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(54) Title: ANTIBODIES TO BOTULINUM NEUROTOXINS

(57) Abstract: The present disclosure provides antibodies that specifically bind to botulinum neurotoxins. The antibodies can be used in methods to specifically bind and, in some embodiments, neutralize, botulinum neurotoxin and are therefore also useful in the treatment of pathologies associated with botulinum neurotoxin poisoning.

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ANTIBODIES TO BOTULINUM NEUROTOXINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefit to U.S. provisional application serial no. 63/329,619, filed April 11, 2022, which application is incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED AS A SEQUENCE LISTING XML FILE

[0002] A Sequence Listing is provided herewith as a Sequence Listing XML, (UCSF-580WO_Sequence_listing), created on April 7, 2023 and having a size of 72,306 bytes. The contents of the Sequence Listing XML are incorporated herein by reference in their entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0003] This invention was made with government support under Cooperative Agreement U01AI056493 with the National Institute of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID), Cooperative Agreement U01 AI056493 with NIAID, Grant No. R01AI104579 from NIAID, and contract HDTRAA1-07-C-0030 from Defense Threat Reduction Agency (DTRA). The government has certain rights in the invention.

INTRODUCTION

[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 "bt(dot)cdc(dot)gov/agent/botulism/index(dot)asp"). Botulinum 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). 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. 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. Currently existing equine antitoxins include Botulism Antitoxin Heptavalent (A,B,C,D,E,F,G) – Equine (BAT), which is an FDA-Approved CDC stock for Anti-BoNT treatment following documented or suspective exposure to botulinum neurotoxin serotypes in adults and pediatric patients (Center for Biologics Evaluation and Research, Cangene Corporation, and U.S. Food and Drug Administration. "Fractionated Plasma Products - BAT (Botulism Antitoxin Heptavalent (A, B, C, D, E, F, G) - (Equine)." U.S. Food and Drug Administration Home Page. Center for Biologics Evaluation and Research, 16 July 2016).

[0006] The development of monoclonal antibody (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.

[0007] 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

[0008] The present disclosure provides antibodies that specifically bind to botulinum neurotoxins. These antibodies can be used in methods to specifically bind and, in some embodiments, neutralize, botulinum neurotoxin and are therefore also useful in the treatment of botulism.

[0009] Antibodies that bind to and neutralize and/or otherwise clear botulinum neurotoxin(s) are disclosed herein. Particularly effective neutralization of a BoNT serotype can be achieved by the use of neutralizing antibodies that bind two or more subtypes of the particular neurotoxin serotype with particularly high affinity and/or by combinations of such antibodies. The present disclosure provides antibodies that bind one or more BoNT serotypes BoNT/C, BoNT/D, BoNT/CD, BoNT/DC, BoNT/F, as well as subtypes thereof. BoNT subtypes include but are not limited to pure BoNT/C, pure BoNT/D, and pure BoNT/F. BoNT mosaics include BoNT/CD and BoNT/DC. Compositions comprising neutralizing antibodies that bind two or more BoNT subtypes (e.g., BoNT/C, BoNT/CD, BoNT/D, BoNT/DC, etc.) with high affinity are also provided herein.

[0010] An antibody for Botulinum neurotoxin (BoNT) is provided herein may be a single chain Fv (scFv), a Fab, a $F(ab')_2$, an $(ScFv)_2$, and the like. The antibody may be an IgG. The antibody may also be in a composition comprising a pharmaceutically acceptable excipient (e.g., in a unit dosage formulation).

[0011] Methods of inhibiting and/or neutralizing the activity of BoNT in a mammal may involve administering to a mammal in need thereof a composition comprising at least one neutralizing anti-BoNT antibody as described herein. The composition may include at least two different antibodies, each of which binds to different BoNT subtypes or to different epitopes on a BoNT subtype. The composition may include at least three, at least four, or more different antibodies, each of which may bind to different BoNT epitopes.

[0012] Compositions provided herein may comprise an antibody that specifically bind to a BoNT. The compositions typically include a first antibody that binds one or more serotypes, e.g., an antibody as described above. The compositions can optionally include a second antibody , a third antibody, or a fourth antibody, or more antibodies that bind one or more BoNT serotypes.

[0013] Nucleic acids provided herein encode one or more antibodies that are described herein. Cells containing such nucleic acids are also provided herein. Kits provided for neutralizing a BoNT may include a composition containing one or more antibodies as described herein. The kits optionally also include instructional materials teaching the use of the composition to specifically bind to a BoNT. The composition may be 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/C polypeptide" refers to either a full-length BoNT/C (a neurotoxin produced by *Clostridium botulinum* of the type C serotype) or a fragment thereof (e.g., the heavy chain (HC) fragment). The HC fragment of BoNT/A is an approximately 50 kDa 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/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F etc.).

[0016] The term "BoNT subtype" refers to botulinum neurotoxins of a particular serotype (e.g., A, B, C, D, E, F, G, etc.) that differ from each other sufficiently to produce differential antibody binding. BoNT serotypes A, B, E, and F all exist as multiple subtypes which differ from each other in amino acid sequence to an extent that impacts the ability of monoclonal antibodies (mAbs) and polyclonal serum to bind and neutralize the toxin.

[0017] A "mosaic BoNT", as used herein, refers to a BoNT polypeptide that contains at least two contiguous amino acid sequences, each of which is derived from a different serotype or subtype.

[0018] "Derived from" in the context of an amino acid sequence or polynucleotide sequence (e.g., an amino acid sequence "derived from" BoNT/C) is meant to indicate that the polypeptide or nucleic acid has a sequence that is based on that of a reference polypeptide or nucleic acid (e.g., a naturally occurring BoNT/C or encoding nucleic acid), and is not meant to be limiting as to the source or method in which the protein or nucleic acid is made.

[0019] An "anti-BoNT antibody" refers to an antibody that binds a BoNT polypeptide, specifically binds a BoNT polypeptide with a K_D less than about 10^{-7} M, less than about 10^{-8} M, less than about 10^{-9} M, less than about 10^{-10} M, less than about 10^{-11} M, or less than about 10^{-12} M or less. In certain embodiments, "high affinity" antibodies have a K_D of 5 nM or less, e.g., 100 pM, 80 pM, or less.

[0020] "Neutralization" refers to a measurable decrease in the toxicity and/or circulating level of a Botulinum neurotoxin (e.g., BoNT/C) in *in vitro* testing, animals, or human patient.

[0021] By "treatment" it is meant that at least an amelioration of the symptoms associated with the condition afflicting the host is achieved, where amelioration refers to at least a reduction in the magnitude of a parameter, e.g., symptom, associated with the condition being treated. As such, treatment includes situations where the condition, or at least symptoms associated therewith, are reduced or

avoided. Treatment includes: (i) prevention, that is, reducing the risk of development of clinical symptoms, including causing the clinical symptoms not to develop, e.g., preventing disease progression to a harmful or otherwise undesired state; (ii) inhibition, that is, arresting the development or further development of clinical symptoms, e.g., mitigating or completely inhibiting an active disease.

[0022] "Potency" refers to the degree of protection from challenge with BoNT. This can be measured/quantified for example, as an increase in the LD_{50} of a Botulinum neurotoxin (BoNT). In toxicology, the median lethal dose, LD_{50} (abbreviation for "Lethal Dose, 50%"), or LCt_{50} (Lethal Concentration & Time) of a toxic substance or radiation is the dose required to kill half the members of a tested population. The LD_{50} usually expressed as the mass of substance administered per unit mass of test subject, such as grams of substance per kilogram of body mass. Stating it this way allows the relative toxicity of different substances to be compared, and normalizes for the variation in the size of the animals exposed (although toxicity does not always scale simply with body mass). Typically, the LD_{50} of a substance is given in milligrams per kilogram of body weight. In the case of some toxins, the LD_{50} may be more conveniently expressed as micrograms per kilogram (μg/kg) of body mass.

[0023] 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 5 nM or less.

[0024] The following abbreviations are used herein: BoNT; Botulinum neurotoxin, BoNT/A; BoNT serotype A, BoNT/B; BoNT serotype B, BoNT/C; BoNT serotype C, BoNT/D; BoNT serotype D, BoNT/F; BoNT serotype F, BoNT/G; BoNT serotype G, Fc; fragment crystalizable, Fab'_2; fragment, antigen binding, mAb; monoclonal antibody, IgG; immunoglobulin G, $LD_{50,}$; lethal dose 50%, scFv; single chain variable fragment, V_H; heavy chain variable region, V_k; kappa light chain variable region, PCR; polymerase chain reaction, AgaII or Aga2; yeast agglutinin receptor II, BoNT/A L_C; BoNT/A light chain, BoNT/B L_C; BoNT/B light chain, BoNT/B H_C; C-terminal domain of the BoNT/B heavy chain, pM; picomolar, fM; femtomolar, IU; International Unit, SD-CAA; selective dextrose casamino acids media, SG-CAA; selective galactose casamino acids media, CHO; Chinese hamster ovary cells, FACS; fluorescent activated cell sorting, K_D; equilibrium dissociation constant, k_{on}; association rate constant, k_{off}; dissociation rate constant, MFI: mean fluorescent intensity.

[0025] The terms "polypeptide", "peptide", and "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 usually 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. However, the absence of a dash should not be taken to mean that such peptide bonds or covalent bond to a carboxyl or hydroxyl end group is not present, as it is conventional in representation of amino acid sequences to omit such.

[0026] The term "antibody" (also used interchangeably with "immunoglobulin") encompasses polyclonal and monoclonal antibody preparations where the antibody may be of any class of interest (e.g., IgM, IgG, and subclasses thereof), as well as preparations including hybrid antibodies, altered antibodies, $F(ab')_2$ fragments, F(ab) molecules, Fv fragments, scFv fragments, single chain antibodies, single domain antibodies, chimeric antibodies, humanized antibodies, and functional fragments thereof which exhibit immunological binding properties of the parent antibody molecule. The antibodies may be conjugated to other moieties, and/or may be bound to a support (e.g., a solid support), such as a polystyrene plate or bead, test strip, and the like.

[0027] Immunoglobulin polypeptides include the kappa and lambda light chains and the alpha, gamma (IgG_1, IgG_2, IgG_3, IgG_4), delta, epsilon and mu heavy chains or equivalents in other species. Full-length immunoglobulin "light chains" (usually of about 25 kDa or about 214 amino acids) comprise a variable region of about 110 amino acids at the NH_2-terminus and a kappa or lambda constant region at the COOH-terminus. Full-length immunoglobulin "heavy chains" (of about 50 kDa or about 446 amino acids), similarly comprise a variable region (of about 116 amino acids) and one of the aforementioned heavy chain constant regions, e.g., gamma (of about 330 amino acids).

[0028] An immunoglobulin light or heavy chain variable region is composed of a "framework" region (FR) interrupted by three hypervariable regions, also called "complementarity determining regions" or "CDRs". The extent of the framework region and CDRs have been defined (see, "Sequences of Proteins of Immunological Interest," E. Kabat et al., U.S. Department of Health and Human Services, (1991) and Lefranc et al. IMGT, the international ImMunoGeneTics information system®. Nucl. Acids Res., (2005) 33:D593-D597)). A detailed discussion of the IMGTS system, including how the IMGTS system was formulated and how it compares to other systems, is provided on the World Wide Web at imgt.cines.fr/ textes/ IMGTScientificChart/ Numbering/ IMGTnumberingsTable.html. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs. The CDRs are primarily responsible for binding to an epitope of an antigen. All CDRs and framework provided by the present disclosure are defined according to Kabat et al, *supra*, unless otherwise indicated. The three light chain CDRs, as used herein,

are also referred to as "LCDR1", "LCDR2", and "LCDR3". The three heavy chain variable CDRs, as used herein, are also referred to as "HCDR1", "HCDR2", and "HCDR3".

[0029] An "antibody" thus encompasses a protein having one or more polypeptides that can be genetically encodable, e.g., 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.

[0030] 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.

[0031] Antibodies encompass intact immunoglobulins as well 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 VH-CHI 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 $F(ab')_2$ 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, including, but are not limited to, Fab'_2, IgG, IgM, IgA, scFv, dAb, nanobodies, unibodies, and diabodies.

[0032] Antibodies and fragments of the present disclosure encompass those that are bispecific. Bispecific antibodies or fragments can be of several configurations. For example, bispecific antibodies may resemble single antibodies (or antibody fragments) but have two different antigen binding sites (variable regions). Bispecific antibodies may be produced by chemical techniques (Kranz et al. (1981) *Proc. Natl. Acad. Sci.*, USA, 78: 5807), by "polydoma" techniques (see, e.g., U.S. Pat. No. 4,474,893), or by recombinant DNA techniques. Bispecific antibodies may have binding specificities for at least two different epitopes, at least one of which is an epitope of BoNT. The BoNT binding antibodies and

fragments can also be heteroantibodies. Heteroantibodies are two or more antibodies, or antibody binding fragments (e.g., Fab) linked together, each antibody or fragment having a different specificity.

[0033] 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).

[0034] 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 K_D 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 equilibrium dissociation constant K_D (see, generally, Davies el al. *Ann. Rev. Biochem.* 1990, 59: 439-15 473).

[0035] An "anti-BoNT antibody" refers to an antibody that binds to one or more Botulinum neurotoxin(s) (e.g., BoNT/C, BoNT/CD, etc.). Thus, for example the term "anti-BoNT/C- antibody", as used herein refers to an antibody that specifically binds to a BoNT/C polypeptide (e.g, a BoNT/C polypeptide). An example of an antibody of the present disclosure may bind to an LC domain of a BoNT/C polypeptide.

[0036] Antibodies derived from anti-BoNT antibodies have a binding affinity of about 1.6 x 10^{-8} M or better and can be derived by screening libraries of single chain Fv fragments displayed on phage or yeast constructed from heavy (V_H) and light (V_L) chain variable region genes obtained from mammals, including mice, 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.

[0037] An "epitope" is a site on an antigen (e.g., BoNT) to which an antibody binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed (1996).

[0038] A neutralizing epitope refers to the epitope specifically bound by a neutralizing antibody.

[0039] "Isolated" refers to an entity of interest that is in an environment different from that in which the compound may naturally occur. An "isolated" compound (e.g., an "isolated" antibody) is separated from all or some of the components that accompany it in nature and may be substantially enriched, e.g., may be purified so that the compound is at least about 70% pure, at least about 80% pure, at least about 90% pure, at least about 95% pure, at least about 98% pure, at least about 99%, or greater than 99% pure, or free of impurities, contaminants, and/or components other than the compound. "Isolated" also refers to the state of a compound separated from all or some of the components that accompany it during manufacture (e.g., chemical synthesis, recombinant expression, culture medium, and the like).

[0040] A single chain Fv ("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 are available for converting the 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.

[0041] Recombinant design methods may be used to develop suitable chemical structures (linkers) for converting two 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.

[0042] 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.*

[0043] 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 may be free of residues having large side groups which might interfere with proper folding of the V_H and V_L chains. Thus, suitable linkers 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 has the amino acid sequence $(Gly_4Ser)_3$ (SEQ ID NO:77). Another example of a suitable linker is a linker that has the amino acid sequence comprising 2 or 3 repeats of $[(Ser)_4Gly]$ (SEQ ID NO:78), such as $[(Ser)_4Gly]_3$ (SEQ ID NO:79), 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.).

[0044] The phrase "specifically binds to" 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/C-neutralizing antibodies can be raised to BoNT/C protein(s) that specifically bind to BoNT/C protein(s), and not to other proteins present in a sample. A variety of immunoassay formats may be used

to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase enzyme-linked immunosorbent assay (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.

[0045] 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 substituting 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).

DETAILED DESCRIPTION

[0046] The present disclosure provides antibodies that specifically bind to botulinum neurotoxins. The antibodies and derivatives thereof can be used in methods to specifically bind and, in some embodiments, neutralize, botulinum neurotoxin and are therefore also useful in the treatment of botulism.

[0047] 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 a 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. For each BoNT serotype, there can be multiple subtypes of BoNT. Antibodies of the present disclosure encompass antibodies that specifically bind one serotype (e.g., the BoNT/C serotype) and also antibodies that can bind more than one subtype.

[0048] In certain aspects, antibodies that are particularly efficient in the neutralization of a botulism neurotoxin (BoNT) subtype are disclosed. In certain aspects, these antibodies have a high affinity for BoNT and each of the various antibodies is either highly specific for a serotype/subtype or can cross-react with two, three, or more serotypes/subtypes (e.g., BoNT/C, BoNT/D, BoNT/CD, BoNT/DC and/or BoNT/F). In certain embodiments, neutralizations of BoNT may also be accomplished by using one, two, three, four, 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 (e.g., BoNT/C, BoNT/D, BoNT/CD, BoNT/DC and/or BoNT/F), or by bispecific or polyspecific antibodies with specificities for two, three, or four or more BoNT epitopes, and/or serotypes, and/or subtypes.

