

Chapter 33

BOTULINUM TOXINS

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INTRODUCTION

HISTORY AND MILITARY SIGNIFICANCE

DESCRIPTION OF THE AGENT

Serology
Genetics

PATHOGENESIS

Relation to Other Bacterial Toxins
Stages of Toxicity

CLINICAL DISEASE

DIAGNOSIS

MEDICAL MANAGEMENT

SUMMARY

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INTRODUCTION

The clostridial neurotoxins are the most toxic substances known to science. The neurotoxin produced from *Clostridium tetani* (tetanus toxin) is encountered by humans as a result of wounds and remains a serious public health problem in developing countries around the world. However, nearly everyone reared in the western world is protected from tetanus toxin as a result of the ordinary course of childhood immunizations. Humans are usually exposed to the neurotoxins produced by *Clostridium botulinum* (ie, the botulinum toxins, of which there are seven in all) by means of food poisoning, although there are rare incidents of wound botulism and a colonizing infection of neonates known as infant botulism.¹ Since the incidence of botulinum poisoning by all routes is very rare,

immunization of the general population is not warranted on the basis of cost and the expected rates of adverse reactions to even the best vaccines.

Thus, humans are not protected from botulinum toxins and, because of their relative ease of production and other characteristics, these toxins are likely biological warfare agents. Indeed, the United States itself explored the possibility of weaponizing botulinum toxin after World War II, as is discussed elsewhere in this textbook. Although the United States disavowed any further research on developing the botulinum toxins as biological warfare agents, great concern remains that other nations might employ them, and ongoing research seeks ways to protect our armed forces from their use.

HISTORY AND MILITARY SIGNIFICANCE

Because of the extreme toxicity of botulinum toxin, it was one of the first agents to be considered as a biological weapons agent. Before offensive research on biological warfare was renounced, researchers in the United States worked on the weaponization of botulinum toxin for over two decades. Efforts began early during World War II (Exhibit 33-1). Intelligence information indicated that Germany was attempting to develop botulinum toxin as a cross-channel weapon to be used against invasion forces.² At the time the Allied work began, the composition of the toxic agent produced by *C botulinum* was not clear, nor was the mechanism of lethality in animals and man. Therefore, the earliest goals of research on botulinum toxin were to isolate and purify the toxin and to determine its pathogenesis. Botulinum toxin was referred to as agent X.

Strains that produced each of the five serotypes known at the time were obtained and those expressing the most toxin were selected for use in further study, although most of the research involved serotype A.² Culture conditions required to produce maximal levels of toxin were established, and techniques appropriate for purification and concentration were perfected. An important advance was “crystallization” of the toxin. The preferred method of toxin purification involved an initial acid precipitation from the culture supernatants, followed by redissolving the toxin in an aqueous buffer. At that point, the addition of ammonium sulfate produced toxin in a form called “crystalline.” To a protein chemist of today, this term means a highly purified protein that may be suitable for three-dimensional structure determination. The

scientists of that time thought that their crystalline preparations were pure, but we now know that these preparations were far from pure—although the procedure did put the toxin in a physical state of high stability. The crystalline toxin they produced was authentic neurotoxin with an accompanying protein or proteins (hemagglutinin, in most cases) that stabilizes the toxin from thermal and proteolytic degradation. Further technical advances in analytical protein chemistry were required before the true physical state of the toxin became evident. However, a good deal of work was carried out with this form of the toxin, and nearly all the insights and conclusions derived from the work remain valid.

One of the more lasting legacies of the early botulinum toxin biowarfare research was the development of the botulinum vaccine that is used even today. It was clear that the scientists working with large quantities of the toxin needed to be protected from possible laboratory exposures and that a vaccine would serve them, as well as the armed forces at risk of biological warfare attack. A formalin-inactivated *toxoid* (ie, a toxin that has been treated so as to destroy its toxicity but retain its antigenicity) proved effective in animal studies, and large quantities were prepared for human use.³ A large store of vaccine was shipped to England for possible use by the expeditionary forces, but for reasons that are not elaborated in the official history,² the decision was made not to vaccinate the troops. Many humans have since been vaccinated with this and similarly prepared botulinum toxin vaccines, and clinical experience has indicated that they are safe and effective.

EXHIBIT 33-1

A FOOTNOTE TO HISTORY: WAS BOTULINUM TOXIN USED IN THE ASSASSINATION OF REINHARD HEYDRICH?

