrenic nerves are stimulated supramaximally (*e.g.* at 0.05 Hz with square waves of 0.2 ms duration). Isometric twitch tension is measured with a force displacement transducer (*e.g.*, GrassModel FT03) connected to a chart recorder.

[0145] Purified antibodies are incubated with purified BoNT (*e.g.* BoNT/A1, BoNT/A2, BoNT/B, *etc.*) for 30 min at room temperature and then added to the tissue bath, resulting in a final antibody concentration of about 2.0×10^{-8} M and a final BoNT concentration of about 2.0×10^{-11} M. For each antibody studied, time to 50% twitch tension reduction is determined (*e.g.*, three times for BoNT alone and three times for antibody plus BoNT).. Differences between times to a given (arbitrary) percentage (*e.g.* 50%) twitch

reduction are determined by standard statistical analyses (*e.g.* two-tailed *t* test) at standard levels of significance (*e.g.*, a *P* value of <0.05 considered significant).

VIII. Diagnostic Assays.

[0146] As explained above, the anti-BoNT antibodies of this invention can be used for the *in vivo* or *in vitro* detection of BoNT toxin (*e.g.* BoNT/E toxin) and thus, are useful in the diagnosis (*e.g.* confirmatory diagnosis) of botulism. The detection and/or quantification of BoNT in a biological sample obtained from an organism is indicative of a *Clostridium botulinum* infection of that organism.

[0147] The BoNT antigen can be quantified in a biological sample derived from a patient such as a cell, or a tissue sample derived from a patient. As used herein, a biological sample is a sample of biological tissue or fluid that contains a BoNT concentration that may be correlated with and indicative of a *Clostridium botulinum* infection. Preferred biological samples include blood, urine, saliva, and tissue biopsies.

[0148] Although the sample is typically taken from a human patient, the assays can be used to detect BoNT antigen in cells from mammals in general, such as dogs, cats, sheep, cattle and pigs, and most particularly primates such as humans, chimpanzees, gorillas, macaques, and baboons, and rodents such as mice, rats, and guinea pigs.

[0149] Tissue or fluid samples are isolated from a patient according to standard methods well known to those of skill in the art, most typically by biopsy or venipuncture. The sample is optionally pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

A) Immunological Binding Assays

[0150] The BoNT polypeptide (*e.g.*, BoNT/E, BoNT/B, *etc.*) can be detected in an immunoassay utilizing one or more of the anti-BoNT antibodies of this invention as a capture agent that specifically binds to the BoNT polypeptide.

[0151] As used herein, an immunoassay is an assay that utilizes an antibody (*e.g.* a anti-BoNT/E antibody) to specifically bind an analyte (*e.g.*, BoNT/E). The immunoassay is characterized by the binding of one or more anti-BoNT antibodies to a target (*e.g.* one or

more BoNT/A subtypes) as opposed to other physical or chemical properties to isolate, target, and quantify the BoNT analyte.

[0152] The BoNT marker can be detected and quantified using any of a number of well recognized immunological binding assays (see, *e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168, and the like) For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37: Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991)).

[0153] The immunoassays of the present invention can be performed in any of a number of configurations (*see*, *e.g.*, those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non isotopic Immunoassays* Plenum Press, NY).

[0154] Immunoassays often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte (*e.g.*, an anti- BoNT/E antibody/BoNT/E complex). The labeling agent can itself be one of the moieties comprising the antibody/analyte complex. Thus, for example, the labeling agent can be a labeled BoNT/E polypeptide or a labeled anti-BoNT/E antibody. Alternatively, the labeling agent is optionally a third moiety, such as another antibody, that specifically binds to the BoNT antibody, the BoNT peptide(s), the antibody/polypeptide complex, or to a modified capture group (*e.g.*, biotin) which is covalently linked to BoNT polypepitde or to the anti-BoNT antibody.

[0155] In one embodiment, the labeling agent is an antibody that specifically binds to the anti-BoNT antibody. Such agents are well known to those of skill in the art, and most typically comprise labeled antibodies that specifically bind antibodies of the particular animal species from which the anti-BoNT antibody is derived (*e.g.*, an anti-species antibody). Thus, for example, where the capture agent is a human derived BoNT/E antibody, the label agent may be a mouse anti-human IgG, *i.e.*, an antibody specific to the constant region of the human antibody.

[0156] Other proteins capable of specifically binding immunoglobulin constant regions, such as streptococcal protein A or protein G are also used as the labeling agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see generally* Kronval, *et al.*, (1973) *J. Immunol.*, 111:1401-1406, and Akerstrom, *et al.*, (1985) *J. Immunol.*, 135:2589-2542, and the like).

[0157] Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays are carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 5°C to 45°C.

1) Non competitive assay formats.

[0158] Immunoassays for detecting BoNT neurotoxins (*e.g.* BoNT serotypes and/or subtypes) are, in certain embodiments, either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case, BoNT polypeptide) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (*e.g.*, an anti-BoNT antibody) is bound directly or indirectly to a solid substrate where it is immobilized. These immobilized anti-BoNT antibodies capture BoNT polypeptide(s) present in a test sample (*e.g.*, a blood sample). The BoNT polypeptide(s) thus immobilized are then bound by a labeling agent, *e.g.*, an anti-BoNT/E antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. Free labeled antibody is washed away and the remaining bound labeled antibody is detected (*e.g.*, using a gamma detector where the label is radioactive).

2) Competitive assay formats.

[0159] In competitive assays, the amount of analyte (*e.g.*, BoNT/E) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent (*e.g.*, anti-BoNT/E antibody) by the analyte present in the sample. For example, in one competitive assay, a known amount of BoNT/E is added to a test sample with an unquantified amount of BoNT/E, and the sample

is contacted with a capture agent, *e.g.*, an anti-BoNT/E antibody that specifically binds BoNT/E. The amount of added BoNT/E that binds to the anti-BoNT/E-neutralizing antibody is inversely proportional to the concentration of BoNT/E present in the test sample.

[0160] The anti-BoNT/E antibody can be immobilized on a solid substrate. The amount of BoNT/E bound to the anti-BoNT/E antibody is determined either by measuring the amount of BoNT/E present in a BoNT/E-anti-BoNT/E antibody complex, or alternatively by measuring the amount of remaining uncomplexed BoNT/E.

B) Reduction of Non Specific Binding.

[0161] One of skill will appreciate that it is often desirable to reduce non specific binding in immunoassays and during analyte purification. Where the assay involves, for example BoNT/E polypeptide(s), BoNT/E-neutralizing antibody, or other capture agent(s) immobilized on a solid substrate, it is desirable to minimize the amount of non specific binding to the substrate. Means of reducing such non specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used.

