Chapter 14

BOTULINUM TOXIN

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INTRODUCTION

The seven neurotoxin serotypes (A-G) produced by *Clostridia* species are among the most potent toxins known. These structurally similar neurotoxins are immunologically distinct because neutralizing antibodies for one serotype does not protect against the other six serotypes. Because of their extreme toxicity, neurotoxin from *Clostridium botulinum* was one of the first agents to be considered as a biological weapons agent. Botulinum neurotoxin has been developed as a biological weapon by many countries, including Japan, Germany, the United States, Russia, and Iraq (Figure 14-1).

Botulism in its various forms (foodborne, wound, infant and adult intestinal, and iatrogenic) is a potentially fatal neuroparalytic disease that most often presents as a descending, symmetric flaccid paralysis, which is typically associated with neurotoxin types A, B, and E. Foodborne outbreaks receive considerable public health attention, as the risk of widespread food dissemination of botulinum toxin constitutes a public health emergency and is often fatal if untreated. However, the most common form of botulinum intoxication in the United States is infant botulism from the intestinal colonization with toxin-producing *Clostridium* in infants younger than 1 year of age. For a recent 10-year period (2002–2011), of 1,379 reported cases of botulism in the United States, the greatest number was infant botulism (68%), followed by wound botulism (18%), and foodborne botulism (12%).

HISTORY

In the early 1930s during its occupation of Manchuria, Japan formed a biological warfare research program. The largest facility in this program, which was located in Pingfang, was known as Unit 731. General Shiro Ishii, the Japanese military medical commander of Unit 731, admitted to feeding lethal cultures of *C. botulinum* to prisoners. US researchers began working on weaponization of botulinum toxin in the 1940s, and Allied intelligence indicated that Germany attempted to develop botulinum toxin as a weapon to be used against invasion forces. At the time, neither the composition of the toxic agent produced by *C. botulinum* nor its mechanism of injury were fully known. Therefore, the earliest research goals were to isolate and purify the toxin and determine its pathogenesis, with the latter work conducted at Camp Detrick. The potential of botulinum neurotoxin as an offensive biological weapon was also investigated. The US code name given to botulinum neurotoxin at that time was “agent X.”

Following President Richard M Nixon’s executive orders in 1969–1970, as explicitly stated in National Security Decision Memoranda 35 and 44, all biological agent stockpiles in the US offensive biological program, including botulinum neurotoxin, were destroyed. The 1975 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction prohibited the production of offensive toxins. Although the Soviet Union signed and ratified this convention, its biological warfare program, which included botulinum neurotoxin research, biological weapons development, and production, continued and was expanded in the post-Soviet era. The Soviet Union reportedly tested botulinum-filled weapons at the Soviet site Aralsk-7 on Vozrozhdeniye (Renaissance) Island in the Aral Sea and attempted to use genetic engineering technology to transfer complete toxin genes into other bacteria. In April 1992, President Boris Yeltsin publicly declared that his country had covertly continued a massive offensive.

Figure 14-1. Representations of the structure of BoNT/A. *Left panel*: the protein is composed of a ~50 kDa light chain (LC, blue-green) and a ~100 kDa heavy chain. The heavy chain is composed of three distinct structural domains: two C-terminal ganglioside binding domains are used in recombinant vaccines, HCc (red) and HCn (yellow), and an N-terminal translocation domain (HN, green). The LC functions as a zinc-dependent endopeptidase. *Right panel*: in this rendering of the molecule, the belt-like portion of the heavy chain (red) is more clearly seen to wrap around the LC (blue). Data sources: (1) Lacy DB, Tepp W, Cohen AC, et al. Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat Struct Biol. 1998;5:898–902. (2) Swaminathan S, Eswaramoorthy S. Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. Nat Struct Biol. 2000;7:693–699.

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biological warfare buildup, which included developing botulinum toxin as a weapon. Yeltsin’s assertions gave credence to the claims of Biopreparat laboratory director Vladimir Pasechnik, who defected to the United Kingdom in 1989.\textsuperscript{20} Also in 1992, Colonel Kanatjan Alibekov (Kenneth Alibek),\textsuperscript{19} the former deputy director of Biopreparat (a Soviet agency whose primary function was to develop and produce biological weapons of mass casualties), defected to the United States and eventually described in considerable detail the Soviet biological weapons program in his book \textit{Biohazard}.\textsuperscript{19}

Iraq, which also signed the 1975 convention, expanded its biowarfare program in 1985. Ten years later, it admitted to the United Nations Special Commission inspection team to having produced 19,000 liters of concentrated botulinum neurotoxin for use in specially designed missiles, bombs, and tank sprayers in 1989 and 1990.\textsuperscript{16,21} Of this preparation, 10,000 liters were used to fill 13 SCUD missiles with a 600-km range and 100 181-kg (R-400) bombs (each bomb could hold 83 liters of toxin solution). However, Iraq did not use biological agents during the Persian Gulf War or Operation Iraqi Freedom, and it has maintained that its biological weapon stockpiles were destroyed.\textsuperscript{22} No biological weapons were discovered in Iraq in wartime from 2003 through 2011.\textsuperscript{23}

The Aum Shinrikyo, a Japanese cult formed in 1987 by Shoko Asahara, attempted to develop biological weapons after its political party was defeated in the 1990 election campaign. Known for its deadly 1995 sarin attack in the Tokyo subway, Aum Shinrikyo also attempted to produce botulinum neurotoxin. Five days before the sarin attack, three briefcases containing portable disseminating devices generating water vapor were found in the Kasumigaseki subway station.\textsuperscript{24} At his 1996 trial, Asahara said he believed the cases contained botulinum neurotoxin, although the toxin was not detected in the devices. With 50,000 followers worldwide and an estimated $1 billion in financial resources, the cult had the capability to develop biological toxins for use as weapons, and the intent to do so.\textsuperscript{25} Although no cult members were specialists in biological weapons development, microbiologists, medical doctors, and other scientists were among the followers. In their thorough analysis of the Aum Shinrikyo’s efforts to develop biological and chemical weapons, Richard Danzig and his colleagues postulate that the group specifically failed to develop a viable botulinum toxin because of the following reasons:\textsuperscript{25}

- inability to acquire an appropriate strain of \textit{C. botulinum} capable of producing sufficient concentrations of active botulinum toxin (five different strains were isolated by the cult);
- inability to achieve specific culture conditions (eg, appropriate fermentation broth, appropriate anaerobic environment) required for toxin production;
- presence of bacterial contamination, as \textit{Bacillus subtilis natto} was identified in the cult’s \textit{C. botulinum} product (as an aerobic bacteria, also indicates inappropriate anaerobic cultural conditions);
- toxin degradation that may have occurred during postfermentation recovery or storage; and
- suboptimal concentrations of toxin (if present in any amount) that were disseminated that did not cause harm.