Reinhard Heydrich, head of the Gestapo and Security Service in Germany during World War II, was arguably second only to Hitler as the chief perpetrator of the Holocaust. He was assassinated in Prague in the spring of 1942 by Czech patriots who were trained and equipped by the British. The fatal injury resulted from the detonation of a bomb, which drove fragments through a seat in Heydrich's car and into his left flank, injuring the lung, diaphragm, and spleen.

The surgical care he received was surprisingly good, even when judged by today's standards. Heydrich's initial postoperative course was satisfactory, although he was modestly febrile and there was drainage from the wound of entrance. His condition worsened suddenly on the seventh postoperative day and he died early the next day.

An autopsy showed no apparent gross or microscopic cause of death; specifically, there was no missed injury, evidence of peritonitis, abscess, wound tract infection, or retained foreign bodies; and the heart and lungs appeared normal.¹ The senior German pathologists in attendance wrote that "...death occurred as a consequence of ... bacteria and possibly by poisons carried ... by the bomb splinters...."^{2(p17)} Although when we use modern terminology their assessment can be interpreted to mean that death was due to septic shock or multiple organ failure, looking at the incident from the vantage point of 50 years also allows for a more diabolical interpretation.

The extraordinary efforts made by the British and Americans to develop biological weapons in World War II are not generally known. For instance, by 1944, it would have been possible for the Allies to drop tens of thousands of bombs containing *Bacillus anthracis* (ie, anthrax) spores on major German cities.³ Other potential biological warfare agents were also being investigated, among them the neurotoxins of *Clostridium botulinum*. It is here that Heydrich's death becomes relevant. Although we have no official written documentation, the chief scientist in charge of the British biological warfare program, Paul Fildes, is recorded as having made remarks to colleagues that can only be interpreted to mean that he and, by implication, a biological warfare agent, played a role in Heydrich's death: "[I] had a hand [in Heydrich's death]"^{3(p94)} and "[Heydrich] was the first notch on my pistol."^{3(p94)}

There is reason to believe that Fildes's research group was actively developing botulinum toxin as a weapon. That the British were very knowledgeable about the potential use of botulinum toxin in war is apparent from their request to the Canadian government for several hundred thousand doses of toxoid as a defense against possible German use. Although not carrying the weight of written documentation, Fildes's recorded statements, together with the known British interest in botulinum toxin, have led two British historians to propose that the bomb used to assassinate Heydrich contained botulinum toxin in addition to the usual explosive charge.³

How likely is it that botulinum toxin played a role in Heydrich's death? Certain observations are possible:

- The bomb used in the assassination was not of standard issue but instead was of distinctly unusual design: the upper third of a British hand grenade had been cut off and the open end and sides wrapped with tape.² This strange modification becomes understandable if a way was needed to contaminate its contents with a foreign substance.
- Heydrich's clinical course does not explain his death. Although infection was likely to accompany his injury, his sudden deterioration and death do not conform to the usual expression of fatal sepsis. It is noteworthy that infection was not a prominent finding at autopsy. Heydrich's death is actually much more suggestive of a massive pulmonary embolism, yet his heart and lungs were said to be normal.¹
- Heydrich's death is not especially suggestive of botulism. The clinical course of wound botulism (albeit with a more-rapid onset) probably comes close to what should have happened if Heydrich's wound was actually contaminated with botulinum toxin. However, the apparent absence of such expected signs and symptoms as ptosis, diplopia, dysphonia, dysarthria, dysphagia, facial paralysis, and generalized muscular weakness culminating in respiratory insufficiency developing over several days speak against botulism.

The answer will probably never be known, although the British archives for this period, which are scheduled to be opened early in the 21st century, may contain relevant information.

(1) Davis RA. The assassination of Reinhard Heydrich. *Surg Gynecol Obstet*. 1971;August:304-318. (2) Ramsey WG, ed. The assassination of Reinhard Heydrich. *After the Battle*. 1979;24:cover 2-37. (3) Harris R, Paxman J. *A Higher Form of Killing*. New York, NY: Hill and Wang; 1982.

DESCRIPTION OF THE AGENT

C botulinum and *C tetani* are spore-forming, anaerobic bacteria found worldwide in soil. As mentioned previously, however, the organisms produce their toxicoses in very different manners. Victims of tetanus present clinical symptoms of a rigid tetanic paralysis, whereas the victims of botulinum poisoning present with a radically different symptom: peripheral, flaccid paralysis. Poisoning by tetanus toxin is a result of wound contamination and is an infectious disease like cholera or diphtheria, in which invading organisms multiply within the body and produce their toxin. The disease is very old, with descriptions and drawings of victims going back to the Middle Ages.