C) Substrates.

[0162] As mentioned above, depending upon the assay, various components, including the BoNT polypeptide(s), anti-BoNT antibodies, *etc.*, are optionally bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.*, glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass, silica, plastic, metallic or polymer bead. The desired component may be covalently bound, or noncovalently attached through nonspecific bonding.

[0163] A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride

(PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

[0164] In preparing the surface, a plurality of different materials may be employed, *e.g.*, as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non specific binding, simplify covalent conjugation, enhance signal detection or the like.

[0165] If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, (1970) *J. Biol. Chem.* 245 3059.

[0166] In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082.

D) Other Assay Formats

[0167] BoNT polypeptides or anti-BoNT antibodies (*e.g.* BoNT/E neutralizing antibodies) can also be detected and quantified by any of a number of other means well

known to those of skill in the art. These include analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

[0168] Western blot analysis and related methods can also be used to detect and quantify the presence of BoNT polypeptides in a sample. The technique generally comprises separating sample products by gel electrophoresis on the basis of molecular weight, transferring the separated products to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind either the BoNT polypeptide. The antibodies specifically bind to the biological agent of interest on the solid support. These antibodies are directly labeled or alternatively are subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-human antibodies where the antibody to a marker gene is a human antibody) which specifically bind to the antibody which binds the BoNT polypeptide.

[0169] Other assay formats include liposome immunoassays (LIAs), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see, Monroe *et al.*, (1986) *Amer. Clin. Prod. Rev.* 5:34-41).

E) Labeling of anti-BoNT (*e.g.*, anti-BoNT/E) antibodies.

[0170] Anti-BoNT antibodies can be labeled by any of a number of methods known to those of skill in the art. Thus, for example, the labeling agent can be, *e.g.*, a monoclonal antibody, a polyclonal antibody, a protein or complex such as those described herein, or a polymer such as an affinity matrix, carbohydrate or lipid. Detection proceeds by any known method, including immunoblotting, western analysis, gel-mobility shift assays, tracking of radioactive or bioluminescent markers, nuclear magnetic resonance, electron paramagnetic resonance, stopped-flow spectroscopy, column chromatography, capillary electrophoresis, or other methods which track a molecule based upon an alteration in size and/or charge. The particular label or detectable group used in the assay is not a critical aspect of the invention. The detectable group can be any material having a detectable physical or

chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads^{TM}), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, LacZ, CAT, horse radish peroxidase, alkaline phosphatase and others, commonly used as detectable enzymes, either as marker gene products or in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

[0171] The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on the sensitivity required, ease of conjugation of the compound, stability requirements, available instrumentation, and disposal provisions.

[0172] Non radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

[0173] The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

[0174] Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a

scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, *e.g.*, by microscopy, visual inspection, via photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing appropriate substrates for the enzyme and detecting the resulting reaction product. Finally, simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

[0175] Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of BoNT peptides. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

IX. Pharmaceutical Compositions.

[0176] The BoNT-neutralizing antibodies of this invention are useful in mitigating the progression of botulisum produced, *e.g.*, by endogenous disease processes or by chemical/biological warfare agents. Typically compositions comprising one or preferably two or more different antibodies are administered to a mammal (*e.g.*, to a human) in need thereof.

[0177] We have discovered that particularly efficient neutralization of a botulism neurotoxin (BoNT) subtype is achieved by the use of neutralizing antibodies that bind two or more subtypes of the particular BoNT serotype with high affinity. In certain embodiments, this can be accomplished by using two or more different antibodies directed against each of the subtypes and/or neutralizing antibodies that bind two or more BoNT subtypes (*e.g.*, BoNT/A1, BoNT/A2, BoNT/A3, *etc.*) with high affinity.

[0178] It was also a surprising discovery that when one starts combining neutralizing antibodies that the potency of the antibody combination increases dramatically. This increase makes it possible to generate a botulinum antibody compositions of the required potency for therapeutic use. It was also surprising that as one begins combining two and three monoclonal antibodies, the particular BoNT epitope that is recognized becomes less important. Thus, in certain embodiments, this invention contemplates

compositions comprising at least two, more preferably at least three high affinity antibodies that bind non-overlapping epitopes on the BoNT.

[0179] In certain embodiments, this invention contemplates compositions comprising two or more, preferably three or more different antibodies selected from the group consisting of 2A10, 3E1, 3E2, 3E3, 3E4, 3E4.1, 3E5, 3E6, 3E6.1, 4E11, 4E13, 4E16, 4E16.1, 4E17, 4E17.1, n A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1, B12.2, 1B18, 2B18.1, 4B19, and 1B22, an/or antibodies comprising one or more CDRs from these antibodies, and/or one or more antibodies comprising mutants of these antibodies.

[0180] The BoNT-neutralizing antibodies of this invention are useful for parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and lozenges. The antibodies comprising the pharmaceutical compositions of this invention, when administered orally, are preferably protected from digestion. This is typically accomplished either by complexing the antibodies with a composition to render them resistant to acidic and enzymatic hydrolysis or by packaging the antibodies in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art.

[0181] The pharmaceutical compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of one or more BoNT-neutralizing antibody dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of BoNT/A-neutralizing antibody in these formulations can vary widely,

and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[0182] Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 to 10 mg per patient per day. Dosages from about 1 mg up to about 200 mg per patient per day can be used. Methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

[0183] The compositions containing the BoNT-neutralizing antibodies of this inventon or a cocktail thereof are generally administered for therapeutic treatments. Preferred pharmaceutical compositions are administered in a dosage sufficient to neutralize (mitigate or eliminate) the BoNT toxin(s) (*i.e.*, reduce or eliminate a symptom of BoNT poisoning (botulism)). An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

[0184] Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the antibodies of this invention to effectively treat the patient.

X. Kits For Diagnosis or Treatment.

[0185] In another embodiment, this invention provides for kits for the treatment of botulism or for the detection/confirmation of a *Clostridium botulinum* infection. Kits will typically comprise one or more anti-BoNT antibodies (*e.g.*, BoNT-neutralizing antibodies for pharmaceutical use) of this invention. For diagnostic purposes, the antibody(s) can optionally be labeled. In addition the kits will typically include instructional materials disclosing means of use BoNT-neutralizing antibodies in the treatment of symptoms of botulism. The kits may also include additional components to facilitate the particular application for which the kit is designed. Thus, for example, where a kit contains one or more anti-BoNT antibodies for detection of diagnosis of BoNT subtype, the antibody can be labeled, and the kit can additionally contain means of detecting the label (*e.g.* enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a sheep anti-human antibodies, or the like). The kits may additionally include

buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

[0186] In certain embodiments, kits provided for the treatment of botulisum comprise one or more BoNT neutralizing antibodies. The antibodies can be provided separately or mixed together. Typically the antibodies will be provided in a steril pharmacologically acceptoable excipient. In certain embodiments, the antibodies can be provided pre-loaded into a delivery device (*e.g.*, a disposable syringe).