A successful bioterrorist attack on large numbers of people with botulinum neurotoxin would overwhelm the public health system. The medical intervention required to assist patients with botulism includes mechanical ventilation and urgent attendant healthcare. If the Rajneeshee cult (the followers of the Bhagwan Shree Rajneesh who had carried out a biological attack to influence a local election) had used botulinum toxin instead of \textit{Salmonella typhimurium} on salad bars in its 1984 attack in The Dalles, Oregon,\textsuperscript{26–28} many of the 751 persons who contracted Salmonella gastroenteritis would likely have died; the neurological sequelae of hundreds of patients with botulinum toxin poisoning would have quickly overwhelmed community medical resources.\textsuperscript{29}

The potential consequences of a botulinum toxin-induced mass casualty disaster can be estimated by reviewing the March 2006 foodborne botulism outbreak in Thailand.\textsuperscript{30} This event signifies a profound national public health mass casualty event, and successful patient recovery is not trivial. In this outbreak, home-canned bamboo shoots were consumed in a village, where 209 individuals consumed a common meal. Of 163 individuals examined in a hospital, 140 were hospitalized and 42 developed respiratory failure and required mechanical ventilation.\textsuperscript{30} Sufficient antitoxin was donated to Thailand by various international health organizations to treat 90 patients. This antitoxin was administered to patients with the most severe symptoms, but treatment was delayed 5 to 9 days from exposure. The median duration of hospital admission was 6 days for patients without mechanical ventilation and 25 days for patients with mechanical ventilation.\textsuperscript{31} A long incubation time was associated with a better prognosis.\textsuperscript{32} The massive public health response undertaken by the Thai healthcare system, the global health community, and others outside of healthcare, including many embassies, airlines, and commercial
partners, is credited for no mortalities.\textsuperscript{30} Besides the need for adequate ventilators and healthcare support, successful treatment of such large numbers of severely affected patients required intervention from neurologists, pulmonologists, intensivists, cardiologists, infectious disease specialists, and rehabilitation and referral services.\textsuperscript{31} Other supportive personnel required for a mass casualty botulism event included pharmacists, respiratory care officials, and psychological services personnel.\textsuperscript{32,33}

In 2005, Wein and Liu\textsuperscript{35} described in detail how a bioterrorism attack using botulinum neurotoxin could be perpetrated on the nations’ milk supply. They describe a mathematical model representative of California’s dairy industry with milk traveling from cows to consumer in a supply chain: milk is processed via separation, pasteurization, homogenization, and vitamin fortification; and eventually distributed to the public.\textsuperscript{35} Naturally occurring salmonellosis outbreaks from milk and milk products affecting more than 200,000 persons have occurred, leading to a realistic assessment of such vulnerability in the national milk distribution system.\textsuperscript{36,37} The ability to spread botulinum neurotoxin via a liquid media, if present in sufficient concentration, makes this agent a logical choice for such a scenario. Modeling of botulinum in a liquid dispersal medium is not new and has been posited for terrorist use in a water fountain,\textsuperscript{38} based on microbiological contamination at a recreational facility.\textsuperscript{39} However, Wein and Liu’s modeling goes further than toxicity generation, pinpointing critical entry points of neurotoxin into the milk supply, estimating the amount of toxin required, and identifying weaknesses in current detection technology.\textsuperscript{35,39} The Wein and Liu paper generated considerable debate\textsuperscript{40} by the possible security risk it exposed.\textsuperscript{41} Stewart Simonson, former assistant secretary for public health emergency preparedness at the US Department of Health and Human Services, regrets the publication decision.\textsuperscript{42} It has been demonstrated that the milk supply is likely not at a risk for botulism contamination during the presterilization process because standard pasteurization at 72°C for 15 seconds inactivates at least 99.99% of BoNT/A and BoNT/B and at least 99.5% of their respective complexes.\textsuperscript{43}

**DESCRIPTION OF THE AGENT**

*Clostridium* species bacteria are sporulating, obligate anaerobic, gram-positive bacilli. The spores of *C. botulinum* are ubiquitous, distributed widely in soil and marine sediments worldwide, and often found in domestic grazing animals’ intestinal tracts.\textsuperscript{44–48} Under appropriate environmental or laboratory conditions, spores can germinate into vegetative cells that will produce toxin. *C. botulinum* grows and produces neurotoxin in the anaerobic conditions frequently encountered in food canning or preservation. The spores are hardy, and special efforts in sterilization are required to ensure that the spores are inactivated.\textsuperscript{49} Modern commercial procedures have virtually eliminated food poisoning by botulinum toxin. However, the leading cause of foodborne botulism is attributed to home-canned foods (particularly vegetables such as beans, garlic, peppers, carrots, and corn that are pH >4.6) or food items improperly prepared by restaurants.\textsuperscript{50–52}

*C. botulinum* produces eight antigenic types of neurotoxins denoted by the letters A through H. Seven neurotoxins (A-G) are structurally similar (approximately 150 kDa in mass) but immunologically distinct.\textsuperscript{53,54} However, there is some serum cross-reactivity among the serotypes because they share some sequence homology with one another as well as with tetanus toxin.\textsuperscript{55,56} The unique strain *Clostridium baratii* produces only serotype F,\textsuperscript{57} and the *Clostridium butyricum* strain, serotype E.\textsuperscript{58}

**Botulism Neurotoxin Production**

Spore germination and subsequent growth of toxin-producing bacteria occur in improperly preserved foods,\textsuperscript{60–72} decaying animal carcasses and vegetable
matter, and microbiology laboratories. A terrorist with the proper expertise and resources could obtain a toxin-producing strain of *C. botulinum*. Various scientific journals, textbooks, and Internet sites provide information on how to isolate and culture anaerobic bacteria and, specifically, how to produce botulinum toxin. A major cause of botulism is the ingestion of foods contaminated with *C. botulinum* and preformed toxin. The food supply remains vulnerable to a botulinum toxin attack.