In contrast, poisoning by botulinum toxin (ie, the disease we call *botulism*) seems to have been more rare, especially during ancient times. Although there may have been ancient cases of wound botulism, there is little or no evidence for such infections until much more recent times. Food poisoning due to botulinum toxin emerged as a problem when food preservation became a widespread practice. Since the outbreaks were so dramatic, they soon received the attention of scientists and the etiology of the poisoning was elucidated. It is now clear that *C botulinum* grows and produces neurotoxin in the anaerobic conditions frequently encountered in the canning or preservation of foods. The spores are very hardy, and special efforts in sterilization are required to ensure that the organisms are inactivated and unable to grow and synthesize their toxin. Modern commercial procedures have virtually eliminated the problem of food poisoning by botulinum toxin, and most of the cases now seen are associated with home-canned foods or meals produced by restaurants.

One other mode of botulinum toxin poisoning has a significant number of cases in the United States: infant botulism.¹ These cases involve an ongoing colonization of the intestines of infants, usually in the first year of life, by the usually benign *C botulinum* organism. Apparently, the flora of newborns, their intestinal environment, or both is such that the organism can grow and produce toxin; there are no well-documented cases of intestinal infections in adult humans.

Serology

The initial identification of botulinum toxin as the etiologic agent in poisoning came after isola-

tion of organisms from the victims, followed by growth in the laboratory and demonstration of toxigenicity by injection of animals with culture filtrates. After inactivation, the culture filtrates were used to raise (ie, produce) toxin-neutralizing antiserum. This antiserum was used to confirm poisoning by *C botulinum* until victims with similar symptoms appeared, but the antiserum did not neutralize the toxin in animal experiments. However, when the entire process of antiserum production was repeated with the new isolates, neutralization was observed. It soon became clear that medicine was dealing with a family of toxins that produced related poisoning sequelae, but that differed immunologically. Thus, seven distinct serotypes of botulinum toxin have now been isolated, designated A through G. Interestingly, not all serotypes have been associated with poisoning of humans. Serotypes A, B, E, and F have been clearly identified in numerous human poisoning episodes. Serotype G is the most recently isolated toxin and has only been identified in a few outbreaks. For serotypes C and D, respectively, only a single anecdotal human case of intoxication has been reported. These serotypes have been found in outbreaks involving various animals including chickens and mink in domestic settings and ducks in wild environments. Why it is that humans are typically not poisoned by serotypes C and D is not clear.

Because the clostridial toxins are so potent, they have been the subjects of many studies by laboratories throughout the world. In nearly every case, multiple strains have been isolated that produce the same serotype of botulinum toxin. (Strangely, however, *C tetanus* strains all produced the same serotype of tetanus toxin.) Many of the strains are available from microbiological repositories such as the American Type Culture Collection. However, due to the ubiquitous nature of the organisms, we could simply isolate anaerobic organisms from the soil in nearly any country and expect to obtain one or more serotypes of toxin-producing *C botulinum*. In fact, there is recent evidence that other clostridia can carry and express the genes for the botulinum neurotoxins.⁴ In addition, with the emergence of molecular genetics as a readily available technology, nearly any laboratory with such expertise could move the gene for botulinum toxin into other organisms. Although such research is forbidden by law in most western nations, including the United States, there is no international legal prohibition of such work.

Genetics

Our understanding of many important details of the action of botulinum and tetanus toxins has been slow in progressing. However, enormous strides have been made during the last 5 years, and science is now closing in on a detailed description of how these toxins work. The most important breakthrough involved cloning and sequencing of the genes for tetanus and all seven serotypes of botulinum neurotoxins.⁵⁻¹⁴ With that information, the amino acid sequences could be deduced, and this led to other important insights into the molecular mechanisms of action.

The family of structural genes for the clostridial neurotoxins is unrestricted in its location, being both chromosomal and extrachromosomal. The structural gene for tetanus toxin is on a plasmid, as is probably the structural gene for botulinum toxin serotype G.¹⁵ The structural genes for botulinum toxin serotypes C and D are found on bacteriophages.^{16,17} The remainder are believed to be chromosomal in location, but this is not definitely proven.