[0049] Compositions comprising at least two, at least three, at least four of the antibodies disclosed herein are also provided. In certain embodiments, the composition may comprise two or more antibodies that bind overlapping (partial or complete overlapping) or non-overlapping epitopes on the BoNT.

[0050] Compositions provided herein may further comprise antibodies selected from those described in PCT Pub. Nos. WO 07/094754, WO 05/016232, WO 09/008916, and WO 2010/014854), each of which PCT publication is herein incorporated by reference in its entirety.

[0051] As indicated above, the antibodies provided by the present disclosure bind to one or more botulinum neurotoxin serotypes C, D, or F (or mosaics thereof) and in some embodiments, neutralize the neurotoxin. Neutralization, in this context, refers to a measurable decrease in the toxicity and/or circulating level of the target neurotoxin. Such a decrease in toxicity can also 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 reduction can be determined in vivo, e.g., as an LD_{50} in a test animal (e.g., mouse) BoNT 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. The rate of clearance of BoNT mediated by a test antibody, or combination of test antibodies, can be measured (e.g., in mice) by administering labeled BoNT (e.g., radiolabeled BoNT) and measuring the levels of BoNT in the serum and the liver and other organs over time in the presence or absence of test antibody or antibodies (see, e.g., Ravichandran et al. (2006) *J Pharmacol Exp Ther* 318: 1343-1351).

[0052] The present disclosure also contemplates an antibody that specifically binds an epitope shared by two or more (e.g., two, three, four, five, six, or seven) BoNT serotypes and/or subtypes and/or mosaics, e.g., BoNT polypeptides that share at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or at least about 99%, amino acid sequence identity over the complete holotoxin, over the light chain only, over the translocation domain only, or over the C-terminal third of the protein that includes the receptor-binding domain.

[0053] As the antibodies of the present disclosure act to specifically bind to, and in some embodiments, neutralize botulinum neurotoxins, they are useful in the treatment of pathologies associated with botulinum neurotoxin poisoning. The treatments comprise administering to the poisoned subject

(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.

[0054] 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 progression) or even prophylactically (a use the military envisions for soldiers going in harm's way). Treatment with the neutralizing antibody can be provided as an adjunct to other therapies (e.g., antibiotic treatment).

[0055] The antibodies provided by this disclosure can also be used for the rapid detection/diagnosis of botulism and thereby supplement and/or replace previous laboratory diagnostics.

[0056] This disclosure also provides the epitopes specifically bound by botulinum neurotoxin antibodies described herein. Such epitopes are found at or near the N-terminus of the alpha helix-1 on the LC domain. These epitopes can be used to isolate, and/or identify and/or screen for other antibodies BoNT neutralizing antibodies as described herein.

I. Botulinum neurotoxin (BoNT)-binding antibodies

[0057] Anti-BoNT antibodies may be defined based on their affinity to one or more BoNT serotypes/subtypes. Numbering system used herein for toxins is based on Lacy et al. (1999) *J. Mol. Biol.* 291:1091-1104. A number of subtypes are known for each BoNT serotype. Thus, for example, BoNT/A subtypes include, but are not limited to, BoNT/Al, BoNT/A2, BoNT/A3, and the like. 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. 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, e.g. A3, is produced by a strain called Loch Maree that killed a number of people in an outbreak in Scotland. Similarly, a number of subtypes are known for BoNT/F. These include, but are not limited to, BoNT/F1, BoNT/F2, BoNT/F3, and the like, with amino acid sequence difference between subtypes as high as 36%. The subject antibodies encompass antibodies with high affinity to one or more BoNT serotypes/subtypes (e.g., BoNT/C, BoNT/D, and/or BoNT/F).

[0058] Serotypes that can be bound by the subject antibodies include BoNT/C (UniProt: P18640), BoNT/D (UniProt: P19321), mosaics thereof, or BoNT/F (UniProt: A7GBG3). Other BoNT subtypes/serotypes include BoNT/C1, BoNT/CD, BoNT/DC, BoNT/F1, BoNT/F2, BoNT/F3, BoNT/F4, BoNT/F5, BoNT/F6, and BoNT/F7.

[0059] The subject antibodies encompass high affinity antibodies that are cross-reactive with two or more subtypes within a serotype (such as BoNT/F1, BoNT/F2, BoNT/F3, BoNT/F4, BoNT/F5,

BoNT/F6, and BoNT/F7). The disclosure further provides antibodies that are cross-reactive with two or more serotypes (such as BoNT/C and BoNT/D).

[0060] Moreover, without being bound to a particular theory, these cross-reactive antibodies can be more efficient in neutralizing Botulinum neurotoxin, particularly when used in combination with one or more different neutralizing antibodies.

[0061] The antibodies of the present disclosure can be used individually, and/or in combination with each other, and/or in combination with other known anti-BoNT antibodies (see, e.g., Application Pub. No: 20080124328, 20020155114, 20040175385, 20020155114, and PCT Pub. Nos. WO 07/094754, WO 05/016232, WO 09/008916, and WO 2010/014854, which are incorporated herein by reference for all purposes). These antibodies can be used individually, and/or in combination with each other, and/or in combination with other known anti-BoNT antibodies to form bispecific or polyspecific antibodies.

[0062] Amino acid sequences of CDRs, Framework regions, VH, and VL chains of various antibodies disclosed herein are provided in Tables 1-4. CDRs listed in Tables 1 and 2 are defined according to Kabat et al., Sequences of Proteins of Immunological Interest," E. Kabat et al., U.S. Department of Health and Human Services, (1991). It will be appreciated that the amino acid sequence of a CDR can also be defined using alternative systems, which will be readily apparent to and applied by the ordinarily skilled artisan (see, e.g., Lefranc et al. IMGT, the international ImMunoGeneTics information system. Nucl. Acids Res., (2005) 33:D593-D597)). A detailed discussion of the IMGTS system, including how the IMGTS system was formulated and how it compares to other systems, is provided on the World Wide Web at imgt.cines.fr/ textes/ IMGTScientificChart/ Numbering/ IMGTnumberingsTable.html.

[0063] 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:77) to form a single-chain Fv antibody. The various CDRs and/or framework regions can be used to form human antibodies, chimeric antibodies, antibody fragments, polyvalent antibodies, and the like.

[0064] An antibody encompassed by the present disclosure may have the binding specificity (*i.e.,* in this context, the same CDRs, or substantially the same CDRs) of an antibody set forth in Table 4. An antibody encompassed by the present disclosure may therefore include one or more CDRs as set forth in a V_H or V_L sequence shown in Table 4 and, additionally, may have at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% identity, or up to 100% to a full-length V_H or V_L sequence shown in Table 4. In certain embodiments, an antibody of the present disclosure may include a VH chain comprising the HCDRs1-3 of an antibody listed in Table 1 and heavy chain framework regions 1-4, where each framework region has a sequence identity of at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% to the sequence of a corresponding framework region listed in Table 3. In certain embodiments, an antibody of the present disclosure may include a VL chain comprising the LCDRs1-3 of an antibody listed in Table 2 and light

chain framework regions 1-4, where each framework region has a sequence identity of at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% to the sequence of a corresponding framework region listed in Table 3.

[0065] Anti-BoNT antibodies of the present disclosure have a binding affinity (K_D) for a BoNT protein of 10^{-7} M or lower, 10^{-8} M or lower, 10^{-9} M or lower, 10^{-10} M or lower, 10^{-11} M or lower, or 10^{-12} M or lower. Some examples of K_Ds for BoNT/C or BoNT/D fall in the following ranges: between about 2×10^{-12} M to about 5×10^{-10} M, between about 5×10^{-10} to about 1×10^{-9} M, between 1×10^{-9} to 5×10^{-9} M, between 5×10^{-9} to 1×10^{-8} M, between 4×10^{-9} to 2×10^{-8} M.

[0066] In some cases, an antibody of the present disclosure has a K_D with a Botulinum neurotoxin of about 5 nM or less. In some cases, the antibody binds to BoNT/C, BoNT/D, a mosaic thereof, or BoNT/F with high affinity.

[0067] An antibody encompassed by the present disclosure may have a binding affinity (K_D) for a BoNT/C, BoNT/D, BoNT/DC, and/or BoNT/CD protein in the following ranges: between about 1×10^{-12} M to about 1×10^{-9} M.

[0068] An antibody encompassed by the present disclosure may comprise a VH chain comprising HCRDs1-3 and a VL chain comprising LCRDs1-3 of the 8DC4.4 antibody and may have binding affinity for (i) BoNT/C in the range of to about 1×10^{-10} M to about 2×10^{-10} M; (ii) BoNT/CD in the range of to about 1×10^{-11} M to about 5×10^{-11} M; (iii) BoNT/DC in the range of about 1×10^{-11} M to about 3×10^{-11} M; (iii) BoNT/D in the range of to about 1×10^{-11} M to about 2×10^{-11} M.

[0069] An antibody encompassed by the present disclosure may have a binding affinity (K_D) for a BoNT/F protein and/or subtypes thereof in the following ranges: between about 1×10^{-13} to about 5×10^{-13} M, between about 5×10^{-13} to about 1×10^{-12} M, between about 1×10^{-12} to about 5×10^{-12} M, between about 5×10^{-12} to about 1×10^{-11} M, between about 1×10^{-11} to about 5×10^{-11} M, between about 5×10^{-11} to about 1×10^{-10} M, between about 1×10^{-10} to about 5×10^{-10} M, between about 5×10^{-10} to about 1×10^{-9} M, between 1×10^{-9} to about 5×10^{-9} M, between about 5×10^{-9} to about 1×10^{-8} M, between about 1×10^{-8} to about 5×10^{-8} M, between about 5×10^{-8} to about 1×10^{-7} M. Certain antibodies can have a K_D for BoNT/F and/or subtypes thereof in the range between about 1×10^{-13} to about 3×10^{-8} M.

[0070] As noted above, the antibody may also be defined by the serotypes and/or subtypes with which it is cross-reactive. Some antibodies have an affinity that is specific for only one serotype or subtype. Others are cross-reactive for two or more subtypes and/or serotypes, for example BoNT/C, BoNT/D, BoNT/CD, BoNT/DC, and/or BoNT/F and its subtypes thereof. Antibodies can also be reactive across two or more serotypes (e.g., BoNT/C, BoNT/D, BoNT/CD, BoNT/DC, and/or BoNT/F). Similarly, a number of mosaics are also known for BoNT/C and BoNT/D. The subject antibodies encompass high

affinity antibodies that are cross-reactive with two or more mosaics or subtypes within a serotype (e.g., BoNT/C, BoNT/D, BoNT/CD and/or BoNT/DC).

[0071] The antibody of the present disclosure may also be defined by the epitope or the domain of BoNT bound by the antibody. The antibodies provided here may encompass those that bind to one or more epitopes or a specific domain of a BoNT.

[0072] In some cases, the antibody that binds to a Botulinum neurotoxin comprises a V_H chain comprising a CDR1, CDR2 and CDR3 and a V_L chain comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, or the Hu6F11 antibody. In some cases, the antibody comprises a V_H chain comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, or the Hu6F11 antibody. In some cases, the antibody comprises a V_L chain comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, or the Hu6F11 antibody. In some cases, the antibody that binds to a Botulinum neurotoxin comprises a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, or the Hu6F11 antibody. In some cases, the antibody is 8DC4.4. In some cases, the antibody is 6F5.4. In some cases, the antibody is Hu6F13.4. In some cases, the antibody is Hu6F11.

[0073] In some cases, the antibody that binds to a Botulinum neurotoxin comprises a V_H chain comprising a heavy chain framework region 1 (H-FR1), CDR1, CDR2 and CDR3 and a V_L chain comprising a CDR1, CDR2 and CDR3 of the 4C4.4 antibody. In some cases, the antibody that binds to a Botulinum neurotoxin comprises a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody. In some cases, the antibody is 4C4.4.

[0074] In some cases, the antibody comprises a V_H chain comprising the amino acid sequence of the V_H chain of the 8DC4.4 antibody, the 4C4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, or theHu6F11 antibody. In some cases, the antibody comprises a V_L chain the amino acid sequence of the V_L chain of the 8DC4.4 antibody, the 4C4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, or theHu6F11 antibody.

[0075] In some cases, the antibody binds to BoNT/C and/or BoNT/D. In some cases, the antibody that binds to BoNT/C and/or BoNT/D comprises a V_H chain comprising V_H CDRs 1-3 and a V_L chain comprising V_L CDRs 1-3 of the 8DC4.4 antibody. In some cases, the antibody that binds to BoNT/C and/or BoNT/D comprises a V_H chain comprising V_H CDRs 1-3 and H-FR1 and a V_L chain comprising V_L CDRs 1-3 of the 4C4.4 antibody. In some cases, the antibody that binds to BoNT/C and/or BoNT/D comprises a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 8DC4.4 or 4C4.4 antibody.

[0076] In some cases, the antibody binds to BoNT/F. In some cases, the antibody that binds to BoNT/F comprises a V_H chain comprising V_H CDRs 1-3 and a V_L chain comprising V_L CDRs 1-3 of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody. In some cases, the antibody that binds to BoNT/F comprises a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

[0077] The present disclosure provides an antibody that binds to a Botulinum neurotoxin, wherein the antibody is selected from the group consisting of:

[0078] a) an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:2, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:4, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:6, and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:9, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:11, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:13;

[0079] b) an antibody comprising a VH chain comprising a H-FR1 having the amino acid sequence set forth in SEQ ID NO:15, a HCDR1 having the amino acid sequence set forth in SEQ ID NO:16, a HCDR2 having the amino acid sequence set forth in SEQ ID NO18, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:20, and a VL chain comprising a LCDR1 having amino acid sequence SEQ ID NO:23, a LCDR2 having amino acid sequence SEQ ID NO:25, and a LCDR3 having amino acid sequence SEQ ID NO:27;

[0080] c) an antibody comprising a VH chain comprising HCDR1 having the amino acid sequence set forth in SEQ ID NO:30, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:32, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:34 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:36, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:37, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:39;

[0081] d) an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:42, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:44, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:46 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:49, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:50, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:52; and

[0082] c) an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:55, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:57, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:59 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:61, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:63, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:65.

[0083] In some cases, the antibody that binds to a Botulinum neurotoxin (e.g., BoNT/C, BoNT/D, BoNT/CD, and/or BoNT/DC) comprises a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:2, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:4, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:6 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:9, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:11, a LCDR3 having the amino acid sequence set forth in SEQ ID NO:13. In some cases, the antibody comprises a V_H chain having the amino acid sequence set forth in SEQ ID NO:67. In some cases, the antibody comprises a V_L chain having amino acid sequence set forth in SEQ ID NO:68.

[0084] In some cases, the antibody that binds to a Botulinum neurotoxin (e.g., BoNT/C, BoNT/D, BoNT/CD, and/or BoNT/DC) comprises a VH chain comprising a H-FR1 having the amino acid sequence set forth in SEQ ID NO:15, a HCDR1 having the amino acid sequence set forth in SEQ ID NO:16, a HCDR2 having the amino acid sequence set forth in SEQ ID NO18, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:20 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:23, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:25, and a LCDR3 having amino acid sequence SEQ ID NO:27. In some cases, the antibody comprises a V_H chain having amino acid sequence SEQ ID NO:69. In some cases, the antibody comprises a V_L chain having amino acid sequence SEQ ID NO:70.

[0085] In some cases, the antibody that binds to a Botulinum neurotoxin (e.g., BoNT/F) comprises a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:30, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:32, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:34 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:36, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:37, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:39. In some cases, the antibody comprises a V_H chain having the amino acid sequence set forth in SEQ ID NO:71. In some cases, the antibody comprises a V_L chain having the amino acid sequence set forth in SEQ ID NO:72.

[0086] In some cases, the antibody that binds to a Botulinum neurotoxin (e.g., BoNT/F) comprises a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:42,

a HCDR2 having the amino acid sequence set forth in SEQ ID NO:44, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:46 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:49, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:50, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:52. In some cases, the antibody comprises a V_H chain having amino acid sequence SEQ ID NO:73. In some cases, the antibody comprises a V_L chain having amino acid sequence SEQ ID NO:74.

[0087] In some cases, the antibody that binds to a Botulinum neurotoxin (e.g., BoNT/F) comprises a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:55, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:57, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:59 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:61, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:63, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:65. In some cases, the antibody comprises a V_H chain having amino acid sequence SEQ ID NO:75. In some cases, the antibody comprises a V_L chain having amino acid sequence SEQ ID NO:76.