**PATHOGENESIS**

The seven neurotoxins have different specific toxicities and durations of persistence in nerve cells. All botulinum toxin serotypes inhibit acetylcholine release, but they act through different intracellular protein targets, exhibit different durations of effect, and have different potencies. All seven toxins may potentially cause botulism in humans given a large enough exposure. Botulinum neurotoxin can enter the body via the pulmonary tract (inhalational botulism), the gastrointestinal tract (foodborne and infant botulism, adult intestinal [adult infectious] botulism), direct injection (iatrogenic botulism), and from infected wounds (wound botulism). Infant, adult intestinal, and wound botulism are also referred to as infective botulism, as toxin produced by *C. botulinum* either colonizes the lumen of the intestinal tract or is produced in a wound. Upon absorption, the circulatory system transports the toxin to peripheral cholinergic synapses, primarily targeting neuromuscular junctions. The toxin binds to high-affinity presynaptic receptors and is transported into the nerve cell through receptor-mediated endocytosis. In the nerve cell, it functionally blocks neurotransmitter (acetylcholine) release, thereby causing neuromuscular paralysis. Other neurotransmitters co-located with acetylcholine may also be inhibited, and noncholinergic cells may also be affected. The estimated human dose (assuming a weight of 70 kg) of type A toxin lethal to 50% of an exposed population (the LD₅₀) is estimated, based on animal studies, to be approximately 0.09 to 0.15 µg by intravenous administration, 0.7 to 0.9 µg by inhalation, and 70 µg by oral administration.

**CLINICAL DISEASE**

Untreated botulism is frequently fatal. The rapidity of the onset of symptoms, as well as the severity and duration of the illness, is dependent on the amount and serotype of toxin. In foodborne botulism, symptoms appear several hours to within a few days (range 2 hours to 10 days) after contaminated food is consumed. In most cases the onset of symptoms occurs within 12 to 72 hours postexposure. In one study, the median incubation period for the onset of symptoms from all toxin serotypes was 1 day. However, the median time to onset of symptoms for serotype E was shorter (range 0–2 days) compared to toxin serotypes A (range 0–7 days) and B (range 0–5 days); most individuals with toxin serotype E had symptoms within 24 hours of ingestion. Symptoms from foodborne botulism from toxin serotype A generally are more severe than from toxin serotypes B and E.

As a neuromuscular illness, botulism presents as an acute, symmetrical, descending, and flaccid paralysis. However, early symptoms may be nonspecific and difficult to associate with botulinum intoxication. Individuals with foodborne botulism often present initially with gastrointestinal symptoms such as nausea, vomiting, abdominal cramps, and diarrhea. Initial neurologic symptoms usually involve the cranial nerves, with symptoms of blurred vision, diplopia, ptosis, and photophobia, followed by signs of bulbar nerve dysfunction such as dysarthria, dysphonia, and dysphagia. Onset of muscle weakness ensues in the following order: muscles involving head control, muscles of the upper extremities, respiratory muscles, and muscles of the lower extremities. Weakness of the extremities generally occurs in a proximal-to-distal pattern, and is generally symmetric. However, asymmetric extremity weakness may occasionally be observed, occurring in 9 of 55 botulism cases in one review.

Respiratory muscle weakness can result in respiratory failure, which may be abrupt in onset. In one study, the median time between the onset of intoxication symptoms and intubation was 1 day. Other commonly reported symptoms included fatigue, sore throat, dry mouth, constipation, and dizziness. Botulism is not associated with sensory nerve deficits. However, one review of botulism from toxin serotype A or B showed that 8 of 55 cases reported symptoms of paresthesias.

Death is usually the result of respiratory failure or secondary infection associated with prolonged mechanical ventilation. In general, intoxication with toxin serotype A results in a more severe disease, often with bulbar and skeletal muscle impairment, and thus the need for mechanical ventilation. Intoxication with toxin serotype B or E is more often associated with symptoms of autonomic dysfunction, such as internal ophthalmoplegia, nonreactive dilated pupils, and dry mouth.
Paralysis from botulism can be long lasting. Mechanical ventilation may be required for 2 to 8 weeks with foodborne botulism, with paralysis lasting as long as 7 months. Symptoms of cranial nerve dysfunction and mild autonomic dysfunction may persist for more than a year.

The following symptom triad should suggest a botulism diagnosis: (1) an acute, symmetric, descending, flaccid paralysis with prominent bulbar palsies in (2) an afebrile patient with (3) a normal sensorium. The bulbar palsies of botulism consist of the “four Ds”: diplopia, dysarthria, dysphonia, and dysphagia. Five classic symptoms have also been used to diagnose botulism: (1) nausea and vomiting, (2) dysphagia, (3) diplopia, (4) dry mouth, and (5) fixed dilated pupils. However, individuals may not exhibit all five symptoms; a review from the country of Georgia reported that only 2% of patients (13/481) presented with all five criteria.

Epidemiological history of injection of black tar heroin (wound botulism), laboratory work with botulinum toxins, or therapeutic use (eg, for cervical dystonia or cosmetic purposes) of botulinum neurotoxin preparations not approved by the Food and Drug Association (FDA) may also support the diagnosis of botulism. Patients with botulism may have a delay in diagnosis because of vague complaints or symptoms only present early in the illness or from misdiagnosis of paralytic symptoms. In one study, less than half of the patients with botulism requiring hospitalization were diagnosed and admitted during the initial physician contact. Alternate diagnoses may include drug overdose or intoxication (particularly with injection drug users having wound botulism) malingering, conversion disorder, stroke, myasthenia gravis, viral syndromes, and others. Clinicians should be aware of early symptoms of botulism in adults (ie, blurred vision, diplopia, and/or ptosis) and infants. In addition, delays to diagnosis are associated with more prolonged hospital course and an increased need for ventilatory support.