The isolation of clostridia not classified as *C botulinum*, yet expressing botulinum toxin and involved in human disease,^{18,19} raises several interesting questions. Does the gene for botulinum neurotoxin move from species to species in the clostridia family? Have the toxin genes always been resident in clostridia other than *C botulinum* and either not expressed or expressed at low levels, therefore remaining unrecognized? Why is the gene for tetanus toxin not found (thus far) in other clostridial species? Why are there multiple serotypes of botulinum, but not of tetanus toxin? Have we found all existing serotypes of botulinum toxin, or are there additional serotypes lurking in the environment waiting to be discovered? And probably the most intriguing question of all: What is the real function of these neurotoxins? Surely not to poison humans. Humans are not predators of clostridia and can hardly be viewed as prey, either. The neurotoxins probably serve some important function in the natural environment or life cycle of clostridia, and humans just happen to get in the way.

PATHOGENESIS

As mentioned earlier, botulinum toxins are the most poisonous substances known. The dose that is lethal to 50% of the population exposed (ie, the LD₅₀) has been estimated²⁰ to be approximately 1 ng/kg. This is similar to LD₅₀s reported for most laboratory animal species when the toxin is administered intravenously, subcutaneously, or intraperitoneally. All of the botulinum toxins are slightly less toxic when exposure is by the pulmonary route: a recent estimate for the human LD₅₀ by inhalation is 3 ng/kg.²¹

The extreme toxicity of the botulinum toxins would lead us to believe that they must have some highly potent and efficient mechanism of action. This probability made botulinum toxin the subject of work by many laboratories, especially after we learned that it is a neurotoxin. Experiments with *in vitro* neuromuscular models established that the toxin acts presynaptically to prevent the release of acetylcholine. In many of those same models, very high doses of botulinum toxin will block the release of neurotransmitters other than acetylcholine, but there seems to be a marked toxin specificity involving acetylcholine.²²

Relation to Other Bacterial Toxins

With the development of such techniques as sodium dodecyl sulfate (SDS) polyacrylamide gel

electrophoresis, it became evident that the crystalline toxin was really an aggregate of proteins, and that the molecular species responsible for poisoning was a single protein of about MW 150,000. However, because the molecular events underlying neurotransmitter release were poorly understood, little progress was made in understanding the details involved in botulinum toxin's pathogenesis during the 1970s and 1980s. As is true for science in general, it was research in related areas that provided the framework for the next round of advances with botulinum toxin. Studies with another microbial toxin, that produced by *Corynebacterium diphtheriae*, provided several important insights that some scientists believed could be applied to botulinum toxin. Most importantly, the realization that diphtheria toxin is an enzyme permitted researchers to understand how certain toxins could be so much more potent than others. Curare, for example, binds to the acetylcholine receptor and acts in a stoichiometric relationship to exert toxicity. Diphtheria toxin, being an enzyme, can act many times over on (or, more properly, *in*) a cell and therefore exert a much more powerful effect (for a given number of molecules) than a stoichiometrically acting toxin. Because botulinum toxin is much more potent than even diphtheria toxin, many laboratories embarked on a search for the (putative) enzymatic activity expressed by botulinum toxin.

Stages of Toxicity

The second insight provided by the work on diphtheria toxin was the recognition that microbial toxins have structural domains (or subunits) that serve common general functions related to a three-stage mechanism of action: binding, internalization, and enzymatic activity.²³ Figure 33-1 depicts these stages.

All three functions are normally required for expression of toxicity in cells or animals, but under certain experimental circumstances these functions can be blocked or overcome. Thus, if the enzyme domain of a toxin is removed or specifically inactivated, the toxin is rendered inactive and could be used as a vaccine. It could bind to a target cell and enter the interior, but without its functional component, no toxicity would result. A derivative of diphtheria toxin called CRM-197 is a perfect example of this type of alteration. On the other hand, the active enzyme domain by itself is virtually non-toxic if it is added to cells or given to animals. The enzyme domain alone is unable to recognize and bind specifically to target cells. If, however, the enzyme domain is linked to other cell-binding ligands, the toxicity can be redirected to new target cells. This type of construction is the basis for many attempts to treat cancer or other diseases (eg, the fusion of diphtheria toxin fragment A, its enzymatic component, with tumor-directed antibodies).