[0187] The kits can optionally include instructional materials teaching the use of the antibodies, recommended dosages, conterindications, and the like.

[0188] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

What is claimed is:

1. A neutralizing antibody for Botulinuym neurotoxin (BoNT), said antibody comprising:

at least one VH complementarity determining region (CDR) selected from the group consisting of a 2A10 VH CDR, a 3E1VH CDR, a 3E2VH CDR, a 3E3VH CDR, a 3E4VH CDR, a 3E4.1VH CDR, a 3E5VH CDR, a 3E6VH CDR, a 3E6.1VH CDR, a 4E11VH CDR, a 4E13VH CDR, a 4E16VH CDR, a 4E16.1VH CDR, a 4E17VH CDR, a 4E17.1VH CDR, an A12 VH CDR, a 6A12 VH CDR, a B1.1 VH CDR, a B6 VH CDR, a B6.1 VH CDR, a B8 VH CDR, a B8.1 VH CDR, a B11 VH CDR, a B11C3 VH CDR, a B11E8 VH CDR, a B12 VH CDR, a B12.1 VH CDR, a B12.2 VH CDR, a 1B18 VH CDR, a 2B18.1 VH CDR, a 4B19 VH CDR, and a 1B22 VH CDR; and/or

at least one VL complementarity determining region selected from the group consisting of a 2A10 VL CDR, a 3E1VL CDR, a 3E2VL CDR, a 3E3VL CDR, a 3E4VL CDR, a 3E4.1VL CDR, a 3E5VL CDR, a 3E6VL CDR, a 3E6.1VL CDR, a 4E11VL CDR, a 4E13VL CDR, a 4E16VL CDR, a 4E16.1VL CDR, a 4E17VL CDR, a 4E17.1VL CDR, an A12 VL CDR, a 6A12 VL CDR, a B1.1 VL CDR, a B6 VL CDR, a B6.1 VL CDR, a B8 VL CDR, a B8.1 VL CDR, a B11 VL CDR, a B11C3 VL CDR, a B11E8 VL CDR, a B12 VL CDR, a B12.1. VL CDR, a B12.2 VL CDR, a 1B18 VL CDR, a 2B18.1 VL CDR, a 4B19 VL CDR, and a 1B22 VL CDR.

2. The antibody of claim 1, wherein said antibody comprises

the VH CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22; and/or

the VL CDRs CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

3. The antibody of claim 2, wherein said antibody comprises:

the VH and VL CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

4. The antibody of claim 2, wherein said antibody comprises the VH and VL domains of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

5. The antibody of claim 1, wherein at least one of said antibodies is a single chain Fv (scFv).

6. The antibody of claim 1, wherein at least one of said antibodies is an IgG.

7. The antibody of claim 1, wherein at least one of said antibodies is a Fab.

8. The antibody of claim 1, wherein at least one of said antibodies is a $(Fab')_2$.

9. The antibody of claim 1, wherein at least one of said antibodies is a $(scFv')_2$.

10. The antibody of claim 1, wherein said antibody is selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

11. The antibody of claim 1, wherein said antibody is in a pharmaceutically acceptable excipient.

12. The antibody of claim 1, wherein said composition is a unit dosage formulation.

13. A method of inhibiting the activity of Botulinum neurotoxin in a mammal, said method comprising administering to a mammal in need thereof

a composition comprising at least one neutralizing anti-BoNT antibody, said antibody comprising:

at least one VH complementarity determining region (CDR) selected from the group consisting of a 2A10 VH CDR, a 3E1VH CDR, a 3E2VH CDR, a 3E3VH CDR, a 3E4VH CDR, a 3E4.1VH CDR, a 3E5VH CDR, a 3E6VH CDR, a 3E6.1VH CDR, a 4E11VH CDR, a 4E13VH CDR, a 4E16VH CDR, a 4E16.1VH CDR, a 4E17VH CDR, a 4E17.1VH CDR, A12 VH CDR, 6A12 VH CDR, B1.1 VH CDR, B6 VH CDR, B6.1 VH CDR, B8 VH CDR, B8.1 VH CDR, a B11 VH CDR, a B11C3 VH CDR, a B11E8 VH CDR, a B12 VH CDR, a B12.1. VH CDR, a B12.2 VH CDR, a 1B18 VH CDR, a 2B18.1 VH CDR, a 4B19 VH CDR, and a 1B22 VH CDR; and/or

at least one VL complementarity determining region selected from the group consisting of 2A10 VL CDR, a 3E1VL CDR, a 3E2VL CDR, a 3E3VL CDR, a 3E4VL CDR, a 3E4.1VL CDR, a 3E5VL CDR, a 3E6VL CDR, a 3E6.1VL CDR, a 4E11VL CDR, a 4E13VL CDR, a 4E16VL CDR, a 4E16.1VL CDR, a 4E17VL CDR, a 4E17.1VL CDR, A12 VL CDR, 6A12 VL CDR, B1.1 VL CDR, B6 VL CDR, B6.1 VL CDR, B8 VL CDR, B8.1 VL CDR, a B11 VL CDR, a B11C3 VL CDR, a B11E8 VL CDR, a B12 VL CDR, a B12.1. VL CDR, a B12.2 VL CDR, a 1B18 VL CDR, a 2B18.1 VL CDR, a 4B19 VL CDR, and a 1B22 VL CDR.

14. The method of claim 13, wherein said antibody comprises

the VH CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22; and/or

the VL CDRs CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH

CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

15. The method of claim 13, wherein said antibody comprises:

the VH and VL CDRs of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

16. The method of claim 13, wherein said antibody comprises the VH and VL domains of an antibody selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1, B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

17. The method of claim 13, wherein at least one of said antibodies is a single chain Fv (scFv).

18. The method of claim 13, wherein at least one of said antibodies is an IgG.

19. The method of claim 13, wherein at least one of said antibodies is a Fab.

20. The method of claim 13, wherein at least one of said antibodies is a $(Fab')_2$.

21. The method of claim 13, wherein at least one of said antibodies is a $(scFv')_2$.

22. The method of claim 13, wherein said antibody is selected from the group consisting of 2A10, 3E1VH CDR, 3E2VH CDR, 3E3VH CDR, 3E4VH CDR, 3E4.1VH CDR, 3E5VH CDR, 3E6VH CDR, 3E6.1VH CDR, 4E11VH CDR, 4E13VH CDR, 4E16VH CDR, 4E16.1VH CDR, 4E17VH CDR, 4E17.1VH CDR, A12, 6A12, B1.1,

B6, B6.1, B8, B8.1, B11, B11C3, B11E8, B12, B12.1., B12.2, 1B18, 2B18.1, 4B19, and 1B22.