Although foodborne botulism is the most likely route of exposure for botulism from either natural causes or a bioterrorist event, botulism acquired on the battlefield is most likely to occur from botulinum toxin inhalation, a route of exposure that does not naturally occur. The duration from exposure to the onset of symptoms for inhalational botulism is similar to that observed with ingestion of botulinum toxin, generally ranging from 24 to 36 hours to several days postexposure. Clinical symptoms resulting from inhalational intoxication are similar to botulism acquired from toxin ingestion.

The first reported inhalation-acquired botulism in humans occurred in 1962 in a German research laboratory. Three laboratory workers experienced symptoms of botulinum intoxication after conducting a postmortem examination of laboratory animals that had been exposed to botulinum toxin type A. Hospitalized 3 days after their exposure, the workers were described as having (a) “a mucous plug in the throat,” (b) difficulty in swallowing solid food, and (c) “the beginning of a cold without fever.” The symptoms had progressed on the 4th day, and the patients complained of “mental numbness,” extreme weakness, and retarded ocular motions. Their pupils were moderately dilated with slight rotary nystagmus, and their speech became indistinct and their gait uncertain. The patients were given antitoxin serum on the 4th and 5th days. Between the 6th and 10th days after exposure, the patients experienced steady reductions in their visual disturbances, numbness, and difficulties in swallowing. They were discharged from the hospital less than 2 weeks after the exposure, with a mild general weakness as their only remaining symptom. Botulism acquired through illicit drug (cocaine) inhalation has also been reported, and the incubation period was difficult to calculate because of frequent drug use.

Other forms of botulism (wound, infant, and iatrogenic botulism) have similar symptoms as foodborne botulism, but they may have some variation in clinical presentation or clinical findings. Wound botulism is associated with an infected wound. However, the wound botulism associated with injecting drug use (usually an abscess) may not be grossly infected (15% to 50% cases), the wound site (usually at injecting sites) may be at an unusual location (ie, base of the tongue), and fever is generally not present. The onset of symptoms in wound botulism associated with injecting drug use (mainly black tar heroin) has ranged from 2 to 14 days, and in wound botulism not associated with injecting drug use (ie, traumatic wounds) from 2 to 18 days (mean of 5 to 7 days). Early symptoms in infant botulism are constipation or change in stool pattern, irritability, poor feeding, lethargy, and cranial nerve findings. Cranial findings in infant botulism may initially be mild and overlooked, and generally manifest as a weak cry, poor suck or gag reflex, sluggish and incomplete pupillary response, ophthalmoplegia, and ptosis. Mortality from infant botulism with supportive intensive care (even before availability of antitoxin for infants) is generally less than 1%. In iatrogenic botulism associated with therapeutic BoNT products (but not reported with lower doses given for cosmetic purposes), onset of symptoms generally occurred within hours to 3 weeks after the last injection.
DIAGNOSIS

The differential diagnosis of botulism includes other diseases with paralysis symptoms:

- Diphtheria (may cause paralysis but also exhibits fever, typical pharyngeal or nasal mucosal lesions, cervical adenopathy, cardiac conduction abnormalities).
- Guillain-Barré syndrome (usually ascending paralysis, paresthesias common, elevated cerebrospinal fluid [CSF] protein [may be normal early in illness], electromyogram findings). The CSF findings are usually normal in botulism, but mild elevation of CSF protein between 50 and 60 mg/dL has been noted in a minority of botulism patients. Most patients (90%) with the Miller Fisher syndrome variant of Guillain-Barré syndrome also have serum antibodies to the gangliosides GQ1b and GT1a.
- Myasthenia gravis (dramatic improvement with edrophonium chloride, autoantibodies present, electromyogram findings). Botulism cases may have a positive response to edrophonium chloride (26%), but the response is generally not dramatic.
- Tick paralysis (ascending paralysis, paresthesias common, usually does not involve cranial nerves; detailed exam often shows presence of tick).
- Lambert-Eaton syndrome (commonly associated with carcinoma, particularly lung carcinomas; deep tendon reflexes absent; usually does not involve cranial nerves; electromyogram findings similar to botulism).
- Stroke or central nervous system mass lesion (paralysis usually asymmetric, brain imaging abnormal).
- Paralytic shellfish poisoning (history of shellfish ingestion; paresthesias of mouth, face, lips, and extremities common).
- Belladonna toxicity, such as atropine (history of exposure, tachycardia, and fever).
- Aminoglycoside toxicity (drug history of aminoglycoside therapy).
- Other neurotoxins, such as snake toxin (history of snake bite, presence of fang punctures).
- Chemical nerve agent poisoning (often associated with ataxia, slurred speech, areflexia, Cheyne-Stokes respiration, and convulsions).

A botulism diagnosis may be suggested by the clinical presentation of an afebrile patient with an acute, symmetric, descending, flaccid paralysis (without sensory deficits) with a normal sensorium. Any occurrence of botulism requires notification of public health officials and an epidemiological evaluation. Electrophysiological studies are helpful in distinguishing botulism from other causes of acute flaccid paralysis and support a presumptive diagnosis of botulism. An electromyogram with repetitive nerve stimulation at 20 Hz to 50 Hz showing facilitation (an incremental response to repetitive stimulation), usually occurring only at 50 Hz, may be helpful in distinguishing botulism from Guillain-Barré syndrome or myasthenia gravis, but not from Lambert-Eaton syndrome. Botulism patients with neuromuscular respiratory failure showed mostly incremental responses to high-rate repetitive nerve stimulation testing of the extensor muscles of the fifth digit in 17 patients, especially in response to >20 Hz stimulation. Electrophysiological testing in botulism may also demonstrate a small evoked muscle action potential response to a single supramaximal nerve stimulus, with normal sensory nerve function and nerve conduction velocity test results. However, electrophysiological tests may be normal in botulism. Approximately 15% of patients with botulism may have normal muscle action potential amplitudes, and as many as 38% of patients may not exhibit facilitation. CSF findings are usually normal in botulism, but mild elevation of CSF protein between 50 and 60 mg/dL has been noted in a minority of botulism patients. Most patients (90%) with the Miller Fisher syndrome variant of Guillain-Barré syndrome also have serum antibodies to the gangliosides GQ1b and GT1a.