[0088] The subject antibody may also be defined by the epitope shared by one or more antibodies. The ability of a particular antibody to recognize the same and/or overlapping epitope as another antibody can be determined by the ability of one antibody to competitively inhibit binding of the second antibody to the antigen. Competitive inhibition of binding may also be referred to as cross-reactivity of antibodies. Any of a number of competitive binding assays can be used to measure competition between two antibodies to the same antigen. For example, a sandwich ELISA assay can be used for this purpose. Additional methods for assaying for cross-reactivity are described later below.

[0089] A first antibody is considered to competitively inhibit binding of a second antibody, if binding of the second antibody to the antigen is reduced by at least 30%, usually at least about 40%, 50%, 60% or 75%, and often by at least about 90%, in the presence of the first antibody using any of the assays used to assess competitive binding.

[0090] Accordingly, antibodies provided by the present disclosure also encompass those that compete for binding to a BoNT with an antibody that includes one or more of the V_H CDRs (e.g., one or more of HCDR1, HCDR2, and HCDR3) set forth in Table 1 and/or one or more of the V_L CDRs (e.g., one or more of LCDR1, LCDR2, and LCDR3) set forth in Table 2.

[0091] In some cases, the antibody that specifically binds to a Botulinum neurotoxin competes for binding to a BoNT with an antibody comprising a V_H chain comprising a CDR1, CDR2 and CDR3 and a V_L chain comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, and/or theHu6F11 antibody. In some cases, the antibody comprises a V_H chain comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4

antibody, and/or theHu6F11 antibody. In some cases, the antibody comprises a V_L chain comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, and/or theHu6F11 antibody. In some cases, the antibody is 8DC4.4. In some cases, the antibody is 6F5.4. In some cases, the antibody is Hu6F13.4. In some cases, the antibody is Hu6F11.

[0092] In some cases, the antibody that specifically binds to a Botulinum neurotoxin competes for binding to a BoNT with an antibody comprising a V_H chain comprising a heavy chain framework region 1 (H-FR1), CDR1, CDR2 and CDR3 and a V_L chain comprising a CDR1, CDR2 and CDR3 of the 4C4.4 antibody. In some cases, the antibody is 4C4.4.

[0093] In some cases, the antibody that specifically binds to a Botulinum neurotoxin competes for binding to a BoNT with an antibody comprising a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 8DC4.4 antibody, the 4C4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, and/or theHu6F11 antibody. In some cases, the antibody comprises a V_H chain comprising the amino acid sequence of the V_H chain of the 8DC4.4 antibody, the 4C4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, and/or theHu6F11 antibody. In some cases, the antibody comprises a V_L chain the amino acid sequence of the V_L chain of the 8DC4.4 antibody, the 4C4.4 antibody, the 6F5.4 antibody, the Hu6F13.4 antibody, and/or theHu6F11 antibody. In some cases, the antibody is 8DC4.4. In some cases, the antibody is 4C4.4. In some cases, the antibody is 6F5.4. In some cases, the antibody is Hu6F13.4. In some cases, the antibody is Hu6F11.

[0094] In some cases, the antibody cross-reacts with and specifically binds to Botulinum neurotoxin serotype BoNT/C, Botulinum neurotoxin serotype BoNT/D, Botulinum neurotoxin serotype BoNT/F, Botulinum neurotoxin serotype BoNT/CD, and Botulinum neurotoxin serotype BoNT/DC, wherein the antibody specifically binds an epitope of a Botulinum neurotoxin that is specifically bound by an antibody comprising a V_H comprising a CDR1, CDR2 and CDR3 and a V_L comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody and/or the 4C4.4 antibody.

[0095] In some cases, the antibody cross-reacts with and specifically binds to Botulinum neurotoxin serotype BoNT/F, wherein the antibody specifically binds an epitope of a Botulinum neurotoxin that is specifically bound by an antibody comprising a V_H comprising a CDR1, CDR2 and CDR3, and a V_L comprising a CDR1, CDR2 and CDR3 of the 6F5.4 antibody, the Hu6F13.4 antibody, and/or the Hu6F11 antibody.

[0096] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody selected from the group consisting of:

[0097] a) an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:2, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:4, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:6, and a VL chain comprising a

LCDR1 having the amino acid sequence set forth in SEQ ID NO:9, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:11, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:13;

[0098] b) an antibody comprising a VH chain comprising a H-FR1 having the amino acid sequence set forth in SEQ ID NO:15, a HCDR1 having the amino acid sequence set forth in SEQ ID NO:16, a HCDR2 having the amino acid sequence set forth in SEQ ID NO18, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:20, and a VL chain comprising a LCDR1 having amino acid sequence SEQ ID NO:23, a LCDR2 having amino acid sequence SEQ ID NO:25, and a LCDR3 having amino acid sequence SEQ ID NO:27;

[0099] c) an antibody comprising a VH chain comprising HCDR1 having the amino acid sequence set forth in SEQ ID NO:30, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:32, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:34 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:36, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:37, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:39;

[00100] d) an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:42, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:44, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:46 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:49, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:50, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:52; and

[00101] e) an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:55, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:57, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:59 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:61, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:63, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:65.

[00102] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:2, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:4, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:6 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:9, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:11, a LCDR3 having the amino acid sequence set forth in SEQ ID NO:13. In some cases, the

antibody comprises a V_H chain having the amino acid sequence set forth in SEQ ID NO:67. In some cases, the antibody comprises a V_L chain having amino acid sequence set forth in SEQ ID NO:68.

[00103] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprising a VH chain comprising a H-FR1 having the amino acid sequence set forth in SEQ ID NO:15, a HCDR1 having the amino acid sequence set forth in SEQ ID NO:16, a HCDR2 having the amino acid sequence set forth in SEQ ID NO18, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:20 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:23, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:25, and a LCDR3 having amino acid sequence SEQ ID NO:27. In some cases, the antibody comprises a V_H chain having amino acid sequence SEQ ID NO:69. In some cases, the antibody comprises a V_L chain having amino acid sequence SEQ ID NO:70.

[00104] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:30, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:32, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:34 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:36, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:37, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:39. In some cases, the antibody comprises a V_H chain having the amino acid sequence set forth in SEQ ID NO:71. In some cases, the antibody comprises a V_L chain having the amino acid sequence set forth in SEQ ID NO:72.

[00105] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprising VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:42, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:44, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:46 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:49, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:50, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:52. In some cases, the antibody comprises a V_H chain having amino acid sequence SEQ ID NO:73. In some cases, the antibody comprises a V_L chain having amino acid sequence SEQ ID NO:74.

[00106] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprising a VH chain comprising a HCDR1 having the amino acid sequence set forth in SEQ ID NO:55, a HCDR2 having the amino acid sequence set forth in SEQ ID NO:57, a HCDR3 having the amino acid sequence set forth in SEQ ID NO:59 and a VL chain comprising a LCDR1 having the amino acid sequence set forth in SEQ ID NO:61, a LCDR2 having the amino acid sequence set forth in SEQ ID NO:63, and a LCDR3 having the amino acid sequence set forth in SEQ ID NO:65. In some cases, the

antibody comprises a V_H chain having amino acid sequence SEQ ID NO:75. In some cases, the antibody comprises a V_L chain having amino acid sequence SEQ ID NO:76.

[00107] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprises a variable heavy chain (VH) polypeptide comprising a V_H CDR1 of the antibody 8DC4.4, a V_H CDR2 of the antibody 8DC4.4, and a V_H CDR3 of the antibody 8DC4.4; and a variable light chain (VL) polypeptide comprising a V_L CDR1 of the antibody 8DC4.4, a V_L CDR2 of the antibody 8DC4.4, and a V_L CDR3 of the antibody 8DC4.4.

[00108] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprises a variable heavy chain (VH) polypeptide comprising a heavy chain framework region 1 (H-FR1) comprising the amino acid sequence of H-FR1 of 4C4.4, a V_H CDR1 of the antibody 4C4.4, a V_H CDR2 of the antibody 4C4.4, and a V_H CDR3 of the antibody 4C4.4; and a variable light chain (VL) polypeptide comprising a V_L CDR1 of the antibody 4C4.4, a V_L CDR2 of the antibody 4C4.4, and a V_L CDR3 of the antibody 4C4.4.

[00109] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprises a variable heavy chain (VH) polypeptide comprising a V_H CDR1 of the antibody 6F5.4, a V_H CDR2 of the antibody 6F5.4, and a V_H CDR3 of the antibody 6F5.4; and a variable light chain (VL) polypeptide comprising a V_L CDR1 of the antibody 6F5.4, a V_L CDR2 of the antibody 6F5.4, and a V_L CDR3 of the antibody 6F5.4.

[00110] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprises a variable heavy chain (VH) polypeptide comprising a V_H CDR1 of the antibody Hu6F13.4, a V_H CDR2 of the antibody Hu6F13.4, and a V_H CDR3 of the antibody Hu6F13.4; and a variable light chain (VL) polypeptide comprising a V_L CDR1 of the antibody Hu6F13.4, a V_L CDR2 of the antibody Hu6F13.4, and a V_L CDR3 of the antibody Hu6F13.4.

[00111] In some cases, the antibody competes for binding to a Botulinum neurotoxin with an antibody comprises a variable heavy chain (VH) polypeptide comprising a V_H CDR1 of the antibody Hu6F11, a V_H CDR2 of the antibody Hu6F11, and a V_H CDR3 of the antibody Hu6F11; and a variable light chain (VL) polypeptide comprising a V_L CDR1 of the antibody Hu6F11, a V_L CDR2 of the antibody Hu6F11, and a V_L CDR3 of the antibody Hu6F11.

Table 1: Amino acid sequence of VH CDRs of anti-BoNT antibodies

mAb	CDR1 (VH)	CDR2 (VH)	CDR3 (VH)
8DC4.4	SDNYFWT (SEQ ID NO:2)	HISYSGRSYTNLSLKR (SEQ ID NO:4)	ERLPPGRGYDMDV (SEQ ID NO:6)
4C4.4	NSPYFWN	YIYWSGSTNYNPSLKS	DQGGGTVVKENWFDP

	(SEQ ID NO:16)	(SEQ ID NO:18)	(SEQ ID NO:20)
6F5.4	HWMT (SEQ ID NO:30)	NINLDGTEKFYVDSVKG (SEQ ID NO:32)	LQWGERDGWLSP (SEQ ID NO:34)
Hu6F13.4	SYWMH (SEQ ID NO:42)	FINPINGGIIYNEKFRF (SEQ ID NO:44)	AGYYGSSRHFDV (SEQ ID NO:46)
Hu6F11	NYGMS (SEQ ID NO:55)	SISSGGSYIYYPDSVKG (SEQ ID NO:57)	RKGYDSLYWYFDV (SEQ ID NO:59)

Table 2: Amino acid sequence of VL CDRs of anti-BoNT antibodies

mAb	CDR1 (VL)	CDR2 (VL)	CDR3 (VL)
8DC4.4	RASRDISIYLN (SEQ ID NO:9)	AASNLET (SEQ ID NO:11)	QQSYNTPLT (SEQ ID NO:13)
4C4.4	QASQDIGKHLN (SEQ ID NO:23)	GASNLKT (SEQ ID NO:25)	QQSYSTPPT (SEQ ID NO:27)
6F5.4	RASQSIGRSLN (SEQ ID NO:36)	DASALHS (SEQ ID NO:37)	QQTNDLPVT (SEQ ID NO:39)
Hu6F13.4	RASQGIRNYLA (SEQ ID NO:49)	DASNLET (SEQ ID NO:50)	LQTSSTPWT (SEQ ID NO:52)
Hu6F11	RASQGIRSYLA (SEQ ID NO:61)	AASTLQS (SEQ ID NO:63)	QQYNSYPLT (SEQ ID NO:65)

Table 3: Amino acid sequence of Framework regions of VH and VL chains of anti-BoNT antibodies

Antibody	VH or VL	Framework 1	SEQ ID NO	Framework 2	SEQ ID NO	Framework 3	SEQ ID NO	Framework 4	SEQ ID NO
8DC4.4	VH	QVQLQESGPGLVKPSQTLSLTCTVSGGSIS	1	WIRQSPGKGLEWLG	3	RLTISVDTSKNQFSLNLNSVTAADTAVYYCAR	5	WGQGTTVTVSS	7
8DC4.4	VL	DIVMTQSPSSVSASVGDRVTITC	8	WYQQKPGKAPKLLIY	10	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	12	FGGGTKLEIKR	14
4C4.4	VH	QVQLQESGPGLVKPSQTLSLTCTVSGASIS	15	WFRQFPGKGLEWIG	17	RLAMSVDTSKNQFSLKLTSATAADTAVYYCAR	19	WGQGTLVTVSS	21
4C4.4	VL	DIVMTQSPSSLSASVGDRLTMTC	22	WYQQKAGKPPKLLIY	24	GVPSRFSAGVSWTDFTFTISNVQPEDVATYYC	26	FGQGTKVEIKR	28
6F5.4	VH	EVQLVPSGGNLVQPGGSLRLSCAATGPIDY	29	WVRQAPGQGLEWVA	31	RFTVSRDNRKSSVFLQMNNLRVDDTAVYYCAR	33	WGQGTLVTVSS	21
6F5.4	VL	DIVMTQSPPSLSASVGDRVRLTC	35	WYQQKPGKAPKLLIY	10	GVPSRFSGSGSGTDFTLTISGLQPEDFATYYC	38	FGGGTKLEIKR	40
Hu6F13.4	VH	QVQLVQSGAEVKKPGASVKVSCKASGYSFT	41	WVRQAPGQGLEWIG	43	RATLTVDTSISTAYMELSRLRSDDTAVYYCAR	45	WGQGTLLTVSS	47
Hu6F13.4	VL	ETTLTQSPSSLSASVGDRVTITC	48	WYQQKPGKAPKLLIY	10	GVPSRFSGSGSGTDFTFTISSLQPEDFATYYC	51	FGQGTKVDIKR	53
Hu6F11	VH	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	54	WVRQAPGKGLEWVA	56	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	58	WGQGTLVTVSS	21
Hu6F11	VL	DVVMTQSPSSLSAFVGDRVTITC	60	WFQQKPGKAPKLLIY	62	GVPSRFSGSGSGTEFTLTVSGLQPEDFATYYC	64	FGGGTRLEIKR	66

Table 4: Amino acid sequence of VH and VL chains of anti-BoNT antibodies

Antibody	VH or VL	Variable Chain Sequence	SEQ ID NO
8DC4.4	VH	QVQLQESGPGLVKPSQTLSLTCTVSGGSISSDNYFWTWIRQSPGKGLEWLGHISYSGRSYTNLSLKRRLTISVD TSKNQFSLNLNSVTAADTAVYYCARERLPPGRGYDMDVWGQGTTVTVSS	67
8DC4.4	VL	DIVMTQSPSSVSASVGDRVTITCRASRDISIYLNWYQQKPGKAPKLLIYAASNLETGVPSRFSGSGSGTDFTLTI SSLQPEDVATYYCQQSYNTPLTFGGGTKLEIKR	68
4C4.4	VH	QVQLQESGPGLVKPSQTLSLTCTVSGASISNSPYFWNWFRQFPGKGLEWIGYIYWSGSTNYNPSLKSRLAMS VDTSKNQFSLKLTSATAADTAVYYCARDQGGGTVVKENWFDPWGQGTLVTVSS	69
4C4.4	VL	DIVMTQSPSSLSASVGDRLTMTCQASQDIGKHLNWYQQKAGKPPKLLIYGASNLKTGVPSRFSAGVSWTDF TFTISNVQPEDVATYYCQQSYSTPPTFGQGTKVEIKR	70
6F5.4	VH	EVQLVPSGGNLVQPGGSLRLSCAATGPIDYHWMTWVRQAPGQGLEWVANINLDGTEKFYVDSVKGRFTV SRDNRKSSVFLQMNNLRVDDTAVYYCARLQWGERDGWLSPWGQGTLVTVSS	71
6F5.4	VL	DIVMTQSPPSLSASVGDRVRLTCRASQSIGRSLNWYQQKPGKAPKLLIYDASALHSGVPSRFSGSGSGTDFTL TISGLQPEDFATYYCQQTNDLPVTFGGGTKLEIKR	72
Hu6F13.4	VH	QVQLVQSGAEVKKPGASVKVSCKASGYSFTSYWMHWVRQAPGQGLEWIGFINPINGGIIYNEKFRFRATLT VDTSISTAYMELSRLRSDDTAVYYCARAGYYGSSRHFDVWGQGTLLTVSS	73
Hu6F13.4	VL	ETTLTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTI SSLQPEDFATYYCLQTSSTPWTFGQGTKVDIKR	74
Hu6F11	VH	EVQLVESGGGLVKPGGSLRLSCAASGFTFSNYGMSWVRQAPGKGLEWVASISSGGSYIYYPDSVKGRFTISR DNAKNSLYLQMNSLRAEDTAVYYCARRKGYDSLYWYFDVWGQGTLVTVSS	75
Hu6F11	VL	DVVMTQSPSSLSAFVGDRVTITCRASQGIRSYLAWFQQKPGKAPKLLIYAASTLQSGVPSRFSGSGSGTEFTLT VSGLQPEDFATYYCQQYNSYPLTFGGGTRLEIKR	76

II. Potency of Botulinum neurotoxin (BoNT)-binding antibodies

[00112] 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 LD_{50}s/mg (human) and 55,000 mouse LD_{50}s /mg (horse).