Binding

What about (a) the receptor for botulinum toxin, (b) the means by which the toxin enters neurons, and (c) the toxin's enzymatic activity? First, the receptor. For many years, the observation that complex sphingolipids prevented toxicity was taken to indicate that gangliosides were receptors for the toxins. However, there is as much evidence suggesting that gangliosides *are not* the receptor (or receptors) as there is that they *are*.²⁴ An important study²⁵ published in 1986 presented good evidence that the different serotypes of botulinum toxin do not share the same receptor. It is not yet clear if there are distinct receptors for each serotype.

As this is written (1996), it is fair to say that science does not know the structure of the receptors for either botulinum or tetanus toxins. A report published in 1994²⁶ suggesting that a synaptic vesicle-associated protein called synaptotagmin may be the receptor for botulinum toxin serotype B. This intriguing work has yet to be confirmed, and

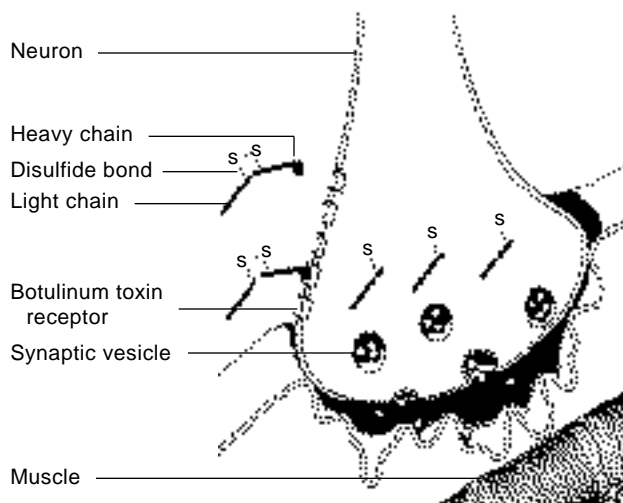


Fig. 33-1. The three stages in the mechanism of action of dichain microbial toxins (ie, two protein chains connected by a disulfide bond). In the first stage, a domain (the sphere at the end of the heavy chain) at the carboxyl end of the holotoxin recognizes and binds to a receptor or acceptor on the surface of the target neuron. The internalization stage follows, which results in delivery of the light chain of the toxin into the cytoplasm. The internalization stage is directed by a domain adjacent to the receptor-recognition domain on the heavy chain. Whether all or part of the heavy chain also enters the cytoplasm remains unclear, but the light chain certainly gains entry into that compartment. Finally, once in the cytoplasm, the light chain acts like an enzyme and catalyzes specific reactions described in the text, preventing both (a) the vesicle from fusing to the membrane and (b) neurotransmitter release.

as it stands, it lacks one key piece of information. The researchers elegantly demonstrate that serotype B binds to synaptotagmin, but the crucial evidence showing that synaptotagmin binding results in internalization and toxicity is not yet available.

Internalization

A substantial body of evidence indicates that botulinum toxin enters neurons by the general pathway used by several other bacterial toxins, a number of polypeptide hormones or growth factors, and even some viruses.²⁷ This pathway has come to be known as receptor-mediated endocytosis (RME).

Briefly, ligands are concentrated on the cell surface by virtue of binding to receptors that are localized in specialized regions called coated pits. The pits invaginate, becoming vesicles, and are transported to one or more sites in the cell interior, car-

rying along the contents. At various stages of vesicle trafficking, some of the contents escape or are released into the cytoplasm, where, in the case of bacterial toxins, they act on the intracellular substrates. This action leads to toxicity.

One general feature of the process is a gradual decrease in the intravesicular pH as a result of a protein pump in the membrane; this may drop as low as pH 4.5. Certain drugs or compounds called lysosomotropic amines prevent this pH drop and, in nearly all cases, inhibit the release of ligands. Because of this known effect, inhibition of a specific biological process by lysosomotropic amines is widely accepted as a hallmark of the presence of an RME process. Indeed, this is the case for botulinum toxin. The presence of lysosomotropic drugs will inhibit, or at least delay, the onset of botulinum toxin paralysis²³; this fact has led most scientists in the field to believe that the toxin enters the neurons by RME.

