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Clostridium botulinum Neurotoxins — Applications in Medicine and Potential Agents of Bioterrorism

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Abstract

Botulism is one of the distinctive diseases known to humankind. Botulism is characterized by a flaccid paralysis progressing to suffocation and death in severe cases that do not receive adequate treatment. Botulism is caused by clostridial neurotoxins with extraordinary potency and neurospecificity. The disease is a true toxemia, in which the neurotoxins are the responsible agents, and the bacteria are not directly involved in paralytic symptoms. Botulinum neurotoxin (BoNT) is produced by strains of neurotoxigenic clostridia, including *Clostridium botulinum*, as well as rare strains of *Clostridium baratii* and *Clostridium butyricum*.

During the past two decades, because of new knowledge about the cellular biology and pharmacology of BoNT, this toxin has become an important tool in cell biology and for the understanding of disease and has stimulated much interest in its actions on the human nervous system. Interest in BoNT has also been raised by awareness of its possible use in bioterrorism, yet the most remarkable discovery that has resulted from investigation of BoNT is its use as a pharmaceutical for the treatment of a myriad of neuronal and hyperactive muscle disorders.

Botulism

The hallmark of botulism is a bilateral descending weakness and paralysis with an extremely long duration of paralysis of months to years in severe cases. Botulism in humans can weaken or paralyze every skeletal muscle in the body. BoNT inhibits acetylcholine exocytosis at parasympathetic and sympathetic neuromuscular synapses. Patients with signs of botulism must be monitored for respiratory difficulties. Although the sensory system and central nervous system (CNS) mentation are generally unaffected, there have been sporadic reports of sensory abnormalities in botulism cases. Botulism incidence is quite rare, and it may be misdiagnosed as more common paralytic diseases, such as Guillain-Barré syndrome, myasthenia gravis, tick paralysis, diphtheritic neuropathy, Lambert-Eaton syndrome, or certain nervous system infections. No specific antidote is currently available for preventing botulism or reversing paralysis once receptor binding and internalization ensues. Early passive administration of antibodies can decrease the severity of disease and slow its progression, resulting in a shorter hospital stay and more rapid recovery. Recovery from severe hospitalized cases of botulism requires supportive care with particular attention to respiratory capacity. Complete recovery requires the regeneration and restoration of functional neuromuscular junctions with resumption of neurotransmission. Recovery is generally complete, with no chronic complications.

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Neurotoxigenic Clostridia

One of the most interesting features of clostridial organisms is their formation of a wide diversity of toxins. The clostridia produce more types of protein toxins than any other group of microorganisms; more than 20 protein toxins have been identified from clostridia, some with very high potencies in animals and humans. At least 15 species of *Clostridium* are known to produce protein toxins. These agents include neurotoxins, lipases, lecithinases, hemolysins, enterotoxins, cytotoxins, collagenases, permeases, necrotizing

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toxins, proteinases, hyaluronidases, DNases, ADP-ribosyltransferases, neuraminidases, and others that are uncharacterized and simply known as "lethal toxins." Clostridia cause a number of diseases, including the well-known syndromes of gas gangrene and other intestinal infections. In contrast to many other bacterial pathogens, *Clostridium botulinum* causes a true toxemia, in which the neurotoxin is solely responsible for pathogenic effects on the human host. These neurotoxins may be elicited during infections, such as in wounds; infections of the intestinal tract, as in infant botulism; or in traditional foodborne botulism, through oral consumption of BoNT in foods.

Members of the genus *Clostridium* have traditionally been classified as anaerobic, gram-positive, rod-shaped, fermentative bacteria that form endospores and obtain energy for growth by fermentation of organic substrates. The clostridia are a large and diverse group of prokaryotes. The ninth edition of *Bergey's Manual of Systematic Bacteriology* presented 83 species in the genus *Clostridium*, and in a recent compilation, the number of *Clostridium* species extends to 177. The genus is clearly in need of more precise classfication and phylogenetic analysis. Clostridia were formerly classified mainly by phenotypic properties and more recently by determination of 16S rRNA and intervening gene sequences. Classification of bacteria on the basis of genetic and protein relatedness is useful for determining the evolutionary relationships of bacterial groups, but it may not be practical to use this approach for rapid identification of neurotoxigenic clostridia. Most investigators will continue to classify neurotoxigenic clostridia into only a few species on the basis of the characteristic toxic symptoms in animals and the medical importance of their toxins.

The isolation and identification of neurotoxigenic clostridia presents practical difficulties. Clostridia are anaerobes and culturing requires media of low redox potential and elimination of oxygen and toxic oxygen metabolites. An anaerobic jar, or preferably, an anaerobic glove box, is needed for cultivation and manipulations. Prompt collection of foods or clinical samples and rapid analysis is necessary to prevent exposure to oxygen and reduction in viability of clostridial organisms. In general, *C. botulinum* and *Clostridium tetani* are more sensitive to oxygen than certain other pathogenic clostridia, such as *Clostridium sporogenes* and *Clostridium perfringens*. Oxygen sensitivity restricts the habitats of vegetative cells to anaerobic environments, though endospores may survive in aerobic environments. Clostridial endospores are present in most environmental, food, and fecal samples and can be isolated using appropriate techniques. Because of the resistance properties of endospores, competitor vegetative organisms can be eliminated by heat or chemical treatments. Incubation of the source materials at 60 to 80°C for 10 to 20 min is usually sufficient to select for clostridial endospores. Mixed cultures are usually present in clinical specimens or foods, and oxygen-utilizing bacteria and fungi will utilize oxygen and provide an environment favorable for survival and growth of clostridia. When clostridia are enriched in complex media from foods, fecal materials, or other complex sources, they may be outgrown by competitor organisms. Selective media have been useful for isolation of *C. botulinum* types A and B (group I), which are most commonly implicated in human disease.

C. botulinum synthesizes seven immunologically distinguishable serotypes of BoNTs, designated A through G. BoNTs can be neutralized by polyvalent antitoxins obtained by immunization of animals with toxoids or recombinant BoNT fragments or by combinations of monoclonal antibodies isolated against BoNTs. A specific serotype of BoNT is neutralized by polyvalent antisera to the causative toxin type but retains toxicity on incubation with heterologous antisera. This practice defines a serotype. A definitive identification of *C. botulinum* or diagnosis of botulism depends on the detection of BoNT, usually by intraperitoneal or intravenous injection in mice with proper controls, including neutralization with monovalent antiserum. Because certain strains of *C. botulinum* produce more than one serotype of BoNT, this can complicate analysis. BoNT detection is often enhanced by proteolytic activation with trypsin or other suitable proteolytic enzymes.

Epidemiologic analysis of botulism outbreaks can be assisted by typing methods for neurotoxigenic clostridia. Several methods have been used for genotyping, including restriction analysis of isolated genomic DNA, PCR, restriction fragment length polymorphism, random amplification of polymorphic DNA, and pulse field gel electrophoresis (PFGE). Of the various methods, many laboratories are using PFGE, as it appears to be the most discriminating and generally provides consistent analyses. Sequencing of 16s rRNA and intervening sequences is also useful in taxonomic and strain identification. As genome sequences become available, identification by molecular methods will become important for identification and characterization of *C. botulinum* and other clostridia.

Botulinum Neurotoxin Structure and Function

BoNTs comprise a related family of neurotoxins that are produced as single-chain protein molecules of ca. 150 kDa. They achieve their characteristic high toxicities of 10^7 to 10^8 mouse 50%