[00113] Based on calculation, a commercially desirable antitoxin may generally have a potency greater than about 10,000 to 100,000 LD_{50}s/mg. Combinations of the antibodies described herein *(e.g.,* two antibodies, or three antibodies) can meet this potency. Thus, this disclosure provides a combination of antibodies (*e.g.,* two or three antibodies) that specifically bind to BoNT, and in some embodiments neutralize BoNT, at a potency of at least about 10,000 mouse LD_{50}s/mg of antibody, at least about 15,000 mouse LD_{50}s/mg of antibody, or at least about 20,000 mouse LD_{50}s/mg of antibody, at least about 25,000 mouse LD_{50}s/mg of antibody.

III. Preparation of anti-BoNT antibodies

Recombinant expression of anti-BoNT antibodies

[00114] Using the information provided herein, the botulinum neurotoxin binding antibodies of the present disclosure are prepared using standard techniques well known to those of skill in the art.

[00115] For example, the polypeptide sequences provided herein (*see, e.g.,* Tables 1-4) can be used to determine appropriate nucleic acid sequences encoding the anti-BoNT 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.

[00116] 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 can be 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.

[00117] 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.

[00118] 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.

[00119] 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), plant, 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.

[00120] 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 *et al* (1989) *supra*.

[00121] 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 and the L-arabinose (araBAD) operon (Better (1999) *Gene Exp Systems* pp95-107 Academic Press, Inc., San Diego, CA). 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 *et al* (1989) *supra* for details concerning selection markers, *e.g.*, for use in *E. coli*.

[00122] 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*. *E. coli* systems may also be used.

[00123] 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). Alternatively, nucleic acid encoding the anti-BoNT antibodies may be operably linked to a secretion signal sequence such as pelB so

that the anti-BoNT antibodies are secreted into the medium in correctly-folded form (Better *et al* (1988) *Science* 240: 1041-1043).

[00124] Methods of transfecting and expressing genes in mammalian cells are known in the art (see e.g., Birch and Racher *Adv. Drug Deliv. Rev.* 2006, 58: 671-685). 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).

[00125] The culture of cells used in the present disclosure, 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).

[00126] 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.

[00127] The anti-BoNT antibody gene(s) (*e.g.,* anti-BoNT scFv gene) may be 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 anti-BoNT antibodies are found, for example, in Amersdorfer *et al.* (1997) *Infect. Immunity,* 65(9): 3743-3752, and the like.

Preparation of whole polyclonal or monoclonal antibodies

[00128] The anti-BoNT antibodies of the present disclosure include individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Certain antibodies may be selected to bind one or more epitopes bound by the antibodies described herein. 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.

Polyclonal antibody production

[00129] Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (*e.g.,* BoNT/C, BoNT/D, BoNT/DC, BoNT/F *etc.*), subsequences including, but not

limited to subsequences comprising epitopes specifically bound by antibodies expressed by clones disclosed herein, e.g., 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. 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 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).

[00130] Antibodies that specifically bind to the epitopes described herein can be selected from polyclonal sera using the selection techniques described herein.

Monoclonal antibody production

[00131] 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.

[00132] Summarized briefly, monoclonal antibody production using hybridomas may proceed by injecting an animal with an immunogen (*e.g.,* BoNT/C, BoNT/D, BoNT/DC, BoNT/F, *etc.*) subsequences including, but not limited to subsequences comprising epitopes specifically bound by antibodies expressed by clones 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 antibodies *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secretes 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.

[00133] 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 (e.g., mammalian) host. The

antibodies of the present disclosure are used with or without modification, and include chimeric antibodies such as humanized murine antibodies.

[00134] Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules which bypass the generation of hybridomas are contemplated for the present BoNT binding antibodies and fragments. DNA is cloned into a bacterial expression system. One example of a suitable technique uses a bacteriophage lambda vector system having a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional Fab fragments for those which bind BoNT. Such BoNT binding agents (Fab fragments with specificity for a BoNT polypeptide) are specifically encompassed within the BoNT binding antibodies and fragments of the present disclosure. Other methods for screening and production of antibodies may employ one or more of display systems such as phage display, yeast display, ribosome, etc., and an antibody production system such as that derived from transgenic mice.

[00135] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an antibody of the present disclosure. The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a V_H of a subject antibody. The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a V_L of a subject antibody. The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a V_H and a V_L of a subject antibody. In some instances, a subject nucleic acid comprises a nucleotide sequence encoding V_H CDR1, CDR2, and CDR3 of a subject antibody and/or a V_L CDR1, CDR2, and CDR3 of a subject antibody.

[00136] In some cases, the isolated nucleic acid comprises the nucleotide sequence encoding an amino acid sequence of a) a V_H of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_H of the antibody 8DC4.4; and b) a V_L of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_L of the antibody 8DC4.4.

[00137] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable heavy chain (V_H) polypeptide comprising a V_H CDR1 selected from a V_H of the antibody 8DC4.4, a V_H CDR2 comprising a V_H CDR2 selected from a V_H of the antibody 8DC4.4, and a V_H CDR2 selected from a V_H of the antibody 8DC4.4. In such cases, a variable heavy chain (V_H) polypeptide is encoded by the nucleic acid.

[00138] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable light chain (V_L) polypeptide comprising a V_L

CDR1 selected from a V_L of the antibody 8DC4.4, a V_L CDR2 comprising a V_L CDR2 selected from a V_L of the antibody 8DC4.4, and a V_L CDR2 selected from a V_L of the antibody 8DC4.4. In such cases, the variable light chain (V_L) polypeptide is encoded by the nucleic acid.

[00139] In some cases, the isolated nucleic acid comprises the nucleotide sequence encoding an amino acid sequence of: a) a V_H of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_H of the antibody 4C4.4; and b) a V_L of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_L of the antibody 4C4.4.

[00140] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable heavy chain (V_H) polypeptide comprising a H-FR1 comprising the amino acid sequence of H-FR1 of 4C4.4, a V_H CDR1 selected from a V_H of the antibody 4C4.4, a V_H CDR2 comprising a V_H CDR2 selected from a V_H of the antibody 4C4.4, and a V_H CDR2 selected from a V_H of the antibody 4C4.4. In such cases, a variable heavy chain (V_H) polypeptide is encoded by the nucleic acid.

[00141] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable light chain (V_L) polypeptide comprising a V_L CDR1 selected from a V_L of the antibody 4C4.4, a V_L CDR2 comprising a V_L CDR2 selected from a V_L of the antibody 4C4.4, and a V_L CDR2 selected from a V_L of the antibody 4C4.4. In such cases, the variable light chain (V_L) polypeptide is encoded by the nucleic acid.

[00142] In some cases, the isolated nucleic acid comprises the nucleotide sequence encoding an amino acid sequence of: a) a V_H of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_H of the antibody 6F5.4; and b) a V_L of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_L of the antibody 6F5.4.

[00143] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable heavy chain (V_H) polypeptide comprising a V_H CDR1 selected from a V_H of the antibody 6F5.4, a V_H CDR2 comprising a V_H CDR2 selected from a V_H of the antibody 6F5.4, and a V_H CDR2 selected from a V_H of the antibody 6F5.4. In such cases, a variable heavy chain (V_H) polypeptide is encoded by the nucleic acid.

[00144] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable light chain (V_L) polypeptide comprising a V_L CDR1 selected from a V_L of the antibody 6F5.4, a V_L CDR2 comprising a V_L CDR2 selected from a V_L of the antibody 6F5.4, and a V_L CDR2 selected from a V_L of the antibody 6F5.4. In such cases, the variable light chain (V_L) polypeptide is encoded by the nucleic acid.

[00145] In some cases, the isolated nucleic acid comprises the nucleotide sequence encoding an amino acid sequence of: a) a V_H of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_H of the antibody Hu6F13.4; and b) a V_L of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_L of the antibody Hu6F13.4.

[00146] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable heavy chain (V_H) polypeptide comprising a V_H CDR1 selected from a V_H of the antibody Hu6F13.4, a V_H CDR2 comprising a V_H CDR2 selected from a V_H of the antibody Hu6F13.4, and a V_H CDR2 selected from a V_H of the antibody Hu6F13.4. In such cases, a variable heavy chain (V_H) polypeptide is encoded by the nucleic acid.

[00147] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable light chain (V_L) polypeptide comprising a V_L CDR1 selected from a V_L of the antibody Hu6F13.4, a V_L CDR2 comprising a V_L CDR2 selected from a V_L of the antibody Hu6F13.4, and a V_L CDR2 selected from a V_L of the antibody Hu6F13.4. In such cases, the variable light chain (V_L) polypeptide is encoded by the nucleic acid.

[00148] In some cases, the isolated nucleic acid comprises the nucleotide sequence encoding an amino acid sequence of: a) a V_H of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_H of the antibody Hu6F11; and b) a V_L of an antibody comprising a CDR1, CDR2 and CDR3, wherein the CDR1, CDR2, and CDR3 are selected from the V_L of the antibody Hu6F11.

[00149] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable heavy chain (V_H) polypeptide comprising a V_H CDR1 selected from a V_H of the antibody Hu6F11, a V_H CDR2 comprising a V_H CDR2 selected from a V_H of the antibody Hu6F11, and a V_H CDR2 selected from a V_H of the antibody Hu6F11. In such cases, a variable heavy chain (V_H) polypeptide is encoded by the nucleic acid.

[00150] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence encoding an amino acid sequence of a variable light chain (V_L) polypeptide comprising a V_L CDR1 selected from a V_L of the antibody Hu6F11, a V_L CDR2 comprising a V_L CDR2 selected from a V_L of the antibody Hu6F11, and a V_L CDR2 selected from a V_L of the antibody Hu6F11. In such cases, the variable light chain (V_L) polypeptide is encoded by the nucleic acid.

[00151] The nucleic acid can be a recombinant vector, as described above, which provides for amplification and/or expression (synthesis) of the encoded antibody. The recombinant vector can be suitable for expression in prokaryotic and/or eukaryotic cells.

[00152] The present disclosure also provides a cell, e.g., a genetically modified cell, that comprises a subject nucleic acid. A subject genetically modified cell can be a prokaryotic cell (e.g., a bacterial cell); or a eukaryotic cell (e.g., an insect cell; a mammalian cell, such as a mammalian cell line suitable for *in vitro* cell culture; a yeast cell; etc.), where the cell may produce the encoded antibody.

IV. Modification of anti-BoNT antibodies

Creation of anti-BoNT (scFv')2 homodimers

[00153] To create anti-BoNT $(scFv')_2$ 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 cysteines. 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/B. Introduction of the correct sequence can be verified by DNA sequencing. The construct may be 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 three repeats of a unit with 4 glycines plus one serine). An scFv 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.

[00154] The $(scFv')_2$ dimer may be created by joining the scFv fragments through a linker, e.g., 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 suitable approach is described by Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA,* 90: 6444-6448 (*see also* WO 94/13804).

[00155] Typically, linkers are introduced by PCR cloning. For example, synthetic oligonucleotides encoding the 5 amino acid linker (Gly_4Ser, SEQ ID NO:80) can be used to PCR amplify the anti-BoNT antibody V_H and V_L genes which are then spliced together to create the anti-BoNT 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.

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

[00156] Anti-BoNT antibodies such as anti-BoNT/C or anti-BoNT/D 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 as described above. An scFv gene can be excised using appropriate restriction enzymes and cloned into another vector as described herein.

[00157] Expressed scFv may include a myc tag at the C-terminus, followed by two glycines, a cysteine, and six histidines to facilitate purification. After disulfide bond formation between the two cystine residues, the two scFv may be 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.

[00158] 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 $(scFv')_2$.

[00159] Anti-BoNT Fab may also be 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/C, BoNT/D, or BoNT/F binding 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 V_H 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.

Selection of antibodies

[00160] Selection of anti-BoNT antibodies (whether produced by phage display, yeast display, immunization methods, hybridoma technology, *etc.*) can involve 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/C, BoNT/D, BoNT/DC, BoNT/CD, BoNT/F, a C-terminal domain of BoNT heavy chain (binding domain) of BoNT holotoxins, recombinant BoNT domains such as H_C (binding domain), H_N (translocation domain), or L_C (light chain), and the like. The antibodies may be selected for specific binding of an epitope recognized by one or more of the antibodies described herein.

[00161] Selection can be by any of a number of methods well known to those of skill in the art. In one example, selection is by immunochromatography (*e.g.,* using immunotubes, Maxisorp, Nunc) against the desired target, *e.g.,* BoNT/C, BoNT/D, BoNT/F, *etc.*. In a related example, 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. Yeast

display libraries are sequentially selected to obtain antibodies that bind with high affinity to all subtypes of BoNT/C. This can be repeated for other subtypes.

[00162] 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).

[00163] 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 may also encode a 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 anti-BoNT antibody is then expressed and purified from the bacterial supernatant using immobilized metal affinity chromatography.

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

[00164] As explained above, selection for increased avidity involves measuring the affinity of an anti-BoNT antibody (e.g., a modified anti-BoNT antibody) for one or more targets of interest (*e.g.,* BoNT subtype(s) or domains thereof. For example, the K_D of a BoNT/C-binding antibody and the kinetics of binding to BoNT/C 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.

[00165] 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 x 10^2 to 5.0 x 10^6 M and k_{off} in the range 1.0 x 10^{-1} to 1.0 x 10^{-6}M. 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} M. Affinities measured in this

manner usually correlate well with affinities measured in solution by fluorescence quench titration. Since increased affinity normally 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. Humanized, human engineered or human antibody production

[00166] The present BoNT binding antibodies and fragments can be humanized or human engineered antibodies. As used herein, a humanized antibody, or antigen binding fragment thereof, is a recombinant polypeptide that comprises a portion of an antigen binding site from a non-human antibody and a portion of the framework and/or constant regions of a human antibody. A human engineered antibody or antibody fragment may be derived from a human or non-human (e.g., mouse) source that has been engineered by modifying (e.g., deleting, inserting, or substituting) amino acids at specific positions so as to alter certain biophysical properties or to reduce any detectable immunogenicity of the modified antibody in a human.

[00167] Humanized antibodies also encompass chimeric antibodies and CDR-grafted antibodies in which various regions may be derived from different species. Chimeric antibodies may be antibodies that include a non-human antibody variable region linked to a human constant region. Thus, in chimeric antibodies, the variable region is mostly non-human, and the constant region is human. Chimeric antibodies and methods for making them are described in Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81: 6841-6855 (1984), Boulianne, et al., *Nature*, 312: 643-646 (1984), and PCT Application Publication WO 86/01533. Although, they can be less immunogenic than a mouse monoclonal antibody, administrations of chimeric antibodies have been associated with human anti-mouse antibody responses (HAMA) to the non-human portion of the antibodies. Chimeric antibodies can also be produced by splicing the genes from a mouse antibody molecule of appropriate antigen-binding specificity together with genes from a human antibody molecule of appropriate biological activity, such as the ability to activate human complement and mediate ADCC. Morrison et al. (1984), *Proc. Natl. Acad. Sci.*, 81: 6851; Neuberger et al. (1984), *Nature*, 312: 604. One example is the replacement of an Fc region with that of a different isotype.

[00168] CDR-grafted antibodies are antibodies that include the CDRs from a non-human "donor" antibody linked to the framework region from a human "recipient" antibody. Generally, CDR-grafted antibodies include more human antibody sequences than chimeric antibodies because they include both constant region sequences and variable region (framework) sequences from human antibodies. Thus, for example, a CDR-grafted humanized antibody may comprise a heavy chain that comprises a contiguous

amino acid sequence (e.g., about 5 or more, 10 or more, or even 15 or more contiguous amino acid residues) from the framework region of a human antibody (e.g., FR-1, FR-2, or FR-3 of a human antibody) or, optionally, most or all of the entire framework region of a human antibody. CDR-grafted antibodies and methods for making them are described in, Jones et al., *Nature*, 321: 522-525 (1986), Riechmann et al., *Nature*, 332: 323-327 (1988), and Verhoeyen et al., *Science*, 239: 1534-1536 (1988)). Methods that can be used to produce humanized antibodies also are described in U.S. Patents 4,816,567, 5,721,367, 5,837,243, and 6,180,377. CDR-grafted antibodies are considered less likely than chimeric antibodies to induce an immune reaction against non-human antibody portions. However, it has been reported that framework sequences from the donor antibodies are required for the binding affinity and/or specificity of the donor antibody, presumably because these framework sequences affect the folding of the antigen-binding portion of the donor antibody. Therefore, when donor, non-human CDR sequences are grafted onto unaltered human framework sequences, the resulting CDR-grafted antibody can exhibit, in some cases, loss of binding avidity relative to the original non-human donor antibody. See, e.g., Riechmann et al., *Nature*, 332: 323-327 (1988), and Verhoeyen et al., *Science*, 239: 1534-1536 (1988).