23. The method of claim 13, wherein said antibody is in a pharmaceutically acceptable excipient.

24. The method of claim 13, wherein said composition comprises a unit dosage formulation.

25. The method of claim 13, wherein said composition comprises at least two different antibodies that each bind different BoNT serotypes.

26. The method of claim 13, wherein said composition comprises at least three different antibodies that each bind different BoNT epitopes.

27. A composition for neutralizing a Botulinum neurotoxin (BoNT), said composition comprising:

a first antibody that binds a BoNT/B or a BoNT/E serotype said first antibody comprising:

at least one VH complementarity determining region (CDR) selected from the group consisting of a 2A10 VH CDR, a 3E1VH CDR, a 3E2VH CDR, a 3E3VH CDR, a 3E4VH CDR, a 3E4.1VH CDR, a 3E5VH CDR, a 3E6VH CDR, a 3E6.1VH CDR, a 4E11VH CDR, a 4E13VH CDR, a 4E16VH CDR, a 4E16.1VH CDR, a 4E17VH CDR, a 4E17.1VH CDR, A12 VH CDR, 6A12 VH CDR, B1.1 VH CDR, B6 VH CDR, B6.1 VH CDR, B8 VH CDR, B8.1 VH CDR, a B11 VH CDR, a B11C3 VH CDR, a B11E8 VH CDR, a B12 VH CDR, a B12.1. VH CDR, a B12.2 VH CDR, a 1B18 VH CDR, a 2B18.1 VH CDR, a 4B19 VH CDR, and a 1B22 VH CDR; and/or

at least one VL complementarity determining region selected from the group consisting of 2A10 VL CDR, a 3E1VL CDR, a 3E2VL CDR, a 3E3VL CDR, a 3E4VL CDR, a 3E4.1VL CDR, a 3E5VL CDR, a 3E6VL CDR, a 3E6.1VL CDR, a 4E11VL CDR, a 4E13VL CDR, a 4E16VL CDR, a 4E16.1VL CDR, a 4E17VL CDR, a 4E17.1VL CDR, A12 VL CDR, 6A12 VL CDR, B1.1 VL CDR, B6 VL CDR, B6.1 VL CDR, B8 VL CDR, B8.1 VL CDR, a B11 VL CDR, a B11C3 VL CDR, a B11E8 VL CDR, a B12 VL CDR, a B12.1. VL CDR, a B12.2 VL CDR, a 1B18 VL CDR, a 2B18.1 VL CDR, a 4B19 VL CDR, and a 1B22 VL CDR; and

a second antibody that binds a BoNT serotype selected from the group consisting of BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, and BoNT/F.

28. A nucleic acid that encodes an antibody comprising:

VH complementarity determining regions CDR1, CDR2, and CDR3 selected from the group consisting of 2A10 VH CDRs, 3E1VH CDRs, 3E2VH CDRs, 3E3VH CDRs, 3E4VH CDRs, 3E4.1VH CDRs, 3E5VH CDRs, 3E6VH CDRs, 3E6.1VH CDRs, 4E11VH CDRs, 4E13VH CDRs, 4E16VH CDRs, 4E16.1VH CDRs, 4E17VH CDRs, 4E17.1VH CDRs, A12 VH CDRs, 6A12 VH CDRs, B1.1 VH CDRs, B6 VH CDRs, B6.1 VH CDRs, B8 VH CDRs, B8.1 VH CDRs, B11 VH CDRs, B11C3 VH CDRs, B11E8 VH CDRs, B12 VH CDRs, B12.1 VH CDRs, B12.2 VH CDRs, 1B18 VH CDRs, 2B18.1 VH CDRs, 4B19 VH CDRs, and 1B22 VH CDRs; and/or

VL complementarity determining regions CDR1, CDR2, and CDR3 selected from the group consisting of 2A10 VL CDRS, 3E1VL CDRS, 3E2VL CDRS, 3E3VL CDRS, 3E4VL CDRS, 3E4.1VL CDRS, 3E5VL CDRS, 3E6VL CDRS, 3E6.1VL CDRS, 4E11VL CDRS, 4E13VL CDRS, 4E16VL CDRS, 4E16.1VL CDRS, 4E17VL CDRS, 4E17.1VL CDRS, A12 VL CDRS, 6A12 VL CDRS, B1.1 VL CDRS, B6 VL CDRS, B6.1 VL CDRS, B8 VL CDRS, B8.1 VL CDRS, B11 VL CDRS, B11C3 VL CDRS, B11E8 VL CDRS, B12 VL CDRS, B12.1. VL CDRS, B12.2 VL CDRS, 1B18 VL CDRS, 2B18.1 VL CDRS, 4B19 VL CDRS, and a 1B22 VL CDRS.

29. A cell containing a nucleic acid that encodes an antibody according to any of claims 1-12.

30. A kit for neutralizing a Botulinum neurotoxin, said kit comprising:

a composition according to claim 27; and

instructional materials teaching the use of said composition to neutralize a Botulinum neurotoxin.