Diagnostic Assays in Botulism

A confirmatory diagnosis of botulism can often be made by demonstrating the presence of toxin in patient specimens, such as the serum, stool, gastric aspirate, vomitus, or wound, using mouse bioassays. Mouse bioassays, which are highly sensitive to botulinum toxin (0.01 ng/mL detection limit), are performed by injecting mice intraperitoneally with the specimen sample suspected to contain toxin (with and without various antitoxins). If toxin is present in the specimen, mice injected with the clinical specimen alone (without the specific antitoxin) will usually die from botulism within 6 to 96 hours, but mice injected with the specimen treated with the specific antitoxin will survive. Specimens for mouse bioassays may be sent...
to the Centers for Disease Control and Prevention (CDC) or other designated state or municipal public health laboratories.120

Diagnosis can also be achieved by anaerobic culture and isolation of Clostridium species toxigenic strains from clinical specimens, including fecal specimens, gastric aspirates, vomitus, or infected wounds. The organism or toxin can also be isolated from the suspect food to help support the diagnosis.

In recent years the CDC has also used real-time polymerase chain reaction (PCR). Endopep-MS (mass spectrometry), and/or an enzyme-linked immunosorbent assay (A, B, E, F) to optimize its evaluation of botulism cases and outbreaks. However, real-time PCR detects DNA in fragments of C. botulinum (not active BoNT), and PCR results require confirmation with the mouse bioassay or other validated test.108 PCR has the advantage of providing results within 24 hours (including serotype of botulism) versus up to 4 days (range 6 to 96 hours) for results from mouse bioassays and 7 to 10 days for cultures. Endopep-MS has a high sensitivity and specificity for detecting botulinum toxin, but generally is used as a secondary assay (ie, if mouse bioassay is negative or cannot be done due to inadequate sample). Other tests in various stages of development for detecting botulinum toxin (ie, lateral flow, endopeptidases, enzyme-linked immunosorbent assays, and electrochemiluminescence assays) have been developed.108

Foodborne Botulism

Toxin assays of specimens from foodborne botulism cases from 1975 to 1988 showed the presence of toxin in specimens from various sites as follows: sera, 53% (126/240); stool, 23% (65/288); and gastric aspirate, 5% (3/63). Specimens were more likely to be positive if obtained soon after toxin ingestion. Toxin assays of sera were positive in more than 60% of specimens obtained within 2 days after toxin ingestion, in 44% of specimens obtained within 3 days of toxin ingestion, but in only 23% of specimens obtained at day 4 or later.98 Toxin assays of sera were more likely to be positive in intoxications from toxin serotype A than from toxin serotypes B and E. Toxin assays of the stool were positive in 50% of specimens obtained within 1 day following toxin ingestion, in 39% of specimens obtained within 3 days of ingestion, but in less than 20% of specimens obtained at day 5 or later.99 Stool and gastric aspirate cultures for C. botulinum resulted in a higher yield of diagnosis than toxin assays.99 Gastric aspirates were positive in 45% of specimens (35/78). Nearly 80% of stool cultures were positive at day 2 postingestion of toxin, with nearly 40% of specimens remaining positive at 7 to 9 days after ingestion. However, in this cohort of patients, laboratory confirmation of botulism could not be obtained in 32% of patients, which reflects the insensitivity of the diagnostic testing, especially when specimens are obtained more than 3 days after toxin ingestion. In these patients, the diagnosis must be based on clinical history, physical examination, electromyography results, epidemiological history (including food consumption), and tests on ingested food samples from epidemiologically linked food.

Inhalation-acquired Botulism

Laboratory confirmation of botulism acquired by inhalation may be difficult, because toxin acquired by inhalational exposure is not generally identifiable in the serum or stool, as in foodborne botulism.122,123 Although not validated, an enzyme-linked immunosorbent assay or PCR test has been reported to detect botulinum toxin (using nasal mucosal swabs) from the nares for up to 24 hours after exposure.122,123 Antibody titers also have limited use in the diagnosis of botulism, because individuals may not develop an antibody response to the small quantity of toxin protein required to cause symptoms. Additionally, cultures of C. botulinum are not helpful for definitive confirmation of inhalation of toxin preparations that do not contain spores of the organism.

Other Forms of Botulism

In wound botulism, serum toxin assays have been reported in one US cohort to be positive in 95% of cases associated with injecting drug use (mainly subcutaneous injection of black tar heroin) and in 83% of cases not associated with injecting drug use, but reported in other cohorts to be lower (range 38% to 68%).108 Toxin assays from the wound were reported positive in a third of cases, and C. botulinum was cultured from the wound in 65% of cases (61% of cases in injecting drug-use-associated and 83% of noninjecting drug-use-associated botulism).108 Laboratory confirmation of infant botulism is often by toxin assay and culture of stool (positive in nearly all cases); toxin in the serum is less sensitive (only 13% [9/67] positive in one US cohort).106,124 Laboratory confirmation of iatrogenic botulism associated with injection of therapeutic preparations of botulinum toxin is by toxin detection in serum.125

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TREATMENT

The current recommended treatment for botulism, although limited, includes antitoxin therapy and supportive care as needed, including mechanical ventilation. Because respiratory failure may begin suddenly, individuals with suspected botulism should be closely monitored, with frequent assessment of the vital capacity and maximal inspiratory force. If ingestion of the implicated food has been recent, removal of unabsober toxins may be hastened with cathartic agents or enemas, provided ileus is not present. Decrease in gastric motility may require parenteral nutritional support. In wound botulism, antibiotic therapy and surgical debridement are recommended to remove the source of toxin production. Wound manipulation preferably should be done after antitoxin therapy administration because it may result in release of toxin into the bloodstream. Aminoglycosides, clindamycin, and magnesium containing medications should be avoided, if possible, as they may potentiate neuromuscular blockade.