[00169] Human engineered antibodies include for example "veneered" antibodies and antibodies prepared using HUMAN ENGINEERING™ technology (U.S. Patent 5,869,619). HUMAN ENGINEERING™ technology is commercially available, and involves altering a non-human antibody or antibody fragment, such as a mouse or chimeric antibody or antibody fragment, by making specific changes to the amino acid sequence of the antibody so as to produce a modified antibody with reduced immunogenicity in a human that nonetheless retains the desirable binding properties of the original non-human antibodies. Techniques for making human engineered proteins are described in Studnicka et al., *Protein Engineering,* 7: 805-814 (1994), U.S. Patents 5,766,886, 5,770,196, 5,821,123, and 5,869,619, and PCT Application Publication WO 93/11794.

[00170] "Veneered" antibodies are non-human or humanized (e.g., chimeric or CDR-grafted antibodies) antibodies that have been engineered to replace certain solvent-exposed amino acid residues so as to further reduce their immunogenicity or enhance their function. As surface residues of a chimeric antibody are presumed to be less likely to affect proper antibody folding and more likely to elicit an immune reaction, veneering of a chimeric antibody can include, for instance, identifying solvent-exposed residues in the non-human framework region of a chimeric antibody and replacing at least one of them with the corresponding surface residues from a human framework region. Veneering can be accomplished by any suitable engineering technique, including the use of the above-described HUMAN ENGINEERING™ technology.

[00171] In a different approach, a recovery of binding avidity can be achieved by "de-humanizing" a CDR-grafted antibody. De-humanizing can include restoring residues from the donor

antibody's framework regions to the CDR grafted antibody, thereby restoring proper folding. Similar "de-humanization" can be achieved by (i) including portions of the "donor" framework region in the "recipient" antibody or (ii) grafting portions of the "donor" antibody framework region into the recipient antibody (along with the grafted donor CDRs).

[00172] For a further discussion of antibodies, humanized antibodies, human engineered, and methods for their preparation, see Kontermann and Dubel, eds., *Antibody Engineering*, Springer, New York, NY, 2001.

[00173] The present antibodies and fragments encompass antibodies having CDRs of human origin, such as antibodies which bind BoNT polypeptides and are encoded by nucleic acid sequences which are naturally occurring somatic variants of human germline immunoglobulin nucleic acid sequence, and fragments, synthetic variants, derivatives and fusions thereof. Such antibodies may be produced by any method known in the art, such as through the use of transgenic mammals (such as transgenic mice) in which the native immunoglobulin repertoire has been replaced with human V-genes in the mammal chromosome. Such mammals appear to carry out VDJ recombination and somatic hypermutation of the human germline antibody genes in a normal fashion, thus producing high affinity antibodies with completely human sequences.

[00174] Human antibodies can also be generated through the in vitro screening of antibody display libraries. See Hoogenboom et al. (1991), *J. Mol. Biol.* 227: 381; and Marks et al. (1991), *J. Mol. Biol.* 222: 581. Various antibody-containing phage display libraries have been described and may be readily prepared. Libraries may contain a diversity of human antibody sequences, such as human Fab, Fv, and scFv fragments that may be screened against an appropriate target. Phage display libraries may comprise peptides or proteins other than antibodies which may be screened to identify selective binding agents of BoNT.

[00175] The development of technologies for making repertoires of recombinant human antibody genes, and the display of the encoded antibody fragments on the surface of filamentous bacteriophage, has provided a means for making human antibodies directly. The antibodies produced by phage technology are produced as antigen binding fragments-usually Fv or Fab fragments-in bacteria and thus lack effector functions. Effector functions can be introduced by one of two strategies: The fragments can be engineered either into complete antibodies for expression in mammalian cells, or into bispecific antibody fragments with a second binding site capable of triggering an effector function.

[00176] Methods for display of peptides on the surface of yeast and microbial cells have also been used to identify antigen specific antibodies. See, for example, U.S. Patent No. 6,699,658. Antibody libraries may be attached to yeast proteins, such as agglutinin, effectively mimicking the cell surface display of antibodies by B cells in the immune system.

[00177] In addition to phage display methods, antibodies may be isolated using ribosome mRNA display methods and microbial cell display methods. Selection of polypeptide using ribosome display is described in Hanes et al., (*Proc. Nat.l Acad. Sc.i USA*, 94:4937-4942, 1997) and U.S. Pat. Nos. 5,643,768 and 5,658,754 issued to Kawasaki. Ribosome display is also useful for rapid large scale mutational analysis of antibodies. The selective mutagenesis approach also provides a method of producing antibodies with improved activities that can be selected using ribosomal display techniques.

VI. Other antibody forms

[00178] Sequence provided herein can be used to generate other antibody forms, including but not limited to nanobodies, UniBodies, and/or affibodies.

VHH and/or nanobodies

[00179] The Camelidae heavy chain antibodies are found as homodimers of a single heavy chain, dimerized via their constant regions. The variable domains of these camelidae heavy chain antibodies are referred to as VHH domains or VHH, and can be either used *per se* as nanobodies and/or as a starting point for obtaining nanobodies. Isolated VHH retain the ability to bind antigen with high specificity (see, e.g., Hamers-Casterman et al. (1993) *Nature* 363: 446-448). VHH domains, or nucleotide sequences encoding them, can be derived from antibodies raised in Camelidae species, for example in camel, dromedary, llama, alpaca and guanaco. Other species besides Camelidae (e.g, shark, pufferfish) can produce functional antigen-binding heavy chain antibodies, from which (nucleotide sequences encoding) such naturally occurring VHH can be obtained, e.g., using the methods described in U.S. Patent Publication US 2006/0211088.

[00180] Human proteins may be used in therapy primarily because they are not as likely to provoke an immune response when administered to a patient. Comparisons of camelid VHH with the V_H domains of human antibodies reveals several key differences in the framework regions of the camelid VHH domain corresponding to the V_H/V_L interface of the human V_H domains. Mutation of these human residues to VHH resembling residues has been performed to produce "camelized" human V_H domains that retain antigen binding activity, yet have improved expression and solubility.

[00181] Libraries of single V_H domains have also been derived for example from V_H genes amplified from genomic DNA or from mRNA came from the spleens of immunized mice and expressed in E. coli (Ward et al. (1989) *Nature* 341: 544-546) and similar approaches can be performed using the V_H domains and/or the V_L domains described herein. The isolated single V_H domains are called "dAbs" or domain antibodies. A "dAb" is an antibody single variable domain (V_H or V_L) polypeptide that specifically binds antigen. A "dAb" binds antigen independently of other V domains; however, as the term is used herein, a "dAb" can be present in a homo- or heteromultimer with other V_H or V_L domains

where the other domains are not required for antigen binding by the dAb, i.e., where the dAb binds antigen independently of the additional V_H or V_L domains.

[00182] As described in U.S. Patent Publication No. 2006/0211088 methods are known for the cloning and direct screening of immunoglobulin sequences (including but not limited to multivalent polypeptides comprising: two or more variable domains--or antigen binding domains--and in particular V_H domains or VHH domains; fragments of V_L, V_H or VHH domains, such as CDR regions, for example CDR3 regions; antigen-binding fragments of conventional 4-chain antibodies such as Fab fragments and scFv's, heavy chain antibodies and domain antibodies; and in particular of VH sequences, and more in particular of VHH sequences) that can be used as part of and/or to construct such nanobodies.

[00183] Methods and procedures for the production of VHH/nanobodies can also be found for example in WO 94/04678, WO 96/34103, WO 97/49805, WO 97/49805 WO 94/25591, WO 00/43507 WO 01/90190, WO 03/025020, WO 04/062551, WO 04/041863, WO 04/041865, WO 04/041862, WO 04/041867, PCT/BE2004/000159, Hamers-Casterman et al. (1993) *Nature* 363: 446; Riechmann and Muyldermans (1999) *J. Immunological Meth.,* 231: 25-38; Vu et al. (1997) *Molecular Immunology,* 34(16-17): 1121-1131; Nguyen et al. (2000) *EMBO J.,* 19(5): 921-930; Arbabi Ghahroudi et al. (19997) *FEBS Letters* 414: 521-526; van der Linden et al. (2000) *J. Immunological Meth.*, 240: 185-195; Muyldermans (2001) *Rev. Molecular Biotechnology* 74: 277-302; Nguyen el al. (2001) *Adv. Immunol.* 79:261, and the like, which are all incorporated herein by reference.

UniBodies

[00184] UniBodies are generated by an antibody technology that produces a stable, smaller antibody format with an anticipated longer therapeutic window than certain small antibody formats. UniBodies may be produced from IgG4 antibodies by eliminating the hinge region of the antibody. Unlike the full size IgG4 antibody, the half molecule fragment is very stable and is termed a UniBody. Halving the IgG4 molecule left only one area on the UniBody that can bind to a target. Methods of producing UniBodies are described in detail in PCT Publication W02007/059782, which is incorporated herein by reference in its entirety (see, also, Kolfschoten et al. (2007) *Science* 317: 1554-1557).

Affibodies

[00185] Affibody molecules are class of affinity proteins based on a 58-amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A. This three helix bundle domain has been used as a scaffold for the construction of combinatorial phagemid libraries, from which affibody variants that target the desired molecules can be selected using phage display technology (see, e.g., Nord et al. (1997) *Nat. Biotechnol.* 15: 772-777; Ronmark et al. (2002) *Eur. J. Biochem.*, 269: 2647-2655.). Details of affibodies and methods of production are known to those of skill (see, e.g., US Patent No. 5,831,012 which is incorporated herein by reference in its entirety).

VII. Assaying for cross-reactivity at an epitope

[00186] The antibodies of the present disclosure encompass those that specifically bind to one or more epitopes recognized by antibodies listed in Table 4. In other words, antibodies are cross-reactive with one or more of these epitopes but may have different sequences. 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).

[00187] This can be ascertained by providing one or more isolated target BoNT polypeptide(s) (*e.g.,* BoNT/C, BoNT/D, and/or BoNT/F, 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 an antibody described herein for binding to the target BoNT peptide. Thus, immunoassays in a competitive binding format can be used for cross-reactivity determinations. For example, a BoNT/C and/or BoNT/D polypeptide may be immobilized to a solid support. Antibodies to be tested (*e.g.,* generated by selection from a phage-display library) added to the assay compete with any antibody listed in Table 4 for binding to the immobilized BoNT polypeptide(s). The ability of test antibodies to compete with the binding of one or more antibodies listed in Table 4 to the immobilized protein(s) are compared. The percent cross-reactivity above proteins is then calculated, using standard calculations.

[00188] If the test antibody competes with one or more of the antibodies listed in Table 4 and has a binding affinity comparable to or greater than a threshold (such as having a K_D equal or less than about 1×10^{-8} M) with the same target then the test antibody is expected to be an anti-BoNT antibody. In some cases, a subject antibody competes for binding to a Botulinum neurotoxin epitope with an antibody comprising VH and/or VL CDRs (e.g., V_H CDR1, CDR2, and CDR3; and/or V_L CDR1, CDR2, and CDR3) of an antibody depicted in Table 4. As one non-limiting example, in some instances, a subject antibody competes for binding to a Botulinum neurotoxin epitope with an antibody comprising VH CDR1, VH CDR2, and VH CDR3 of the antibody designated 8DC4.4. In some instances, a subject antibody competes for binding to a Botulinum neurotoxin epitope with an antibody comprising VL CDR1, VL CDR2, and VL CDR3 of the antibody designated 8DC4.4. As another example, in some cases, a subject antibody competes for binding to a Botulinum neurotoxin epitope with an antibody comprising VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, VL CDR3 of the antibody designated 8DC4.4.

[00189] Cross-reactivity may be performed by using surface plasmon resonance in a BIAcore. In a BIAcore flow cell, the BoNT polypeptide(s) (*e.g.,* BoNT/C, BoNT/D, and/or BoNT/F) are coupled to a sensor chip (*e.g.,* CM5) as described in WO 09/008916, disclosure of which is incorporated herein by reference. 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 is then evaluated using pairs of antibodies at concentrations resulting in near

saturation and at least 100 relative units (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. Antibodies may be said to be cross-reactive if, when "injected" together they show an essentially additive increase (e.g., an increase by at least a factor of about 1.4, an increase by at least a factor of about 1.6, or an increase by at least a factor of about 1.8 or 2).

[00190] Epitope mapping may also be determined by incubating a yeast displayed scFv with a BoNT domain polypeptide followed by incubation with an epitope-tagged scFv. Bound scFv is detected with an antibody recognizing the epitope tag and the level of BoNT domain display quantitated by incubation with anti-SV5.

[00191] Cross-reactivity at the desired epitopes can be 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.*, 8DC4.4, 4C4.4, 6F5.4, Hu6F13.4, and/or Hu6F11, *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.

[00192] 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).

[00193] 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 phosphate-buffered saline (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 can be carried out 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 µl of horse radish peroxidase (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 indicate reactivity of the BoNT peptides in such wells with the test antibodies.

VIII. Assaying for specific binding and neutralizing activity of anti-BoNT antibodies

[00194] Exemplary antibodies of the present disclosure act, individually or in combination, to specifically bind to, and in some embodiments, 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 with 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.

[00195] 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.

[00196] Purified antibodies are incubated with purified BoNT (*e.g.,* BoNT/D, BoNT/C, BoNT/F1, *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).

IX. Diagnostic Assays

[00197] As explained above, the anti-BoNT antibodies of the present disclosure can be used for the *in vivo* or *in vitro* detection of BoNT 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.

[00198] 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. Examples of suitable biological samples include blood (or blood fraction such as serum or plasma), urine, saliva, and tissue biopsies.

[00199] Although the sample is typically taken from a human patient, the assays can be used to detect BoNT antigen in samples 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.

[00200] 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.

Immunological Binding Assays

[00201] The BoNT polypeptide (*e.g.*, BoNT/C, BoNT/D, BoNT/F, *etc.*) can be detected in an immunoassay utilizing only one or more than one of the anti-BoNT antibodies of the present disclosure as a capture agent that specifically binds to the BoNT polypeptide.

[00202] As used herein, an immunoassay is an assay that utilizes only one or more than one antibody (*e.g.*, one or more anti-BoNT antibodies disclosed herein) to specifically bind an analyte. The immunoassay is characterized by the binding of only one or more than one type of anti-BoNT antibody to a target (*e.g.*, one or more BoNT/C or BonT/D subtypes) as opposed to other physical or chemical properties to isolate, target, and quantify the BoNT analyte.

[00203] The BoNT marker can be detected and quantified using any of a number of well recognized immunological binding assays. For example, the antibody of the present disclosure may be immobilized on a substrate (e.g., bead) and/or be the capture antibody in an ELISA. The detection step may take one of many formats known in the art, such as using a labeled secondary antibody or PCR amplification. Where PCR amplification is the method of detection, the antibody is conjugated to a nucleic acid, the antigen may optionally be first attached to a substrate, and the antibody is allowed to be bound to the antigen. The bound antibody-nucleic acid fusion then undergoes PCR amplification of the nucleic acid sequence attached to the antibody. The amplified sequences can in turn be detected via a fluorophore bound to the incorporated nucleotides. The amplified sequences can also be first hybridized to an array before fluorescence is measured to enable multiplexing. Multiplexing encompasses processing and detecting two or more samples and/or two or more analytes in parallel. Details of an assay using

antibody-nucleic acid fusion may be found in US 20060141505, disclosure of which is incorporated by reference.

[00204] Single assay or multiplex assay can also take the form of an array where signal is detected only by electro-stimulation. In this format, the antibody of the present disclosure is conjugated to an electrochemiluminescent moiety and immobilized on an electrode. A signal (e.g., fluorescence) is emitted due to electrical stimulation at a particular electrode. Details of an assay using electrochemiluminescent moiety in an array may be found in US 20100140086, disclosure of which is incorporated by reference.