31. The kit of claim 30, wherein said composition is stored in a disposable syringe.

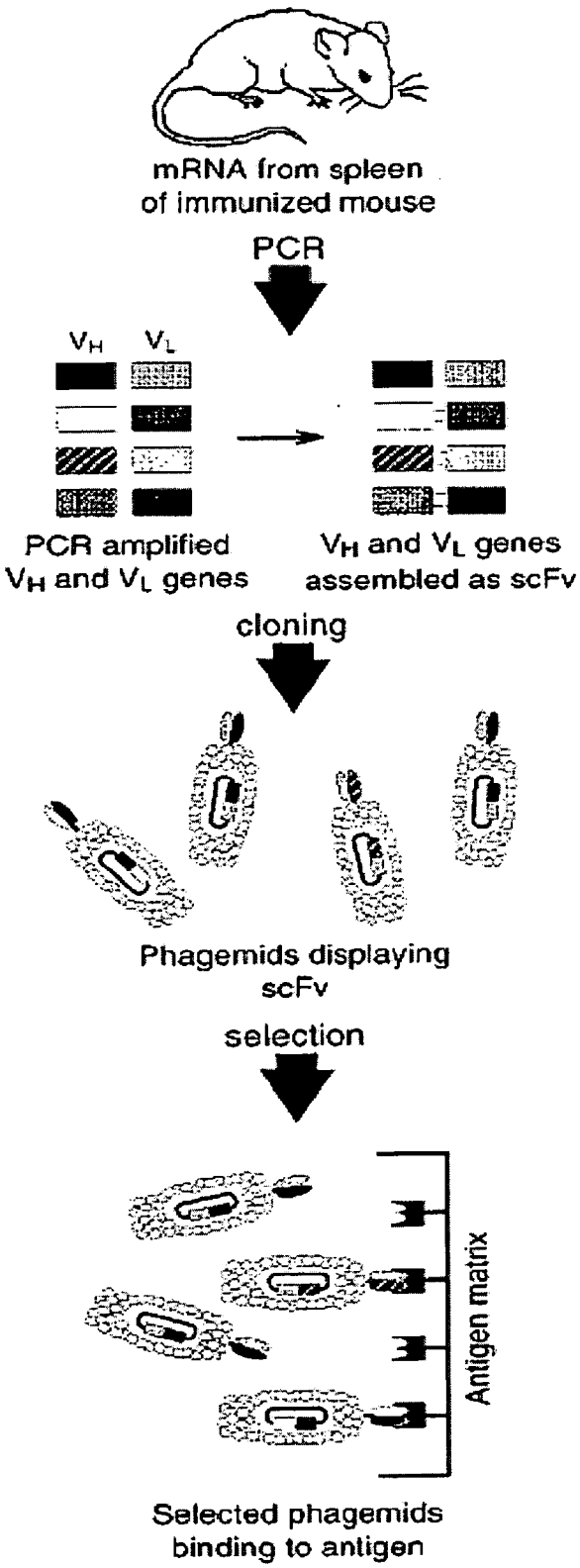

mRNA from spleen
of immunized mouse
PCR
V_H V_L
PCR amplified
V_H and V_L genes
V_H and V_L genes
assembled as scFv
cloning
Phagemids displaying
scFv
selection
Antigen matrix
Selected phagemids
binding to antigen

Fig. 1

Deduced protein sequences of heavy and light chain variable regions of BoNT B binder

VH Clone	Framework1	CDR1	Framework2	CDR2	Framework3	CDR3	Framework4	Gene Family
A12	EVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VIWYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	GYSNYDYYYGMDV	WGQGTTVTVSS	VH3
6A12	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVS	YISSSGSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	VSYVGEYYGMDV	WGQGTTVTVSS	VH3
B1.1	QVQLVQSGAEVKKPGSSVKVSCKASGGSFS	SYAFT	WVRQAPGQGLEWMG	RIVPFFGVPNYAQKFRG	RVTITADKATSTVYMELSSLRSEDTAVYYCAR	DRRTTEDNWNSLWF	WGRGTLVTVSS	VH1
B6	QVQLVQSGAEVKKPGESLVISCKASGYSFSQG	SFWIA	WVRQMPGKGLEWMG	IIYPGDSDTRYSPSFQG	EVNISVDRSINTAYLQWSSLKASDTAMYYCAR	HDSRYYGFYFGMDV	WGQGTTVTVSS	VH5
B6.1	----------	-----	----------	----------	----------	---------- --	----------	VH5
B8	QVQLLESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VIWDGSNKYYADSVKG	RFTISRDNSKDTLYLQMNSLRAEDTAVYYCAR	GYSNYDYYYGMDV	WGQGTTVTVSS	VH3
B8.1	----------	-----	----------	----------	---------N----------	---------- -	----------	VH3
B11	QVQLLQSGAGVVQPGRSLRLSCAASGFIFR	TYGMH	WVRQAPGKGLEWVA	FVSSDGNNKFYSDSVKG	RFTIPRDNAKNTLYLQMNSLETEDTAVYYCAK	DRVPIDCSGGSCFSYGMDV	WGQGTTVTVSS	VH3
B11C3	E---VE-G----------T----L-	-----	----------	----------	---------- ----------	----L----	----------	VH3
B11E8	E---VQ-G----------	-----	----------	----S----------M----	---------- ----------	----------	----------	VH3
B12	QVNLRESGGGVVQPGRSLRLSCAASGFTFS	SYALH	WVRQTPGKGLEWVA	LISYDGSNKYYADSVKG	RFTISRDNSKNMLYLQMNSLRAEDTAVYYCAK	DRSHYGDYVGYLDY	WGQGTLVTVSS	VH3
B12.1	----------	-----	----------	----------	----------	---------- -	----------	VH3
B12.2	----------	-----	----------	----------	----------	---------- -	----------	VH3
1B18	EVQLVQSGGGLVQPGGSRRLSCAASGFTFN	AYWMT	WVRQAPGKGLEWVA	NINLDGTEIYYLDSVKG	RFTVSRDNVANSVFLQMSSLKVEDTAVYFCAR	LEWSGNGWSP	WGQGTLVTVSS	VH3
2B18.1	Q----------	-----	----------	----------	----------	---------- -	----------	VH3
4B19	QVQLVQSGAEVKKPGASVNVSCKASGYTFT	GYYIY	WVRQAPGQGLEWMG	WINPNSGVTKSAQKFQG	RVTMTTDTSTNTAYMELRRLRADDTAVYYCAR	EWTQLHSPYDY	WGQGTTVTVSS	VH1
1B22	QVQLQESGSRLVKPSQTLSLTCGVSGGSIS	SS	WIRQTPSKGLEWIG	YIYHSGSTYYNPSLKS	RVTMSVDKSKNQFSLNMSSVTAADTAVYYCAR	TAFYYENYGFIRCYLDF	WGQGTLVTVSS	VH4

