

ANTIBODIES AGAINST TYPE A BOTULINUM NEUROTOXIN

[0001] Anaerobic bacterium *Clostridium botulinum* produces seven immunologically distinct but structurally similar botulinum neurotoxins (BoNTs) designated BoNT/A-G that are associated with foodborne, infant, and wound botulism (Montecucco and Schiavo, 1994, *Mol. Microbiol.* 13, 1-8; Simpson, 1981, *Pharmacol. Rev.* 33, 155-188). Due to their unique properties, botulinum neurotoxins (BoNTs) have been used to treat a variety of human disorders involving voluntary muscles. BoNT/A was approved in 1989 by the Food and Drug Administration for the treatment of ocular disorders such as strabismus. It has also been used in the treatment of spasmodic torticollis, limb dystonias, vocal disorders, cerebral palsy, gastrointestinal disorders, and tremors (Schantz and Johnson, 1997, *Perspectives in Biology and Medicine* 40, 317-327). Interestingly, BoNT has been found to relieve pain and other syndromes associated with the autonomic nervous system. Injections of BoNT have been shown to induce sweating in certain individuals and relieve migraine headache pain in other individuals. The toxin is known to disrupt the mechanism associated with the release of neuronal substances or neuronal peptides. Besides having the capacity to provide relief from a variety of human diseases, BoNTs also pose a threat as a neurotoxic biological agents.

[0002] Following synthesis, highly active neurotoxin generated by proteolytic cleavage of the CNTs (clostridial neurotoxins). The active neurotoxin composed of two main chains that are connected via a disulfide linkage. The location of the enzymatic subunit of the CNTs has been mapped to the smaller N terminal chain (50-kDa), while the binding and translocation motifs are located within the larger chain (a 100-kD heavy (H) chain). The 50-kD carboxyl-terminal fragment of the H chain (HC) have been postulated to be the receptor binding subunit of the toxin and is thought that this fragment plays a crucial role to direct and pass the enzymatic portion of the CNT across the vesicle membrane. The zinc-endopeptidase catalytic domain of the toxin resides in the 50 kDa N-terminal portion of the active protein. Upon entry into the cells BoNT works at the neurojunction by rendering key neuronal proteins associated with the release of acetyl-choline inactive (Montecucco and Schiavo, 1994, supra; Blasi et al., 1993, *Nature* 365, 160-163). Very recently, the structure of BoNT/A was solved and showed substantial homology among the structure of the C-terminal binding domain of BoNT/A and Tetanus (Lacy et al., 1998, *Nature Struct. Biol.* 5, 898-902; Umland et al., 1998, *Nature Struct. Biol.* 4, 788-792). Unlike the translocation domain of other toxins, the protein fold seen in BoNT/A translocation domain contains a kinked pair of α -helices and a 54 residue that wraps around the enzymatic domain similar to a "belt" (Lacy and Stevens, 1998, *Curr. Opin. Struct. Biol.* 8, 778-784). The role that the "belt" plays in toxicity of BoNT has not been studied. However, it may play an important role during translocation and cleavage of the substrates.

[0003] Although all CNTs are zinc-dependent endopeptidases, they differ in substrate specificity, substrate cleavage-site location, and their sites of action within the central nervous system (Lacy et al., 1998, supra). For example, BoNT serotypes A and E cleave SNAP-25 (synaptosome-associated protein of 25 kDa), while other CNTs cleave

syntaxin or synaptobrevin. BoNTs inhibit cholinergic vesicle docking at neuromuscular presynaptic nerve endings and cause potentially fatal flaccid paralysis, whereas TeNT (tetanus neurotoxin) is transported in a retrograde manner to the spinal cord, resulting in spastic paralysis and death (Montecucco and Schiavo, 1994, supra).

SUMMARY OF THE INVENTION

[0004] Probably due to unusually high toxicity of BoNTs, previous attempts to produce large numbers of high affinity neutralizing monoclonal antibodies (MAbs) against these neurotoxins have been unsuccessful. We reasoned because immunization with non-toxic binding fragment of BoNT/A can induce protective immunity in mice, then it should be possible to generate neutralizing antibodies using this approach. We report herein that immunization with BoNT/A-Hc allowed the generation of MAbs recognizing both the whole BoNT/A and BoNT/A Hc. We characterized these antibodies in detail, demonstrated biochemical detection of BoNT/A and its binding fragment. We used, neutralizing MAbs directed against the BoNT/A-Hc, in combination with theoretically derived predictions of secondary and solvent accessibility of the residues within the BoNT/A-Hc, to locate the principle protective antigenic determinants (PPDs) of BoNT/A-Hc. Binding of the neutralizing MAbs to overlapping truncated recombinant polypeptides corresponding to BoNT/A-Hc were examined. In addition, we tested MAb recognition of two synthetic 25-mer peptides, whose sequences correspond to predicted solvent-exposed loops within the C-terminal end of the BoNT/A-Hc. Finally, we examined the ability of these peptides to elicit antibody production and to determine whether the resultant antibodies protected the immunized mice from BoNT/A challenge. From these experiments, we identified two regions within the Hc that may contribute to a neutralizing epitope. Because of their ability to neutralize BoNT/A, they could be used for mapping binding sites of the toxin, for competitive-based ELISA to predict immunity following vaccination, identify protective epitopes, and they may be important tools for therapeutic purposes.

[0005] Therefore, it is one object of the present invention to provide protective antibodies against BoNT/A. The antibodies of the present invention can be monoclonal or polyclonal antibodies. The present invention also pertains to hybridomas producing antibodies, such as 4A2-2, 6B2-2, and 6C2-4, which bind to an epitope of BoNT/A.

[0006] It is another object of the present invention to provide a method of purifying BoNT/A from an impure solution containing BoNT/A. The method involves contacting the impure solution with an antibody which binds an epitope of BoNT/A, allowing the antibody to bind to BoNT/A to form an immunological complex, and separating the complex from the impure solution. The method of purification can further comprise separating the BoNT/A from the antibody and recovering the BoNT/A. In one embodiment, the separation is conducted by contacting the immunological complex with a saturating amount of peptide comprising the epitope recognized by the antibody of the immunological complex.

[0007] The present invention still further pertains to a method for detecting BoNT/A in a sample. The method involves contacting the sample with an antibody which