[00205] A fluorescent compound may be also added later to the assay for visualization by either Luminex type or other type of detection (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)).

[00206] The immunoassays of the present disclosure 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).

[00207] 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/C antibody/BoNT/C complex, an anti- BoNT/D antibody/BoNT/D complex, an anti- BoNT/DC antibody/BoNT/DC complex, or an anti-BoNT/F antibody/BoNT/F 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/C polypeptide or a labeled anti-BoNT/C 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 polypeptide or to the anti- BoNT antibody.

[00208] The labeling agent encompasses 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/C antibody, the label agent may be a mouse anti-human IgG, *i.e.,* an antibody specific to the constant region of the human antibody.

[00209] 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).

[00210] 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, for example, from about 5 minutes to about 24 hours (e.g., from 5 minutes to 15 minutes, from 15 minutes to 30 minutes, from 30 minutes to 60 minutes, from 1 hour to 4 hours, from 4 hours to 8 hours, from 8 hours to 12 hours, or from 12 hours to 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.

Non competitive assay formats

[00211] Immunoassays for detecting BoNT neurotoxins (*e.g.,* BoNT serotypes and/or subtypes) may be 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 example of a suitable "sandwich" assay, 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 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).

Competitive assay formats

[00212] In competitive assays, the amount of analyte (*e.g.,* BoNT) 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 antibody) by the analyte present in the sample. For example, in one competitive assay, a known amount of BoNT is added to a test sample with an unquantified amount of BoNT, and the sample is contacted with a capture agent, *e.g.,* an anti-BoNT antibody that specifically binds BoNT/C. The amount of added BoNT that binds to the anti-BoNT antibody is inversely proportional to the concentration of BoNT/C present in the test sample.

[00213] The anti-BoNT antibody can be immobilized on a solid substrate. The amount of BoNT bound to the anti-BoNT antibody is determined either by measuring the amount of BoNT present in a BoNT-anti-BoNT antibody complex, or alternatively by measuring the amount of remaining uncomplexed BoNT.

Reduction of Non-Specific Binding

[00214] 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/C polypeptide(s), BoNT/C-binding 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.

Substrates

[00215] 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.

[00216] 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.

[00217] 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, and enhance signal detection or the like.

[00218] 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.

[00219] 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, which is incorporated herein by reference.

Other Assay Formats

[00220] BoNT polypeptides or anti-BoNT antibodies (*e.g.,* BoNT neutralizing antibodies and antibodies that specifically bind to BoNT) 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.

[00221] 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.

[00222] 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).

Labeling of anti-BoNT antibodies

[00223] 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 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 in the various embodiments of the present disclosure. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present disclosure include magnetic beads (*e.g.*, Dynabeads^{TM}), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, Alexa fluor dyes 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, luciferase, 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. For example, an antibody can include a fluorescent label, a chemiluminescent label, a radiolabel, a chromogenic label, or other suitable label.

[00224] 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.

[00225] 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.

[00226] 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.

[00227] 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.

[00228] 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.

X. Compositions

[00229] The BoNT-binding antibodies of this disclosure are useful in preventing or mitigating the progression of botulism produced, *e.g.*, by endogenous disease processes or by chemical/biological warfare agents. Typically, compositions containing one, two, or more different antibodies can be provided as a pharmaceutical composition and administered to a mammal (*e.g.*, to a human) in need thereof.

[00230] As disclosed herein, particularly efficient neutralization of a botulism neurotoxin (BoNT) can be achieved by the use of antibodies that bind two or more BoNT subtypes/serotypes/mosaics with high affinity. This can be accomplished by using one, two or more different antibodies. Where there is more than one type of antibody, each can be directed against a different subtype. One or more of the antibodies can also be cross-reactive. Cross-reactive antibodies can bind two or more BoNT serotypes/subtypes (*e.g.*, BoNT/CD, BoNT/D, BoNT/DC, BoNT/C, BoNT/F *etc.*) with high affinity.

[00231] Different neutralizing antibodies when combined, exhibit a potency that is increased dramatically. This increase makes it possible to generate a botulinum antibody composition of the required potency for therapeutic use. Compositions comprising at least two, at least three, or more high affinity antibodies that bind overlapping or non-overlapping epitopes on the BoNT are contemplated herein.

[00232] In some cases, the compositions of the present disclosure comprise at least one or more, two or more, three or more, four or more, or all of the antibodies disclosed herein. In some cases, the compsition additionally comprises a pharmaceutical carrier.

[00233] The subject composition encompasses compositions that specifically bind to one or more serotypes/subtypes/mosaics. The composition can contain one or more antibodies that are cross-reactive. The composition may also contain any first combination of antibodies described above that specifically bind to one serotype together with a second combination of antibodies that specifically binds to, and in some embodiments, specifically neutralizes a different serotype. The subject composition may contain multiple combinations such that that composition may bind and/or neturalize two, three, or more serotypes/subtypes (e.g., BoNT/C, BoNT/CD, BoNT/D, BoNT/DC, BoNT/F, etc.).

[00234] A composition that specifically binds to, and in some embodiments, neutralizes multiple serotypes may include any of the combinations described above or one or more of the antibodies disclosed in Table 4.

[00235] Where combinations of antibodies are disclosed herein, such combinations can be provided in a single formulation or can be provided as separate formulations in a kit, where the separate formulations may contain a single antibody or more antibodies. Such separate formulations of a kit may be combined prior to administration or administered by separate injection.

[00236] The anti-BoNT antibodies provided by the present disclosure 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 present in the pharmaceutical compositions of the present disclosure, when administered orally, can be 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.

[00237] The present disclosure provides a pharmaceutical composition comprising an antibody comprising a V_H of a subject antibody. The present disclosure provides a pharmaceutical composition comprising an antibody comprising a V_L of a subject antibody. The present disclosure provides a pharmaceutical composition comprising an amino acid sequence of a V_H and a V_L of a subject antibody. In some instances, a subject pharmaceutical composition comprises an amino acid sequence comprising a V_H CDR1, CDR2, and CDR3 of a subject antibody and/or a V_L CDR1, CDR2, and CDR3 of a subject antibody.

[00238] The pharmaceutical compositions of the present disclosure 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 can comprise a solution of one or more anti-BoNT antibody dissolved in a pharmaceutically acceptable carrier, which may be an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like.

[00239] The pharmaceutical composition of the present disclosure, in some cases, comprises: a pharmaceutically acceptable carrier; and at least a first antibody that specifically binds to Botulinum neurotoxin serotype BoNT/C and Botulinum neurotoxin serotype BoNT/D, and their mosaics, wherein the antibody specifically binds an epitope of a Botulinum neurotoxin that is specifically bound by an antibody comprising a V_H comprising a CDR1, CDR2 and CDR3 and a V_L comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody.

[00240] The pharmaceutical composition of the present disclosure, in some cases, comprises: a pharmaceutically acceptable carrier; and at least a first antibody that cross-reacts with and specifically binds to Botulinum neurotoxin serotype BoNT/C and Botulinum neurotoxin serotype BoNT/D, and their mosaics, wherein the antibody specifically binds an epitope of a Botulinum neurotoxin that is specifically bound by an antibody comprising a H-FR1, a V_H comprising a CDR1, CDR2 and CDR3, and a V_L comprising a CDR1, CDR2 and CDR3 of the 4C4.4 antibody.

[00241] The pharmaceutical composition of the present disclosure, in some cases, comprises: a pharmaceutically acceptable carrier; and at least a first antibody that specifically binds to Botulinum neurotoxin serotype BoNT/F, wherein the antibody specifically binds an epitope of a Botulinum neurotoxin that is specifically bound by an antibody comprising a V_H comprising a CDR1, CDR2 and CDR3 and a V_L comprising a CDR1, CDR2 and CDR3 of the 6F5.4 antibody, the Hu6F13.4 antibody, and/or the Hu6F11 antibody.

[00242] In some cases, the pharmaceutical composition comprises a first anti-BoNT antibody that binds a Botulinum neurotoxin C, D, or mosaics BoNT/CD and BoNT/DC. In some cases, the pharmaceutical composition comprises a first anti-BoNT antibody that binds a Botulinum neurotoxin F. In some cases, the BoNT antibody binds more than one subtype of Botulinum neurotoxin.

[00243] In some cases, the pharmaceutical composition comprises at least one or more, two or more, three or more, four or more, or all of the antibodies disclosed herein. In some cases, the pharmaceutical composition comprises an antibody comprising a V_H comprising a CDR1, CDR2 and CDR3, and a V_L comprising a CDR1, CDR2 and CDR3 of the 8DC4.4 antibody and an antibody comprising a H-FR1, a V_H comprising a CDR1, CDR2 and CDR3, and a V_L comprising a CDR1, CDR2 and CDR3 of the 4C4.4 antibody.

[00244] In some cases, the pharmaceutical composition comprises two or more antibodies comprising a V_H comprising a CDR1, CDR2 and CDR3 and a V_L comprising a CDR1, CDR2 and CDR3 of the 6F5.4 antibody, the Hu6F13.4 antibody, and/or the Hu6F11 antibody. In some cases, the pharmaceutical composition additionally comprises a pharmaceutical carrier.

[00245] The pharmaceutical composition of the present disclosure, in some cases, comprises: a pharmaceutically acceptable carrier, a first antibody that binds to BoNT/C and/or BoNT/D, and a second antibody that binds to BoNT/F.

[00246] Non- aqueous pharmaceutically acceptable carriers (excipients) are known to those of skill in the art. Such excipients can comprise any substance that is biocompatible and liquid or soft enough at the subject's body temperature to release the active agent(s) (e.g., Anti-BoNT antibodies) into the subject's bloodstream at a desired rate. Non-aqueous carriers are usually hydrophobic and commonly organic, e. g., an oil or fat of vegetable, animal, mineral or synthetic origin or derivation. The carrier may include at least one chemical moiety of the kind that typifies “fatty" compounds, e. g., fatty acids, alcohols, esters, etc., i. e., a hydrocarbon chain, an ester linkage, or both. "Fatty” acids in this context include, but are not limited to, acetic, propionic and butyric acids through straight-or branched-chain organic acids containing up to 30 or more carbon atoms. The non-aqueous carrier may be immiscible in water and/or soluble in the substances commonly known as fat solvents. The non-aqueous carrier can correspond to a reaction product of a "fatty" compound or compounds with a hydroxy compound, e, g., a mono-hydric, di-hydric, trihydric or other polyhydric alcohol, e.g., glycerol, propanediol, lauryl alcohol, polyethylene or-propylene glycol, etc. These compounds include, but are not limited to, the fat-soluble vitamins, e.g., tocopherols and their esters, e. g., acetates sometimes produced to stabilize tocopherols. Sometimes, for economic reasons, the carrier can comprise a natural, unmodified vegetable oil such as sesame oil, soybean oil, peanut oil, palm oil, or an unmodified fat. Alternatively, the vegetable oil or fat may be modified by hydrogenation or other chemical means which is compatible with the present disclosure. The appropriate use of hydrophobic substances prepared by synthetic means is also envisioned. Non-aqueous excipient compositions can also comprise, in addition to a biocompatible oil, an "antihydration agent" which term as used herein means a substance that retards hydration of the active agent(s) and/or the biocompatible oil or fat and thereby further decreases and/or stabilizes the rate of

release of the active agent(s) from that composition following administration to an animal (e.g., human). A great variety of non-toxic antihydration agents are known. By way of example there are "gelling" agents that, when dispersed, and in some cases heated to dissolve them in the oil, give the body of oil greater visco-elasticity (and therefore greater structural stability) and thereby slow down penetration of the oil by body fluids.

[00247] Illustrative antihydration agents include various polyvalent metal salts or complexes of organic acids, for instance fatty acids having from about 8 or 10 to about 20 or 22 carbon atoms, e. g. aluminum, zinc, magnesium or calcium salts of lauric acid, palmitic acid, stearic acid and the like. Such salts can be mono-, di- or tri- substituted, depending on the valence of the metal and the degree of oxidation of the metal by the acid. Of common usage are the aluminum salts of such fatty acids. Aluminum monostearate and distearate are frequently used anti-hydration agents. Others that are useful include aluminum tristearate, calcium mono-and distearate, magnesium mono-and distearate and the corresponding palmitates, laurates and the like. The concentration of such an antihydration agent, based on the weight of the oil plus that agent, may be between about 1% and about 10% (most typically between about 2% and about 5%), although other concentrations may be suitable in some cases.

[00248] The various 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 anti-BoNT 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. In some instances, the solutions may be stored in lyophilized or frozen form. Examples of suitable anti-BoNT antibody formulations are described in WO 2011/028961.

[00249] Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 mg 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).

[00250] The compositions containing the anti-BoNT antibodies of the present disclosure or a cocktail thereof can be administered for therapeutic and/or prophylactic treatments. Pharmaceutical compositions can be 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.

[00251] 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 the present disclosure to effectively treat the patient.

[00252] The present disclosure thus provides a method of specifically binding to, and in some cases, neutralizing a Botulinum neurotoxin in an individual (e.g., a human; or a non-human mammal), the method generally involving administering to the individual an effective amount of a subject anti-BoNT antibody, or an effective amount of a subject composition comprising a subject anti-BoNT antibody. 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. Administering the antibody, or the composition comprising the antibody, provides for specific binding to and, in some embodiment, neutralization of Botulinum neurotoxin present in the individual. The BoNT poisoning can be due to ingestion of contaminated food products (food botulism), can result from an anaerobic wound infection (wound botulism), or can result from an act of biological warfare or bioterrorism.

[00253] The present disclosure also provides methods of reducing the likelihood that an individual at risk of exposure to Botulinum neurotoxin will experience symptoms of Botulinum neurotoxin poisoning following exposure to the Botulinum neurotoxin (e.g., where the exposure is via inhalation, via ingestion, via a wound infection, or via another route/mode of exposure). Administration of a subject antibody or subject composition reduces the likelihood that the individual will experience symptoms of Botulinum neurotoxin poisoning. Thus, e.g., a subject anti-BoNT antibody, or a subject composition comprising a subject anti-BoNT antibody, can be administered to an individual before the individual has Botulinum neurotoxin poisoning, e.g., before a BoNT is present in the individual. For example, a subject anti-BoNT antibody, or a subject composition comprising a subject anti-BoNT antibody, can be administered to an individual who is at risk of BoNT exposure, e.g., an individual who is at greater risk than the general population of experiencing Botulinum neurotoxin exposure and poisoning. Such individuals include, e.g., military personnel, e.g., military personnel in a combat setting; personnel involved in investigation or clean up of a site suspected of involving Botulinum neurotoxin exposure (e.g., hazardous materials ("hazmat") personnel) and other individuals who are at risk of Botulinum neurotoxin exposure, either accidental or intentional.

XI. Kits for Diagnosis or Treatment

[00254] Kits for the treatment of botulism or for the detection/confirmation of a *Clostridium botulinum* infection are also provided. Kits will typically comprise one or more anti-BoNT antibodies

(*e.g.*, anti-BoNT antibodies in a composition for pharmaceutical use). For diagnostic purposes, the antibody(s) can optionally be labeled. In addition, the kits will typically include instructional materials disclosing means of use anti-BoNT 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 antibody, 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.

[00255] Kits provided for the treatment of botulism may contain one or more anti-BoNT antibodies. The antibodies can be provided separately or mixed together. Typically, the antibodies will be provided in a sterile pharmacologically acceptable excipient. The antibodies can also be provided pre-loaded into a delivery device (*e.g.*, a disposable syringe).

[00256] The kits can optionally include instructional materials teaching the use of the antibodies, recommended dosages, contraindications, and the like.

Examples of Non-Limiting Aspects of the Disclosure

[00257] Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure numbered 1-35 are provided below. As will be apparent to those of skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below:

[00258] 1. An isolated antibody that specifically binds to a Botulinum neurotoxin (BoNT), wherein the antibody competes for binding to BoNT with an antibody comprising:

a) a variable heavy (VH) chain comprising heavy complementarity determining regions 1-3 (HCDRs 1-3) and a variable light (VL) chain comprising light CDRs 1-3 (LCDRs 1-3) of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

b) a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody; or

c) a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody.

[00259] 2. The isolated antibody of aspect 1, wherein the isolated antibody competes for binding to BoNT with an antibody comprising:

a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

[00260] 3. The isolated antibody of aspect 1, wherein the isolated antibody competes for binding to BoNT with an antibody comprising:

a VH chain comprising the amino acid sequence of the VH chain and a VL chain comprising the amino acid sequence of the VL chain of the 4C4.4 antibody, the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

[00261] 4. The isolated antibody of aspect 1, wherein the isolated antibody competes for binding to BoNT with an antibody comprising:

a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody.