Fig. 2

VL Clone	Framework1	CDR1	Framework 2	CDR2	Framework3	CDR3	Framework4	Gene Family
A12	DIQMTQSPSSLSASVGDRVTITC	RASQRISNYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTPPLT	FGGGTKVEIKR	VK1
6A12	DIQMTQSPSSVSASVGDRVTITC	RASQGISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QKANSFPLT	FGGGTKVEIKR	VK2
B1.1	DVVMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTPLT	FGQGTKLEIKR	VK1
B6	DVVMTQSPSSLSASVGDRITITC	QASQDISNFLN	WYQQKPGKAPKLLIR	DASNLET	GVPSRFSGGGSGTHFTFTISSLHPEDIATYFC	QQYDNLPYT	FGQGTKLEIKR	VK1
B6.1	-IQ----------V----	R-S-S--SY--	----E--------Y	S--S-QS	-------S---D--I----Q--F--Y-	--SYST-PYT	----------	VK1
B8	DIQMTQSPSSLSASVGDRVTITC	RASQRISNYLN	WYQQKPGKAPKLLIY	AASSLQS	EVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTPPLT	FGGGTKVDIKR	VK1
B8.1	----------	----------	----------	-------	---------Y----------	--------	----------	VK1
B11	DIVMTQSPDSLSASVGDRVTVTC	RASQSINSWLA	WYQQKPGKAPKLLIY	EASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYDSYWLT	FGGGTKVEIKR	VK1
B11C3	--Q------SV---------I--	----GVSR---	----R-E-------	G---Q-	----------D---------E------	-----FP--	----------	VK1
B11E8	E--I---A---V-P-E-A-LS-	-----VSKF--	-----R-Q--R----	G--TRAT	-I-A----------A-------SE--D---	----NWP1-	--Q--RL----	VK1
B12	DIVMTQSPSTLSASVGDRVTITC	RASQGISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQENSYPRA	FGQGTKLEIKR	VK1
B12.1	AIVLTQPPSVSVAPGKTAAITC	EGNNVGNKNVH	WYQQRPGQAPVLVVH	DDSDRPS	GIPERFSGSNSGNTALTITNRVEAGDEADYYC	QVWDSSSAQVV	FGGGTKLTVLG	VL3
B12.2	ESVLTQPPLVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	ENSKRSS	GIPDRFSGSKSGTSATLGITGLQTGDEADYYC	GTWDSSLSAVV	FGGGTKLTVLG	VL1
1B18	DVVMTQSPSSVSASVGDRVTITC	RASQSISSYLN	WYQQRPGKAPKLLIF	AASSLQS	AVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTPPT	FGQGTKVEIKR	VK1
2B18.1	-I---------I-------S-S-	----------	----K--------Y	KT---E-	G------R----------	------I-	--G--------	VK1
4B19	DIVLTQSPSTLSASVGDRVTITC	RASRSIGKYLN	WYQQRPGKAPKLLIY	AASSLHN	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQAFGFPRT	FGQGTKVEIKR	VK1
1E22	DIQMTQSPSTLSASTGDRVTISC	RASQSISQWLA	WYQQRPGKAPKLLIY	SASTLQT	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYPLT	FGQGTKLEIKR	VK1

Fig. 2 cont'd

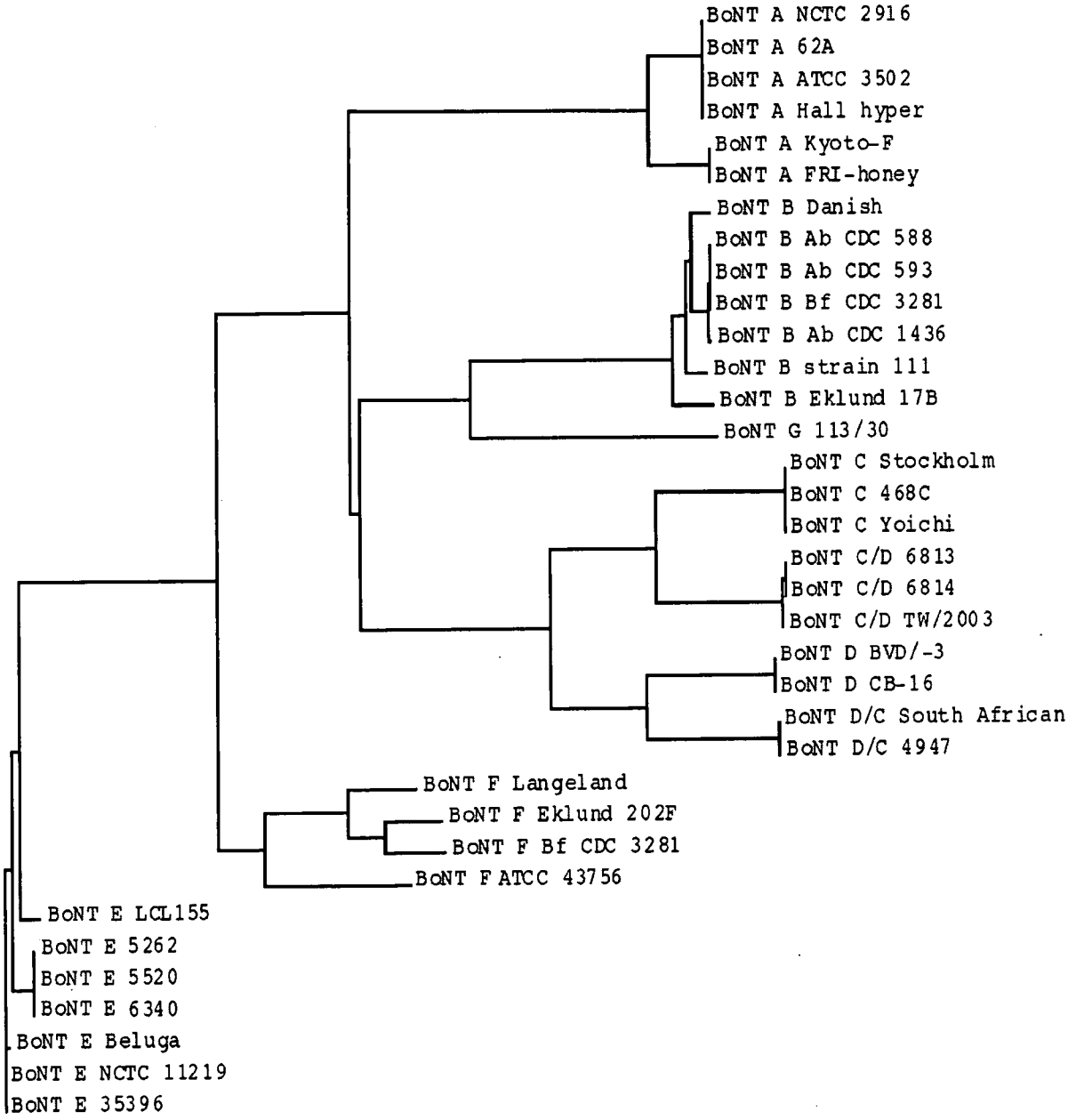
Deduced protein sequences of heavy and light chain variable regions of BoNT/E binder