Antitoxin

Mortality from foodborne botulism before 1950 was approximately 60%, and has been reduced to less than 10% by the use of antitoxin therapy coupled with supportive care (often mechanical ventilation). As botulism antitoxins can only neutralize circulating antitoxin and have no effect on toxin already bound to nerve terminals (antitoxins do not reverse paralysis), antitoxin therapy should be administered as soon as possible. Early antitoxin administration has been associated with a decrease in the duration of illness, number of days of mechanical ventilation, requirement for mechanical ventilation, and duration of hospitalization. Early treatment, especially within 24 hours, is most effective in preventing paralysis progression. Therefore, it is recommended that antitoxin treatment commence with clinical suspicion, before the availability of definitive laboratory test results, and especially when a case is epidemiologically linked to a botulism outbreak. One retrospective analysis of 134 cases demonstrated a 10% mortality rate in patients who received antitoxin within 24 hours of symptom onset versus 15% among those who received late treatment.

Because antitoxin cannot neutralize toxin once it has bound to the nerve receptors, the antitoxin cannot reverse paralysis; it can only prevent paralysis progression. Symptoms may often progress for 12 to 24 hours after antitoxin administration before an effect is observed. Only one dose of antitoxin treatment is usually required for treatment of botulism. However, a second or subsequent doses may be required if symptoms should continue to progress or relapse, as may occur after exposure to extremely high toxin doses, incomplete wound debridement in wound botulism, or persistent colonization in adult intestinal botulism. In such cases, a serum toxin assay is recommended 24 hours after antitoxin administration. Antitoxin levels in four botulism patients given trivalent equine botulism antitoxin had previously demonstrated peak serum levels of antitoxin 10 to 1,000 times higher than the amount required to neutralize serum toxin levels.

In 2010, equine antitoxins available at CDC were replaced with an investigational new drug (IND) despectiated equine heptavalent (A-G) antitoxin product (Cangene Corporation, Winnipeg, Canada) then known as HBAT. It was subsequently FDA approved in 2013 and renamed as BAT (Emergent Biosolutions, Gaithersburg, MD). Despectiated equine antitoxins are made by cleaving the Fc fragments from the horse immunoglobulin G molecules, resulting in only F(ab’)_2 and Fab’ fragments that contain less than 2% horse protein. The decrease in the amount of horse protein may potentially reduce the risk of serum sickness and hypersensitivity reactions as observed with nondespectiated equine botulimum antitoxin products. The decrease in the amount of horse protein may potentially reduce the risk of serum sickness and hypersensitivity reactions as observed with nondespectiated equine botulimum antitoxin products (1% risk with a single vial and 9% risk with 2 to 4 vials). Symptoms in clinical trials in 16 healthy subjects were headache (9%), pruritus (5%), nausea (5%), and urticaria (5%). Safety data from 213/216 adult and 13/15 pediatric subjects who received BAT for suspected or confirmed botulism (5 subjects receiving 2 doses) noted 10% of subjects reported adverse reactions. Most common reactions reported were fever (4%), rash (2%), chills (1%), nausea (1%), and edema (1%). No subject experienced anaphylaxis, but one subject experienced mild serum sickness and one subject experienced hemodynamic instability characterized by bradycardia, tachycardia, and asystole during BAT administration. Rebound intoxication was observed in a case of adult intestinal botulism resulting from toxin serotype F and occurred 10 to 12 days after receiving the BAT. The rebound was attributed to the more rapid clearance of BAT from the circulation due to BAT’s shorter estimated serum half-life, and also due to intestinal colonization and toxin production from Clostridia.
For treatment of infants with botulism resulting from toxin serotypes A and B, an FDA-approved bivalent (A/B) human botulism immune globulin product (BabyBIG/BIG-IV), is available at the California Department of Health. BabyBIG was derived from pooled plasma of adults immunized with an investigational botulinum toxoid. Because it is derived from humans, BabyBIG does not have the high risk of anaphylaxis observed with equine products, nor the risk of lifelong hypersensitivity to equine antigens. A placebo-controlled trial with BabyBIG in treating infant botulism (associated with a mortality less than 2% even with supportive care without antitoxin) demonstrated efficacy by decreased duration of hospital stay, intensive care unit stay, mechanical ventilation, tube feedings, and a $55,000 cost savings per case. A single infusion of BabyBIG has been estimated to neutralize toxin for up to 5 months, based on its prolonged half-life of approximately 28 days. BabyBIG may interfere with the response to live viral vaccines if given shortly before or within 5 months after BabyBIG administration. When botulism in infants is not due to toxin serotypes A or B (or if BabyBIG is not available), HBAT may be considered, as it has been successfully administered to one infant in the United States. Also, a retrospective review of 31 cases of infant botulism in Argentina treated with equine botulinum antitoxin was well tolerated (no serious hypersensitivity reactions) and was associated with a reduction in hospital stay and tube feeding by 24 days and mechanical ventilation by 11 days and a 47% decrease in sepsis.

Animal studies with a heptavalent despeciated antitoxin (IND product developed at the US Army Medical Research Institute of Infectious Diseases that was a basis for BAT development) demonstrated efficacy in preventing and treating botulism in both mice and nonhuman primates against aerosol toxin challenge. The F(ab')₂ heptavalent, despeciated equine antitoxin toxin (known as Hfab-BAT) was well tolerated (no serious hypersensitivity reactions) and was associated with a reduction in hospital stay and tube feeding by 24 days and mechanical ventilation by 11 days and a 47% decrease in sepsis. The Hfab-BAT and the HE-BAT despeciated equine antitoxin (also developed at the US Army Medical Research Institute of Infectious Diseases) are no longer available as IND products, and they have been replaced with the FDA-approved BAT despeciated antitoxin product.