[00262] 5. An isolated antibody that binds to a Botulinum neurotoxin, wherein the antibody comprises:

a) a variable heavy (V_H) chain comprising heavy complementarity determining regions 1-3 (HCDRs 1-3) and a variable light (V_L) chain comprising light CDRs 1-3 (LCDRs 1-3) of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

b) a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4, or the Hu6F11 antibody;

c) a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody;

d) a V_H chain comprising the amino acid sequence of the V_H chain of the 4C4.4 antibody, 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

e) a V_L chain comprising the amino acid sequence of the V_L chain of 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

f) a V_H chain comprising HCDRs 1-3 of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody; or

g) a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

[00263] 6. The isolated antibody of aspect 5, wherein the antibody comprises:

a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

[00264] 7. The isolated antibody of aspect 5, wherein the antibody comprises:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4, or the Hu6F11 antibody.

[00265] 8. The isolated antibody of aspect 5, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody.

[00266] 9. The isolated antibody of any one of aspects 1-8, wherein the antibody is a human antibody or a humanized antibody.

[00267] 10. The isolated antibody of any one of aspects 1-9, wherein the antibody is a single chain Fv (scFv), IgG, Fab, $(Fab')_2$, or $(scFv')_2$.

[00268] 11. The isolated antibody of any one of aspects 1-10, wherein the antibody has a K_D with a BoNT of about 5 nM or less.

[00269] 12.The isolated antibody of any one of aspects 5-11, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody and binds to BoNT/C and/or BoNT/D with high affinity.

[00270] 13.The isolated antibody of any one of aspects 5-11, wherein the antibody comprises:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 or 8DC4.4 antibody and binds to BoNT/C and/or BoNT/D with high affinity.

[00271] 14.The isolated antibody of any one of aspects 5-11, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody and binds to BoNT/C and/or BoNT/D with high affinity.

[00272] 15.The isolated antibody of any one of aspects 5-11, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody and binds to BoNT/F with high affinity.

[00273] 16.The isolated antibody of any one of aspects 5-11, wherein the antibody comprises:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody and binds to BoNT/F with high affinity.

[00274] 17.A composition comprising:

a pharmaceutically acceptable carrier; and

at least a first antibody according to any one of aspects 1-16.

[00275] 18. The composition of aspect 17, comprising a first antibody and a second according to any one of aspects 1-16.

[00276] 19. The composition of aspect 18, wherein the first antibody binds to BoNT/C and/or BoNT/D and the second antibody binds to BoNT/F.

[00277] 20. An isolated nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of any one of the antibodies of any one of aspects 1-16.

[00278] 21. A vector comprising the isolated nucleic acid of aspect 20.

[00279] 22. A first nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of the V_H chain and a second nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of the V_L chain any one of the antibodies of any one of aspects 1-16.

[00280] 23. A first vector comprising the first nucleic acid and a second vector comprising the second nucleic acid of aspect 22.

[00281] 24. A host cell comprising the nucleic acid of aspect 20, the vector of aspect 21, the first and second nucleic acids of aspect 22 or the first and second vectors of aspect 23.

[00282] 25. The isolated antibody according to any one of aspects 1-16, wherein the antibody is labeled.

[00283] 26. A method of specifically binding to a Botulinum neurotoxin in a mammal comprising:

administering to the mammal an effective amount of an antibody of any one of aspects 1-16;

wherein the administering provides for specific binding of Botulinum neurotoxin present in the mammal.

[00284] 27. A method of specifically binding to a Botulinum neurotoxin in a mammal, comprising:

administering to the mammal an effective amount of a composition of any one of aspects 17-19;

wherein the administering provides for specific binding of Botulinum neurotoxin present in the mammal.

[00285] 28. A method for detecting Botulinum neurotoxin in a sample, comprising:

contacting an antibody of any one of aspects 1-16 with the sample,

detecting binding of said antibody to Botulinum neurotoxin in the sample.

[00286] 29. The method of aspect 28, wherein the antibody is labeled.

[00287] 30. The method of aspect 29, wherein said label is a fluorescent label, a chemiluminescent label, a radiolabel, an enzyme, or a chromogenic label.

[00288] 31. The method of any one of aspects 28-30, wherein said sample is blood.

[00289] 32. The method of any one of aspects 28-31, wherein the antibody is immobilized on a substrate.

[00290] 33. The method of any one of aspects 28-32, wherein the antibody is conjugated to a nucleic acid and wherein said detecting comprises a polymerase chain reaction.

[00291] 34. A method of reducing the likelihood that an individual at risk of exposure to Botulinum neurotoxin will experience symptoms of Botulinum neurotoxin poisoning following exposure, the method comprising administering to said individual an effective amount of an antibody of any one of aspects 1-16;

wherein said administering provides for reducing the likelihood that the individual will experience symptoms of Botulinum neurotoxin poisoning.

EXAMPLES

[00292] The following examples are offered to illustrate, but not to limit any embodiments provided by the present disclosure.

[00293] **Example 1**

[00294] *Strains, Media, Antibodies, and Toxin*

YPD medium was used for growth of *Saccharomyces cerevisiae* strain EBY100, SD-CAA, for the selection of pYD2-transformed EBY100 and SG-CAA, and for the induction of scFv expression on the surface of EBY100. *Escherichia coli* strain DH5α was used for the subcloning and preparation of plasmid DNA.

[00295] All IgGs were expressed from Chinese hamster ovary (CHO) cells, while the mouse anti-SV5 antibody was purified from hybridoma cells and labeled with an AlexaFluor-488 (anti-SV5-AF-488) or AlexaFluor-647 (anti-SV5-AF-647) labeling kit (Invitrogen, Carlsbad, CA, USA). The secondary antibody, PE-conjugated goat anti human-Fc, F(ab) was purchased (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

[00296] BoNT/C and BoNT/DC, as pure holotoxin or toxin complexes, were obtained from Metabiologics (sold as BoNT/D but confirmed to be BoNT/DC (Peng, et al. (2012). J.Cell Sci., 125(Pt 13), 3233–3242)); BoNT/CD was obtained as a crude extract complex from USAMRIID, and BoNT/D was obtained as a pure complex from USAMRIID. The determination of the IU potency requires the use of a WHO-standardized antitoxin. We were not able to access such an antitoxin, and thus were unable to determine the IU using the standard assay. To calculate the IU potency from the mouse neutralization data (MNA), an ED_{50} was interpolated from the MNA data as the mAb amount at which 50% of mice survived challenge with that dose of BoNT. This ED_{50} was normalized for a 1 mg amount of mAb and divided by

10,000 mouse LD50s/IU to determine the IU potency. We have previously shown for BoNT/A that such a calculation correlates closely to the IU measured using the reference WHO antitoxin.

[00297] *Yeast-Displayed Human scFv Library Construction and Library Sorting*

To generate human mAbs that bind BoNT/C and /D, total RNA was isolated from the blood of healthy human donors immunized with investigational pentavalent BoNT toxoid (formalin inactivated BoNT/A, BoNT/B, BoNT/C, BoNT/D, and BoNT/E). cDNA synthesis, VH, and Vk gene repertoire preparation and library construction were performed as described previously (Fan, et al. (2015). Toxins, 7(9), 3405–3423).

[00298] Yeast cells were grown in SD-CAA medium and the display of scFv was induced by culturing in SG-CAA with 10% SD-CAA for 48 h. For sorting, the libraries were incubated with 50 nM of BoNT/C or BoNT/DC labeled with Alexa-647 plus SV5-AF-488 at room temperature for 2 h. All subsequent washing and staining steps were performed at 4 °C using ice-cold FACS buffer (phosphate-buffered saline, 0.5% bovine serum albumin, pH 7.4). After washing, yeast clones were flow-sorted on a FACSAria II, and the population with BoNT/C binding was gated and collected. The collected yeast clones were cultured and induced for the next round of sorting. After three rounds of sorting, the collected yeast clones were plated on SD-CAA medium and cultured at 30 °C for 48 h. Binding of individual colonies was confirmed for binding using BoNT/C at progressively reduced concentrations. Unique BoNT/C binding clones were identified by DNA sequencing and best binders were converted into IgGs. Cross-reactive antibodies were isolated using BoNT/DC sorting.

[00299] For the isolation of additional BoNT/C and BoNT/DC specific scFvs, the V-genes of scFvs isolated as described above (mAbs 1C1, 4C2, 4C4, 4C5, 4C10 and 87C78) were converted to IgGs, purified, and IgG labeled with Alexa Fluor (AF)-647. The sorting protocol was then modified by incubation with unlabeled BoNT/C or unlabeled BoNT/DC (50 nM) followed by washing and then incubation with an AF-647-labeled toxin-specific IgG (2 μg/mL) plus anti-SV5-488. The use of labeled IgGs versus labeled BoNT provided a more robust staining and sorting reagent; the use of the cross-reactive IgGs allowed us to sort and analyze binding to all four toxins included in this work. Newly isolated scFvs (8DC1, 8DC2 and 8DC3) were also converted into IgGs.

[00300] *Measurement of K_D Values of Yeast-Displayed scFv*

The K_D values of yeast-displayed scFvs were measured by flow cytometry, as previously described, with modification (Fan, et al. (2015) PloS one, 10(8), e0135306). Briefly, 1×10^6 yeast-displaying scFvs were incubated for 1 h at room temperature in FACS buffer with six different concentrations of BoNT/C BoNT/CD, BoNT/D or BoNT/DC purified holotoxins, or crude extracts, that spanned the range 10-fold above and 10-fold below the expected K_D. Ice-cold FACS buffer was used to wash the samples, and 2 μg/mL each of an AF-647-labeled IgG that bound a different epitope was added

plus anti-SV5-AF-488 at 4 °C and incubated for 60 min. Finally, the yeast clones were washed with ice-cold FACS buffer and the mean fluorescence intensity (MFI) of binding was measured by flow cytometry, as described previously (Fan, et al. (2015) Toxins supra; Dong, et al. (2010) J. Mol. Bio., 397(4), 1106–1118).

[00301] *Epitope Overlap Analysis*

Epitopes were classified based on mAb binding competition to BoNT/C or BoNT/DC, as previously described (Fan, et al. (2015) supra; Fan, et al. (2015) PloS one supra). Briefly, yeast-displayed scFvs were incubated for 60 min with 25 nM of one of the toxins. The ability of other IgGs to bind to the same toxin captured by the yeast-displayed scFvs was detected by incubation for 60 min with 2 μg/mL of Alexa dye APC conjugated IgG and 1 μg/mL of AF-488-labeled SV5 antibody (SV5-AF-488). The ability of the IgG to bind the scFv-captured BoNT/C or BoNT/DC, was determined by flow cytometry. The IgG that bound an overlapping epitope to yeast-displayed scFvs showed no APC signal, while those binding non-overlapping epitopes showed a positive APC signal.

[00302] *Affinity Maturation*

To increase the affinity of mAbs 1C1, 4C4, 4C10, 8DC1 and 8DC4, individual VL chain-shuffled scFv libraries were created. For each scFv library, VK gene repertoires from human immune scFv libraries were amplified by PCR using *Pfu* polymerase (Stratagene Bellingham, WA, USA) and the primers (5′-GGCGGAGGTGGCTCTGGCGGTGGCGGGTCG-3′) and PYDR1(GGTGATGGTGATGATGACCGGTACGCGTAG). To further increase VL diversity, the VL repertoire from a large non-immune scFv phage antibody library transferred from the phagemid vector pHEN1 and cloned into pYD2 was also used (Sheets, et al. (1998) Proc. Nat. Acad. Sci. USA, 95(11), 6157–6162). The VH DNA for each of the scFvs to mature was amplified from the scFv gene in pYD2 using primer PYD-F1 (5′-CCCCTCAACAACTAGCAAAGGCAGCCC-3′), that anneals upstream of the VH gene, and primer (5′-CGACCCGCCACCGCCAGAGCCACCTCCGCC-3′) that anneals in the linker gene between the VH and VL genes. The gel-purified VH gene was mixed with the gel-purified VL repertoires and combined with NcoI- and NotI-digested pYD2 vector DNA. This mixture was used to transform LiAc-treated EBY100 cells by three-fragment gap repair. Library sizes were approximately 10^7. To select higher affinity scFvs, the VL-shuffled library was sorted as described above.

[00303] To create complementarity determining region (CDR) libraries, spiked primers were used to introduce mutations into four or five residues, located in the H1 or H3 loops of each scFv to mature, as described previously (Razai, et al. (2005) J. Mol. Bio., 351(1), 158–169). Spiked oligonucleotides were designed to have a bias for a 25% or 50% wild-type amino acid at each position, according to the codon usage. Library sizes were approximately 10^8.

[00304] To select higher affinity scFvs and maintain cross-reactivity, the VL-shuffled library was sorted as described above for each scFv. To maintain cross-reactivity, sorting was alternated between BoNT/C and BoNT/DC. The K_D values of libraries for both toxins were measured after each round to decide which toxin to use for the following sort. Alternatively, we used a dual staining protocol using recombinant BoNT/C LC-HN plus BoNT/DC and detected each with a different label antibody, e.g., incubating after toxin staining with 1C1 Alexa Fluor-488 (binds to the BoNT/C LC-HN domain only) and 4C2 AF-647 (Binds the HC domain so only BoNT/DC would be detected) plus SV5 antibody (Mouse IgG2a) and goat anti-mouse specific-PE labeled antibody (Jackson Immunoresearch #115-116-146) was used to detect expression.

[00305] *Germline Fitting*

The closest predecessor germlines for each scFv were identified using IMGT/V-Quest (Giudicelli, et al. (2011) Cold Spring Harb. Protoc., 2011(6), 695–715). Sequences were compared to find the divergent amino acids. Then, mutations were restored to its germline amino acid by the QuikChange II-E Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA), as described above for epitope mapping. The K_D values for each construct were determined as yeast-displayed scFvs using flow cytometry. Changes that did not reduce binding affinity were incorporated. After scFv gene optimization, the final clone was used as a template to continue with the next step of affinity maturation

[00306] *Measurement of Solution Phase Affinity at Equilibrium*

For selected IgGs, the solution phase affinity at equilibrium and binding kinetics were measured using flow fluorimetry in a KinExA as previously described (Fan, et al. (2015) PloS one supra; Dong, et al. (2010) supra], except that pure BoNT/C or /DC toxins or crude culture supernatants of BoNT/CD or BoNT/D were used.

[00307] *Measurement of In Vivo Toxin Neutralization*

The mouse neutralization assay was performed as described previously (Nowakowski, et al. (2002) Proc. Nat. Acad. Sci. USA, 99(17), 11346–11350) using female CD-1 mice. Briefly, an equimolar mixture of one to four IgG antibodies (0.5 to 50 µg total antibody) were premixed with a range of mouse LD_{50} of BoNT/C, BoNT/CD, BoNT/DC, or BoNT/D in a total volume of 0.5 mL of gelatin phosphate buffer and incubated at room temperature for 30 min. The mixture was then injected intraperitoneally into cohorts of ten mice. Due to toxin scarcity, studies using BoNT/D involved cohorts of five mice. The animals were observed multiple times daily for clinical signs of botulism. Moribund animals were euthanized. Surviving mice at the study endpoint (5 days) were tabulated.

[00308] **Example 2**

[00309] *BoNT/C and BoNT/D Sequence Analysis and Modeling*

To better understand differences and similarities in the structural epitopes of BoNT/C and BoNT/D, we aligned their amino acid sequences (Webb, et al. (2007) Vaccine, 25(21), 4273–4282) and analyzed their identity by domain. While overall BoNT/C and BoNT/D differed from each other by 49%, the H_N of the two BoNTs and their mosaics differed at most by 32%. This observation suggested that it might be possible to generate cross-reactive mAbs binding the H_N domain of BoNT/C and BoNT/D and their mosaic toxins. This level of difference is similar to the 31.6% difference between BoNT/F1 and BoNT/F7, where cross-reactive and cross-protective antibodies were successfully generated (Fan, et al. (2017) PloS one, 12(3), e0174187).

[00310] To visualize epitope differences between BoNT/C and BoNT/D, a structural model of BoNT/C was generated. Side chain differences between BoNT/C and BoNT/CD, BoNT/DC and BoNT/D were then modeled, and the chemical differences of each amino acid side chain were compared. This approach was previously applied to BoNT/A, BoNT/B, BoNT/E, and BoNT/F.

[00311] The results of the modeling identified regions in the H_N and LC that are identical or homologous in both BoNT/C and BoNT/D and in the mosaic toxins that could provide targets for the generation of cross-reactive antibodies. This result confirmed our hypothesis that amino acid identities among these toxins should provide ample antigenic targets for the development of cross-reactive mAbs capable of binding and neutralizing these BoNT serotypes and variants.