VH Clone	Framework1	CDR1	Framework2	CDR2	Framework3	CDR3	Framework4	Gene Family
2A10	QVQLQQSGAEVKKPGSSVKVSCKASGGTFT	RYTIT	WVRQAPGQGLEWMG	GIIPIFDKANYAQKFQS	RVTFTADASTSTAYMELGSLRPEDTAVYYCAA	YSRGYVHFDY	WGPGTLVTVSS	VH1
3E1	QVQLVESGAEVKKPGSSVKVSCKASGGTFS	NSGFT	WVRQVPGQGLEWMG	GIIPMFGPANYAQKFQG	RVTITADESTRMVTMELRSLRSEDTAVYYCAR	DQGEYTVGMLLYYAMDV	WGEGTTVTVSS	VH1
3E2	QVQLQESGAEVKKPGSSVKVSCKASGGDLN	KYAIT	WLRQAPGQGFEWMG	GITPIFATTNYAQKFQG	RVMITADEVTSTVYMDLSSLGSEDTAIYFCAK	SPRGGIVGTFDT	WGQGTMVTVSS	VH1
3E3	QVQLVESGGGLVKPGESLRLSCAASGFTFS	NTNMN	WVRQAPGKGLEWVS	SISDGGSYRYYAYSVKG	RFTISRDNTKNSLYLQMNSLRAEDTALYYCAR	DEMVHGILVYYGMDV	WGQGTTVTVSS	VH3
3E4	QVQLQESGGGLVQPGGSLRLSCAASGFTFS	SDAMS	WVRQAPGKGLEWVA	AILPSGEATYYADSVKG	RFTISRHSSKNTLYLQMNSLRADDTAVYYCAR	DSYHSRLAAFDI	WGQGTMVTVSS	VH3
3E4.1	----------	-----	----------	----------	----------	----------	----------	VH3
3E5	QVQLVQSGGGVVQPGRPLRLSCAASTFNFR	DFYMS	WIRQAPGKGLEWVS	YIGSSGSALQYADSVKG	RFTISRDNDKNVLYLQMTSLRAEDTAVYYCAR	VASKYHDVLIDGFDI	WGQGTMVTVSS	VH3
3E6	QVQLVQSGGGVVQPGKSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGNKKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDAAVYYCAR	ARLCTSTSCYTFDP	WGQGTLVTVSS	VH3
3E6.1	----------	-----	----------	----------	----------	----------	----------	VH3
4E11	QVQLVQSGGGLVQPGGSLRLSCAASGFRFS	GYSFN	WVRQAPGKGLEWVA	YMSSGGSIKNYADSVKG	RFTISRDNAKNSLYLQVNSLRDEDTALYYCAR	GPPGRPNDAFDI	WGQGTMVTVSS	VH3
4E13	EVQLVQSGGGLVQPGGSLRLSCAASGFTFS	SYAMT	WVRQAPGKGLEWVS	SISVSGDSTYYADSVKG	RFTISRDNSKNTVSLQMNSLRAEDTALYYCAK	GLSKADLFGMDV	WGQGTMVTVSS	VH3
4E16	QVQLQESGPGLVKPSETLSLTCSVSGYSIS	DYYWS	WIRQPPGKGLEWIG	YIYYSGSTNYNPSLKS	RVTISVDTSKNQFSLNLSSVTAADTAVYYCAR	HTSGWSGGAFDI	WGQGTMVTVSS	VH4
4E16.1	----------	-----	----------	----------	----------	----------	----------	VH4
4E17	EVQLVQSGGNLVQPGGSLRLSCAATGFTGS	HRMT	WVRQAPGQGLEWVA	NINLDGTEKFYVDSVKG	RFTVSRDNRKSSVFLQMNNLRVDDTAVYYCAR	LQWGGTNGWLSP	WGQGTLVTVSS	VH3
4E17.1	----------	----	----------	----------	----------	----------	----------	VH3

Fig. 3

VL Clone	Framework1	CDR1	Framework2	CDR2	Framework3	CDR3	Framework4	Gene Family
2A10	DIVMTQSPSFLSASVGDRVTITC	WASQGISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYPLT	FGGGTKVDIKR	VK1
3E1	EIVLTQSPDSLSASVGDRVTITC	RASQGISGYLA	WYQHKAGKAPKLLIY	AASSLQS	GVPSRFSGSGYGTEFTLTISSLQPDDFATYYC	QQYNSYPFT	FGGGTKVEIKR	VK1
3E2	EIVLTQSPSFLSAFVGDRVTITC	RNSQSINNYLN	WYQQKAGKAPKLLIY	AASTLHT	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQSYSIPLT	FGGGTKVEIKR	VK1
3E3	DIVMTQSPDSLSASVGDSVTITC	RASQSFSSSYLA	WYQQKPGQAPRLLIY	AASSRAA	GVPTGSVADGSGTDFTLTISSLQPEDFAAYYC	QQSYSTPYT	FGGGTKVEIKR	VK3
3E4	DIVMTQSPSFLSAFVGDRVTITC	RASQSISNWLA	WYQQKPGKAPKVLIY	KASSLEN	GVPSRFSGSGSGTDFTLTISSLQPDDFATYYC	QQFNAYPLT	FGGGTKVEIKR	VK1
3E4.1	E--L----T---S----A---	----R-GS---	---------NP---	--F---S	--------R---E----S--------F-	---DS--Y-	--Q---L----	VK1
3E5	DVVMTQSPSSLSASIGDRVTFTC	QASQDISNRLN	WYQQKPGKVPKLLIS	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDPLLT	FGGGTKVEIKR	VK1
3E6	DIQMTQSPSSVSASVGDTVTISC	RASQGISSWLA	WYQQKSGQAPTLLIY	AASSLQS	GVPSRFSGSGSGTDFTLIISSLQPEDFATYYC	QQANTFPIT	FGGGTKVEIKR	VK1
3E6.1	----------R-S-T-	Q---D---NT-N	-----P-K--K----	-------	----------T----------	--SYN--P-	--Q---L----	VK1
4E11	ASVLTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKSNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSTGNQL	FGGGTKVTVLG	VL3
4E13	AELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GENSRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSPDSSGIHLV	FGGGTKVTVLG	VL3
4E16	EIVLTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLFY	WASTRES	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	HQYYSSPLT	FGGGTKLEIKR	VK4
4E16.1	-------N----------R-	---------G-----I-	---------I-	-------	----------E---------R----L---	Q----PWT	--Q--------	VK4
4E17	DIVMTQSPSSVSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	GTSNLQS	GVPSGFSGSGSGTDFTLTISSLQPEDFATYYC	QETYSTPPT	FGGGTKLEIKR	VK1
4E17.1	----------L----------S-	------RH-V-	----------	KA-S-A-	-A--R----------D------	-QS--I-L-	-----V----	VK1