In a successful Phase I clinical trial, a product made from three IgG1 monoclonal antibodies (Xoma 3AB) that target different regions of botulinum neurotoxin A and were engineered to neutralize toxin serotype subclasses A1, A2, A3, and A4 was well tolerated when given as a single intravenous injection infused over 1 hour. The product has demonstrated a reduced mortality in mice when given prior to or up to 23 hours after toxin exposure. Although all three monoclonal antibodies were detected for a minimum of 4 weeks after infusion, the protective level of the monoclonal antibodies in humans is currently unknown. Further testing of this product may provide an antitoxin therapy for botulism that offers potential advantages of (1) a longer serum half-life, (2) decreased risk of allergic reactions (contains no residual equine proteins), and (3) mass production potential.

Clinically Relevant Signs of Bioterrorist Attack

The first evidence of a bioterrorist attack with botulinum toxin would likely be reports from hospitals and urgent care medical facilities as they begin to receive victims with symptoms suggestive of botulism. Because antitoxin therapy given early has a greater beneficial effect, the initial diagnosis of botulism is based on clinical presentation with epidemiological associations, with subsequent confirmation by laboratory findings. Neurological signs and symptoms resulting from a toxin-induced blockade of neurotransmission at voluntary motor and cholinergic junctions dominate the clinical manifestation of botulism. A diagnosis of botulism is suggested in individuals presenting with an acute onset of cranial nerve weakness (ie, dip-
lophia, ptosis, dysphonia, dysphagia, and dysarthria). In mild cases, no further symptoms may develop. In more severe cases, individuals may progress and develop descending symmetrical weakness and flaccid paralysis. Because mechanical ventilation may be required for individuals with respiratory failure resulting from paralysis of the respiratory muscles, hospital bioterrorism plans should include contingency plans for additional ventilatory and intensive care unit support for mass intoxication. Antitoxin therapy is indicated in cases of suspected botulism to inactivate and clear toxin from the circulatory system before it can enter peripheral cholinergic nerve cells.

An outbreak of botulism in 2004 illustrates the vulnerability of readily accessible bulk botulinum toxin. Four cases of botulism resulted from the use of toxin serotype A for cosmetic purposes. A vial of raw bulk botulinum toxin (a non-FDA approved formulation) containing between 20,000 and 10 million units of botulinum toxin (a vial of FDA-approved BOTOX [Allergan, Inc, Irvine, CA] contains only 100 units of toxin) was used by an unlicensed physician for cosmetic injections into three patients and himself.148,149 The four individuals were subsequently admitted to medical facilities with symptoms of botulism and faced a long-term recovery.148 One of these patients was iatrogenically injected with as much as 8 million mLD50 (2,857 times the human lethal dose by injection) and has survived with intensive medical care, including long-term mechanical ventilation, although with chronic sequelae.150,152 This patient began improving 4 weeks after the event, and achieved a remarkable recovery 14 weeks from injection.157

Preexposure and Postexposure Prophylaxis

Although passive antitoxin prophylaxis has been effective in protecting laboratory animals from toxin exposure, the limited availability and short-lived protection of antitoxin preparations make preexposure or postexposure prophylaxis with these agents impractical for large numbers of persons.121,137 Administration of equine antitoxin is not recommended for preexposure prophylaxis because of the risk of anaphylaxis from the foreign equine proteins, particularly with repeated doses. These products are not generally recommended for use in asymptomatic persons. In asymptomatic persons with known exposure to botulinum toxin, the risk of anaphylaxis from the equine antitoxin must be weighed against the risk of disease from botulinum toxin. However, botulinum immune globulin is most effective when administered within 24 hours of a high dose aerosol exposure to botulinum toxin.

No FDA-approved vaccines exist to prevent botulism. Of historical note, a bivalent botulinum toxoid (serotypes A and B) had been given to at-risk laboratory workers in the US offensive biological warfare program at Fort Detrick beginning in 1945.152 Between 1945 and 1969, 50 accidental exposures to botulinum toxins (24 percutaneous, 22 aerosol, and 4 ingestion) were reported, but no cases of laboratory-acquired botulism occurred, possibly because of the toxoid immunizations. An IND product, the pentavalent botulinum toxoid (PBT) against botulinum toxin serotypes A through E, had been used since 1959 for persons at risk for botulism (ie, laboratory workers)152–154 but is no longer available as an investigational product on protocol through the CDC. In 2012, due to declining immunogenicity and increased local reactogenicity observed with the requirement for annual booster doses to maintain immunity,155,157 the CDC discontinued its sponsorship of IND that provided PBT to at-risk laboratory workers.158–160 The declining immunogenicity of the PBT was not unexpected, given that the PBT was manufactured more than 30 years earlier. PBT is a toxoid (toxin that has been inactivated) derived from formalin-inactivated, partially purified toxin serotypes A, B, C, D, and E, which was developed by the Department of Defense at Fort Detrick and originally manufactured by Parke-Davis and Company (Detroit, MI). PBT was found to be protective in animal models against challenge with botulinum toxin serotypes A through E,162 including protection in nonhuman primates against aerosol challenge to toxin serotype A.162

PBT was originally given as a primary series of three subcutaneous injections (0.5 mL at 0, 2, and 12 weeks), a booster dose at 12 months, and annual booster doses thereafter.163 PBT was administered to thousands of at-risk persons, and clinical experience has shown the toxoid to be safe and immunogenic. The vaccine has mainly been used for laboratory workers who work directly with botulinum toxin. Approximately 8,000 service members also received the toxoid between January 23 and February 28, 1991, as part of the US force deployed to the Persian Gulf War.164 The main adverse event was local reactions. Adverse events passively reported to the CDC between 1970 and 2002 for more than 20,000 vaccinations included moderate local reactions (edema or induration between 30 mm to 120 mm) in 7% of vaccinees, and severe local reactions (reaction size >120 mm, marked limitation of arm movement, or marked axillary node tenderness) in less than 1%.158 To allow recent vaccinees to complete the primary series of PBT, the investigational new drug protocol remained in effect through May 31, 2012.158
**New Vaccine Research**

Future vaccine candidates for FDA approval will probably not include formalin-inactivated toxoids similar to the formalin-inactivated PBT for several reasons. Production of formalin-inactivated toxoids requires partially purified culture supernatants containing botulinum toxin to be treated with formaldehyde, which must be performed by highly trained staff using a dedicated high-containment laboratory space. Furthermore, the relative impurity of the toxoid (PBT contained only 10% neurotoxoid and 90% is irrelevant material) likely contributed significantly to the need for multiple injections to achieve and sustain protective titers, as well as increased local reactogenicity associated with multiple injections.