[00312] **Example 3**

[00313] *Generation and Characterization of Human Monoclonal Antibodies*

To generate recombinant mAb-based antitoxins to treat type C and D botulism, previously generated yeast-displayed scFv libraries constructed from humans immunized with pentavalent BoNT toxoid (serotypes A, B, C, D, and E) (Garcia-Rodriguez, et al. (2018) Toxins, 10(3), 105) were sorted for binding to BoNT/C and to BoNT/DC. BoNT/DC was used for sorting, as BoNT/D was not commercially available. BoNT/DC is being sold as BoNT/D, but in fact is from a strain that produces BoNT/DC (Peng, et al. (2012) *supra*).

[00314] A panel of 15 unique scFvs binding BoNT/C were isolated by sorting libraries on pure BoNT/C toxin. The mAbs ranged in affinity from 0.19–64 nM, with a mean of 15.6 nM. A panel of 13 scFvs binding BoNT/DC were isolated by sorting libraries on pure BoNT/DC toxin. The mAbs ranged in affinity from 0.5–182 nM, with a mean of 33.6 nM. Yeast-displayed scFvs were then screened for binding to crude culture supernatants prepared from Clostridial strains producing BoNT/CD and BoNT/D. Since the concentration of BoNT in the supernatants is not known, it was not possible to measure a K_D value and only the presence or absence of binding is shown. These studies identified four scFv antibodies, 4C4, 8DC1, 4C10 and 8DC4, that bound to BoNT/C, BoNT/CD, BoNT/DC, and BoNT/D. Two of these cross-reactive scFvs came from sorting on BoNT/C (4C4, 4C10) and two from sorting on BoNT/DC (8DC1,

8DC4). Other mAbs were generated (e.g., 4C2, 87C1, 8DC8) that bound pairs of BoNTs, such as BoNT/C and BoNT/CD, or BoNT/DC and BoNT/D. IgGs were constructed from nine of the scFvs, including the four scFvs binding BoNT/C, BoNT/CD, BoNT/DC, and BoNT/D. As observed with scFvs binding other BoNT serotypes, IgG affinities were generally higher than the scFvs from which they were constructed.

[00315] Example 4

[00316] *Epitope Mapping*

The BoNT domain bound by each mAb was determined by staining yeast-displayed BoNT/C or BoNT/DC light chains (LCs), the translocation domain (H_N), the light chain–translocation domain (LC-H_N), and the binding domain (H_C) with each antibody, as previously described, using either IgG or purified scFv (Levy, et al. (2007) J. Mol. Bio., 365(1), 196–210). Antibodies used include the IgGs 4C2, 4C4.2, 4C10.1 and 8DC4.1. The latter three IgGs are higher-affinity variants of 4C4, 4C10 and 8DC4 which yielded better binding signals. IgGs 4C2, 4C4.2, 4C10.1 and 8DC4.1 bind the BoNT/C H_C, H_N, LC and H_N, respectively. Epitope overlap was determined by incubating BoNT/C or BoNT/DC with yeast-displayed scFvs and probing the captured BoNT with each of the other scFvs or IgGs, as previously described (Fan, et al. (2017) supra). Using these two assays, 13 of the 28 mAbs described above could be mapped to a specific BoNT/C or BoNT/DC domain and a determination could be made as to whether the epitope overlapped with other mAbs. These thirteen mAbs bound seven non-overlapping epitopes: two on the LC, three on the H_N and two on the H_C. The four mAbs (4C4.2, 4C10.1, 8DC1.2, and 8DC4.1) that recognized all four toxins bound to three non-overlapping epitopes, two on the H_N and one on the LC. MAbs 4C4 and 4C10 bound unique epitopes, while 8DC1 and 8DC4 recognized an overlapping epitope. The location of the epitopes of these cross-reactive mAbs on the LC and H_N is consistent with the locations of conserved amino acids in the BoNT models. A fourth antibody, 4C2, bound BoNT/C and BoNT/DC with high affinity, making it an additional candidate for neutralization studies. Thus, these studies identified four mAbs, binding non-overlapping epitopes in each of the domains shared by BoNT/C, BoNT/CD, BoNT/DC and BoNT/D, that could serve as lead antibodies for a recombinant antitoxin for BoNT/C and BoNT/D.

[00317] Example 5

[00318] *Affinity Maturation*

The affinity of the individual mAbs in an antibody combination impacts potency; the higher the affinity, the great the potency (Fan, et al. (2017) supra; Garcia-Rodriguez, et al. (2018) supra). Affinity maturation of each of the cross-reactive antibodies (4C4, 4C10, 8DC1, and 8DC4) was undertaken to improve the affinity, with a goal of a K_D less than 1 nM. To increase the potency of the mAb combinations, we affinity matured each of the cross-reactive mAbs 4C4, 4C10, 8DC1, and 8DC4

that bound BoNT/C, BoNT/CD, BoNT/DC, and BoNT/D. Affinity was increased by diversifying the mAb sequence using light chain shuffling, site-directed mutagenesis, or both (Garcia-Rodriguez, et al. (2007) Nat. Biotechnol., 25(1), 107–116; Lou, et al. (2010) Protein Eng Des Sel., 23(4), 311–319; Razai, et al. (2005) supra). Higher affinity mutants were selected from mutant yeast libraries using FACS. Individual clones were characterized by antibody sequence and scFv affinity. Selected clones were converted to human IgG1 and mAb affinity was measured by using flow fluorimetry (see, e.g., Table 5). Depending on the mAb, this process was iterated one to five times to achieve a K_D for each of the BoNT types of less than 1 nM and optimally less than 100 pM. As shown in, for example, Table 5, this goal was achieved for the four mAbs. Affinities for BoNT/C increased from 3 nM to greater than 1.8 nM to final K_D values of 1.1nM to 147 pM for BoNT/C , and from above 1.4 nM to below 600 pM to final K_D values of 146pM to 87 pM for BoNT/DC, with similar affinity improvements seen for BoNT/CD and BoNT/D. In all cases except for one, affinities were increased using affinity maturation, with overall affinity increases ranging from 2.3-fold to greater than 487-fold.

Table 5: Dissociation Constant (K_D) of antibodies for BoNT/C, BoNT/CD, BoNT/DC, and BoNT/D

	Dissociation Constant, K_D ($\times 10^{-12}$ M)			
Antibody	**BoNT/C**	**BoNT/CD**	**BoNT/DC**	**BoNT/D**
4C4	888	ND	597	ND
4C4.1	570	1400	58	898
4C4.2	35	252	126	254
4C4.4	16	16	87	34
8DC4	3000 (as scFv)	ND	39,000 (as scFv)	ND
8DC4.1	1400	591	1400	1400
8DC4.4	136	117	146	157
8DC4.5	147	38	25	12

[00319] **Example 6**

[00320] *Mouse Neutralization Assays*

For *in vivo* assays, neutralization of BoNT/C and BoNT/C by mAbs 1C1.1, 4C2 and 4C10 was analyzed. Each individual mAb, mAb pair, and the three mAb combination of 1C1.1:4C2:4C10 were evaluated in the mouse neutralization assay, where 50 µg of each mAb or an equimolar mixture of each mAb combination was mixed with BoNT/C and injected intraperitoneally in mice, and the number of mice that survived at 5 days was determined. Survival endpoints are where 50% or more of the mice survive challenge. As observed for other BoNT serotypes (Fan, et al. (2017) supra; Garcia-Rodriguez, et

al. (2018) supra; Fan, et al. (2015) Toxins supra), individual mAbs provided survival endpoints of 60–100% at 20–200 LD_{50}s. The use of mAb pairs increased potency: there were survival endpoints of 100% at 500 LD_{50} with 1C1:4C10, 70% at 2500 LD_{50} with 4C2:4C10, and 70% at 5000 LD_{50} with 1C1:4C2. Adding a third mAb further increased potency. The antibody combination 1C1:4C2:4C10 completely protected mice challenged with 20,000 mouse LD_{50}s. Thus, this three mAb combination was 100 times more potent than the most potent single mAb (1C1.1).

[00321] While the above experiments illustrated the need for multiple antibodies to achieve high potency, the experiments used mAbs that were specifically chosen for their affinity for BoNT/C (190 pM-1.1 nM), rather than their cross-reactive potential. Therefore, we next evaluated the relative potencies of three mAb combinations using cross-reactive mAbs binding BoNT/C, BoNT/CD, BoNT/DC, and BoNT/D (4C4, 4C10 and 8DC4) with and without the addition of mAb 4C2. Initial experiments using BoNT/C as the challenge toxin showed the triple antibody combination of 4C4.2:8DC4.1:4C10.2 to be less effective than the combination of 1C1:4C2:4C10, with little or no survival seen against 5000 LD_{50}. The addition of the mAb 4C2, which binds both BoNT/C and BoNT/DC, increased protection, with 9/10 mice surviving challenge with 20,000 LD_{50}. The reason for the relative lower potency for the combination of three cross-reactive mAbs compared to the mAb combination of 1C1.1:4C2:4C10 is not known. The affinities of these mAbs are not dramatically different, so it is possible that this is due to differences between mAbs in the ability of the mAbs to bind BoNT simultaneously.

[00322] Given the increased potency of the four-mAb combination compared to the three-mAb combination versus BoNT/C, we evaluated decreasing doses of the affinity-matured antibody combination of 4C2:4C4.4:4C10.5:8DC4.4 against 40,000 LD_{50} challenges using BoNT/C, BoNT/CD, and BoNT/DC, and 10,000 LD_{50} using BoNT/D. Complete protection was seen versus BoNT/C, BoNT/CD, and BoNT/DC when 50 µg of total antibody doses were administered. Partial or complete survival against each of these three toxins was seen with antibody doses of 5.0–10 µg. This protective efficacy is comparable to that observed for mAb combinations with other serotypes (Fan, et al. (2017) supra; Garcia-Rodriguez, et al. (2018) supra; Nowakowski, et al. (2002) supra; Tomic, et al. (2019) *Toxins*, *11*(4), 208). However, the potency of this mAb combination was somewhat lower for BoNT/D, where 80% protection was seen using 50 µg of antibody versus 10,000 LD_{50} toxin.

[00323] **Example 7**

[00324] *Antibodies for BoNT/F subserotypes*. Generation of anti BoNT/F antibodies was performed as published in Fan Y, et al. (2017) PLoS ONE 12(3): e0174187.

Table 6: IgG K_D measurements of affinity-matured antibodies for BoNT/F subserotypes by KinExA ($x10^{-12}$ M)

	6F5.2 → 6F5.4		6F11 → hu6F11		6F13 → hu6F13.4	
Origin	human		Mouse → humanized		Mouse → humanized	
Domain bound	HN		HN		HN	
BoNT/F1	3.27	14.85	5.4	0.71	327.3	172.97
BoNT/F2	0.17	0.16	1.47	28.3	26000	215.87
BoNT/F3	3.38	5.19	287.5	8.03	28600	297.39
BoNT/F4	144.3	25.8	85.6	13.7	112.2	404.77
BoNT/F5	141.1	5.88	11.8	21.8	125.4	17.19
BoNT/F6	2.40	0.40	142.6	3.7	822.6	171.65
BoNT/F7	42	3.74	430.6	3.34	458.1	206.10

[00325] 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.

[00326] While the subject antibody, method, and composition have been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. An isolated antibody that specifically binds to a Botulinum neurotoxin (BoNT), wherein the antibody competes for binding to BoNT with an antibody comprising:

a) a variable heavy (V_H) chain comprising heavy complementarity determining regions 1-3 (HCDRs 1-3) and a variable light (V_L) chain comprising light CDRs 1-3 (LCDRs 1-3) of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

b) a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody; or

c) a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody.

2. The isolated antibody of claim 1, wherein the isolated antibody competes for binding to BoNT with an antibody comprising:
a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

3. The isolated antibody of claim 1, wherein the isolated antibody competes for binding to BoNT with an antibody comprising:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

4. The isolated antibody of claim 1, wherein the isolated antibody competes for binding to BoNT with an antibody comprising:
a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody.

5. An isolated antibody that binds to a Botulinum neurotoxin, wherein the antibody comprises:

a) a variable heavy (V_H) chain comprising heavy complementarity determining regions 1-3 (HCDRs 1-3) and a variable light (V_L) chain comprising light CDRs 1-3 (LCDRs 1-3) of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

b) a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4, or the Hu6F11 antibody;

c) a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody;

d) a V_H chain comprising the amino acid sequence of the V_H chain of the 4C4.4 antibody, 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

e) a V_L chain comprising the amino acid sequence of the V_L chain of 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody;

f) a V_H chain comprising HCDRs 1-3 of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody; or

g) a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

6. The isolated antibody of claim 5, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody.

7. The isolated antibody of claim 5, wherein the antibody comprises:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 antibody, 8DC4.4 antibody, the 6F5.4 antibody, Hu6F13.4, or the Hu6F11 antibody.

8. The isolated antibody of claim 5, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody.

9. The isolated antibody of any one of claims 1-8, wherein the antibody is a human antibody or a humanized antibody.

10. The isolated antibody of any one of claims 1-9, wherein the antibody is a single chain Fv (scFv), IgG, Fab, $(Fab')_2$, or $(scFv')_2$.

11. The isolated antibody of any one of claims 1-10, wherein the antibody binds to BoNT with a K_D of about 5 nM or less.

12. The isolated antibody of any one of claims 5-11, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 8DC4.4 antibody; and binds to BoNT/C and/or BoNT/D.

13. The isolated antibody of any one of claims 5-11, wherein the antibody comprises:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 4C4.4 or 8DC4.4 antibody and binds to BoNT/C and/or BoNT/D.

14. The isolated antibody of any one of claims 5-11, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and heavy chain framework region 1 (H-FR1) and a V_L chain comprising LCDRs1-3 of the 4C4.4 antibody and binds to BoNT/C and/or BoNT/D.

15. The isolated antibody of any one of claims 5-11, wherein the antibody comprises:
a V_H chain comprising HCDRs 1-3 and a V_L chain comprising LCDRs 1-3 of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody and binds to BoNT/F.

16. The isolated antibody of any one of claims 5-11, wherein the antibody comprises:
a V_H chain comprising the amino acid sequence of the V_H chain and a V_L chain comprising the amino acid sequence of the V_L chain of the 6F5.4 antibody, Hu6F13.4 antibody, or the Hu6F11 antibody and binds to BoNT/F with high affinity.

17. A composition comprising:
a pharmaceutically acceptable carrier; and
at least a first antibody according to any one of claims 1-16.

18. The composition of claim 17, comprising a first antibody and a second according to any one of claims 1-16.

19. The composition of claim 18, wherein the first antibody binds to BoNT/C and/or BoNT/D and the second antibody binds to BoNT/F.

20. An isolated nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of any one of the antibodies of any one of claims 1-16.

21. A vector comprising the isolated nucleic acid of claim 20.

22. A first nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of the V_H chain and a second nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of the V_L chain any one of the antibodies of any one of claims 1-16.

23. A first vector comprising the first nucleic acid and a second vector comprising the second nucleic acid of claim 22.

24. A host cell comprising the nucleic acid of claim 20, the vector of claim 21, the first and second nucleic acids of claim 22 or the first and second vectors of claim 23.

25. The isolated antibody according to any one of claims 1-16, wherein the antibody is labeled.

26. A method of specifically binding to a Botulinum neurotoxin in a mammal comprising:
administering to the mammal an effective amount of an antibody of any one of claims 1-16;
wherein the administering provides for specific binding of Botulinum neurotoxin present in the mammal.

27. A method of specifically binding to a Botulinum neurotoxin in a mammal, comprising:
administering to the mammal an effective amount of a composition of any one of claims 17-19;
wherein the administering provides for specific binding of Botulinum neurotoxin present in the mammal.

28. A method for detecting Botulinum neurotoxin in a sample, comprising:
contacting an antibody of any one of claims 1-16 with the sample,
detecting binding of said antibody to Botulinum neurotoxin in the sample.

29. The method of claim 28, wherein the antibody is labeled.

30. The method of claim 29, wherein said label is a fluorescent label, a chemiluminescent label, a radiolabel, an enzyme, or a chromogenic label.

31. The method of any one of claims 28-30, wherein said sample is blood.

32. The method of any one of claims 28-31, wherein the antibody is immobilized on a substrate.

33. The method of any one of claims 28-32, wherein the antibody is conjugated to a nucleic acid and wherein said detecting comprises a polymerase chain reaction.

34. A method of reducing the likelihood that an individual at risk of exposure to Botulinum neurotoxin will experience symptoms of Botulinum neurotoxin poisoning following exposure, the method comprising administering to said individual an effective amount of an antibody of any one of claims 1-16;

wherein said administering provides for reducing the likelihood that the individual will experience symptoms of Botulinum neurotoxin poisoning.