Fig. 3, cont'd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BoNT A Hall hyper
BoNT A Kyoto-F
BoNT A FRI-honey
BoNT B Danish
BoNT B Ab CDC 588
BoNT B Ab CDC 593
BoNT B Bf CDC 3281
BoNT B Ab CDC 1436
BoNT B strain 111
BoNT B Eklund 17B
BoNT G 113/30
BoNT C Stockholm
BoNT C 468C
BoNT C Yoichi
BoNT C/D 6813
BoNT C/D 6814
BoNT C/D TW/2003
BoNT D BVD/-3
BoNT D CB-16
BoNT D/C South African
BoNT D/C 4947
BoNT F Langeland
BoNT F Eklund 202F
BoNT F Bf CDC 3281
BoNT F ATCC 43756
BoNT E LCL155
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BoNT E 5520
BoNT E 6340
BoNT E Beluga
BoNT E NCTC 11219
BoNT E 35396

Fig. 4

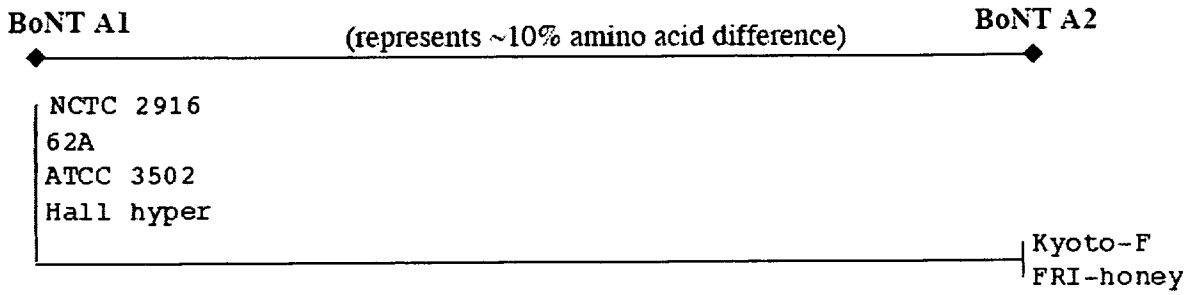
BoNT A1
(represents ~10% amino acid difference)
BoNT A2
NCTC 2916
62A
ATCC 3502
Hall hyper
Kyoto-F
FRI-honey

Fig. 5

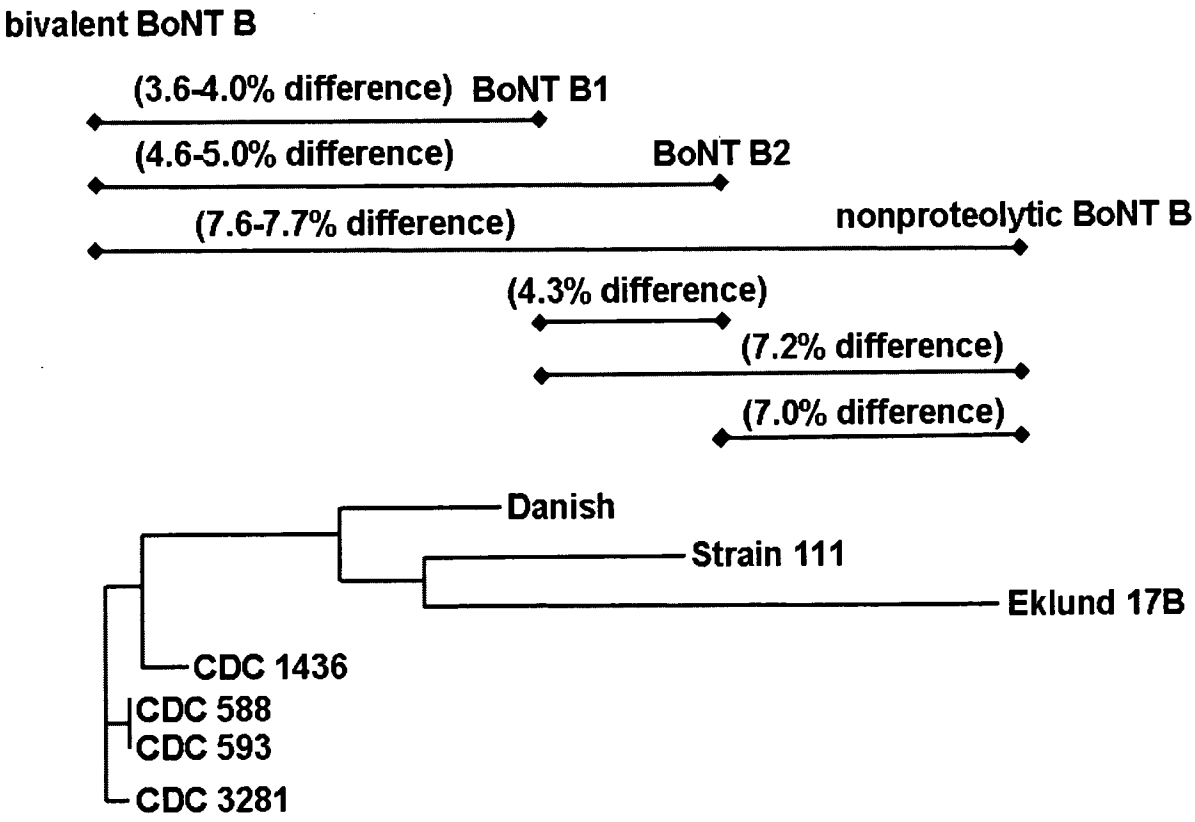
bivalent BoNT B
(3.6-4.0% difference) BoNT B1
(4.6-5.0% difference)
BoNT B2
(7.6-7.7% difference)
nonproteolytic BoNT B
(4.3% difference)
(7.2% difference)
(7.0% difference)
Danish
Strain 111
Eklund 17B
CDC 1436
CDC 588
CDC 593
CDC 3281

Fig. 6

HuC25 KD = 8.44 x 10^{-10} M

*Library constructed by error prone PCR of whole scFv
*3 mutations, 1 VH FMW1 and 2 VL CDR3
*5 fold affinity increase

AR1 KD = 1.69 x 10^{-10} M

*Library constructed by error prone PCR of whole scFv
*1 mutation VH CDR1
*2.8 fold affinity increase

AR2 KD = 6.14 x 10^{-11} M

*VH CDR1 was diversified by spiked oliog
*3 mutations, 2 VH CDR1 and 1 VH CDR2
*2.5 fold affinity increase

AR4 KD = 2.26 x 10^{-11} M

Fig. 7A

3D12 KD = 6.43 x 10^{-10} M

*Library constructed by error prone PCR of whole scFv
*5 mutations, 2 VL CDR1, 2 VL CDR2, and 1 VL CDR3
*45 fold affinity increase

RAZ1 KD = 2.1 x 10^{-11} M

Fig. 7B