The use of pure and concentrated antigen in recombinant vaccines offers the advantages of increased immunogenicity and a decrease in reactogenicity (local reactions at the injection site) over formalin-inactivated toxoids. Recombinant techniques use an immunogenic toxin fragment, which does not have the capability of blocking cholinergic neurotransmitters. Both Escherichia coli and yeast expression systems have been used in recombinant fragment production, primarily the carboxy-terminal fragment (Hc) of the toxin’s heavy chain. Vaccine candidates using recombinant fragments of botulinum toxins against serotypes A, B, C, E, and F were protective in mice. A vaccine recombinant candidate for serotype A was protective in mice against intraperitoneal challenge and produced immunity levels similar to that attained with PBT, but with an increase in safety and decrease in cost per dose. Recombinant vaccines given by inhalational route are also being investigated.

The only botulinum vaccine candidate currently in advanced development is a bivalent recombinant botulinum vaccine (rBV A/B [P. pastoris]) for toxin serotype A and B) developed by the US Army Medical Research Institute of Infectious Diseases for protection against botulinum toxin serotype A (subtype 1) and botulinum toxin serotype B (subtype B1). Animal studies have demonstrated protection against toxin challenge by aerosol and intramuscular challenge with two toxin serotypes. The safety data and immunological responses from Phase 1 and Phase 2 clinical trials and animal data support the continued investigation of the rBV A/B for FDA vaccine licensure.

Another vaccine candidate evaluated involved the insertion of a synthetic carboxy-terminal fragment (Hc) gene of the heavy chain of toxin serotype A into the vector system of the Venezuelan equine encephalitis virus. This vaccine induced a strong antibody response in the mouse model and remained protective in mice against intraperitoneal challenge at 12 months. However, the presence of BoNT variants as subtypes or chimeras challenges the development of any comprehensive “pan-botulinum neurotoxin” vaccines and therapies. BoNT subtypes are natural variants of the prototype BoNT serotype that vary in primary amino acid sequence from 3% to 26% depending on the serotype, whereas BoNT chimeras are natural variants that appear to have derived from recombinant events between two BoNT serotypes. BoNT subtypes have unique activities relative to the prototypical BoNT serotype. One novel approach to vaccine design uses structure-based knowledge to produce a single molecule containing the immunodominant epitopes of multiple, antigenic distinct variants to develop a meningococcal pan-vaccine. A similar research effort may eventually allow the development of a similar pan-vaccine strategy for all seven variants of botulinum neurotoxins.

**New Therapeutic Drug Research**

In addition to the vaccine research for prophylaxis, efforts are underway to find postexposure, pharmacologic treatments. The development of low molecular weight inhibitors is related to work that was designed to pharmacologically “deduce commonalities” among the serotypes. Different molecular targets for inhibitor candidates include those that prevent toxin binding to nerve terminals and internalization or those that inhibit the neurotoxin’s proteolytic activity. One limitation for this approach to be useful is that when the neurotoxin molecule is internalized within the presynaptic endings, the neurotoxin is—as with neutralizing antibodies—no longer susceptible to circulating inhibitors. Thus, effective therapeutic inhibitors must also be internally delivered to nerve termini without creating additional adverse central effects. Another problem concerns the diversity of the substrate binding sites (exosites) and active site structures among the serotypes. This diversity implies that a single inhibitor will not antagonize the substrate binding and subsequent proteolytic activity of all the neurotoxin serotypes.

Active site inhibitor candidates have been extensively reviewed with new candidates appearing frequently in the literature. Within the BotDB resource (http://botdb.abcc.ncifcrf.gov), the BotDBI section has information on more than 60 inhibitor candidates that lists peptides, synthetic and natural compounds, and antibodies. A steady rise over the past 30 years has occurred in the clinical/therapeutic uses of this neurotoxin for vari-
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ous disorders. The FDA-approved uses include treatment of blepharospasm, strabismus, cervical dystonia, upper limb spasticity, axillary hyperhidrosis, detrusor overactivity associated with a neurologic condition, and chronic migraine. Other potential uses are still under investigation including those for wound healing, treatment of cerebral palsy, treatment of lower urinary tract disorders, controlling multiple sclerosis spasticity, and various modalities of pain management. Because of these clinical conditions, very small doses of the neurotoxin may be used for a measurable beneficial effect. One novel clinical application involves the neurotoxin-induced reduction of hyperhidrosis.

SUMMARY

The neurotoxins produced by Clostridia species are among the most potent toxins known. Botulinum toxin has been studied and developed as a biological weapon by many countries, and it should be considered as a bioterrorism threat agent. A mass casualty event caused by botulinum toxin, which has been depicted by a mathematical model, has the potential to cause great harm. Botulism is most commonly caused by neurotoxin types A, B, and E, is a neuroparalytic disease, and often fatal if untreated. Paralysis from botulism can be long-lasting, with concomitant and demanding supportive care requirements. Clinicians should be able to recognize the early signs and symptoms of botulinum intoxication, as early initiation of antitoxin therapy has been associated with decreased duration of mechanical ventilator support and days spent in intensive care, and increased survival. Antitoxin therapy should be initiated based on clinical presentation consistent with botulism and epidemiological history, as results of laboratory confirmatory assays may not be available for days.

For infants with botulism resulting from toxin serotypes A and B, an FDA-approved human antitoxin known as BabyBIG is available at the California Department of Public Health. An FDA-approved despecified equine heptavalent antitoxin product known as BAT (serotypes A-G) is the only antitoxin for adults (and for infants if BabyBIG is not available or botulism not due to serotypes A or B), and is available through the CDC. No FDA-approved vaccine for botulism currently exists. The IND PBT product is no longer available for protection of at-risk laboratory workers because of declining immunogenicity and increased local reactogenicity associated with required annual booster doses. Future vaccine research may lead to a new class of recombinant vaccines to protect against botulism while pharmacologic approaches may lead to viable postexposure drug treatments.

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