Enzymatic Activity

Finally, the enzymatic activity of botulinum toxin. A critical key to the identification of this enzymatic activity was provided by the cloning and sequencing of tetanus toxin and all seven serotypes

of botulinum toxin—work that was performed by several laboratories. Initially, a scientist not in the neurotoxin field noted²⁸ that tetanus toxin has an amino acid sequence in its light chain that is similar to that seen in zinc-dependent proteases. When the amino acid sequences of the botulinum toxins became available, they, too, were seen to have this sequence in the light chain. This remarkable similarity led several laboratories to seek to determine if botulinum and tetanus toxins might exhibit a zinc-dependent proteolytic activity.²⁹

Demonstration of this activity was initially difficult because the toxins are very specific for their substrates. However, it is now clear that (a) the clostridial neurotoxins express proteolytic activity and (b) this activity is absolutely required for toxicity.³⁰ The substrate proteins for this action appear to be part of a hetero-oligomeric assembly associated with the synaptic vesicles. Interestingly, the specific target site for cleavage seems to be different for each serotype of the botulinum toxins: in some cases, different locations on the same protein; in others, different proteins of the assembly.³⁰ The basis of this marked specificity is not yet clear and remains fascinating to scientists interested in neurosecretion.

CLINICAL DISEASE

Botulism is a feared and dramatic disease and is frequently fatal for animals and humans alike. In food poisoning, the symptoms appear several hours to 1 or 2 days after contaminated food is consumed. The earliest symptoms are difficult to associate with poisoning and, depending on their severity, might result in a number of clinical effects: blurred vision, ptosis, dysphagia, dysarthria, and apparent muscle weakness. As the neuromuscular symptoms progress and respiratory distress begins, healthcare providers usually consider botulism. A confirmatory diagnosis comes from mouse bioassays demonstrating toxin in blood or stool, neutralized by the appropriate antisera. Many times, the organism can be isolated from the offending food, and toxin and neutralizing tests can then be run again using food samples. Established effective treatments are few or none, save artificial ventilation and other forms of life support.

Inhalational botulism, the syndrome most likely to be seen on the battlefield, is rare. One incident involving accidental exposure of humans to botulinum toxin occurred in a laboratory in Germany and was reported in 1962.³¹ After conducting a post-

mortem examination of laboratory animals that had been exposed, whole-body, to botulinum toxin type A, three laboratory workers experienced symptoms of botulinum intoxication. Three days after exposure, they described having (a) a “mucous plug in the throat,” (b) difficulty in swallowing solid food, and (c) “the beginning of a cold without fever” and were hospitalized. On the fourth day, their signs were more severe. The patients complained of “mental numbness” and retarded ocular motions; their pupils were moderately dilated with slight rotary nystagmus. Speech became indistinct and gait uncertain as patients complained of extreme weakness. The patients were given antitoxin serum on the fourth and fifth days. Between the sixth and tenth 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 only a mild general weakness remaining. The signs and symptoms of inhalational botulinum intoxication, listed in order of onset, are found in Exhibit 33-2.

EXHIBIT 33-2

SIGNS AND SYMPTOMS OF INHALATIONAL BOTULISM, IN ORDER OF ONSET

Humans ¹ (sublethal dose)	Monkeys ² (lethal dose)
<p>Third day postexposure:</p> <ul style="list-style-type: none"> Mucous in the throat Difficulty swallowing solid food Feeling of catching a cold but without fever <p>Fourth day postexposure:</p> <ul style="list-style-type: none"> Mental numbness Retarded ocular motions Pupils moderately dilated with slight nystagmus Indistinct speech Uncertain gait Extreme weakness 	<ul style="list-style-type: none"> Mild muscular weakness Intermittent ptosis Severe weakness of postural muscles of the neck Occasional mouth breathing Serous nasal discharge Salivation, dysphagia Mouth breathing Rales Anorexia Severe generalized weakness Lateral recumbency

Data sources: (1) Holzer E. Botulism caused by inhalation. *Med Klin.* 1962;41:1735-1740. (2) Franz DR, Pitt LM, Clayton MA, Hanes MA, Rose KJ. Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism. In: Das Gupta B, ed. *Botulinum and Tetanus Neurotoxins and Biomedical Aspects.* New York, NY: Plenum Press; 1993: 473-476.

More data are available on exposure of animals to toxin aerosols. Rhesus monkeys were exposed by inhalation to botulinum toxin, type A, in conjunction with toxoid and hyperimmune globulin efficacy trials.³² Exposure to 5 to 10 monkey LD₅₀ (ie, 5 to 10 times the LD₅₀ for monkeys) resulted in death

in 2 to 4 days. Clinical signs of intoxication were noted 12 to 18 hours before death; they are also listed in order of onset in Exhibit 33-2. Preliminary studies with small numbers of animals (N = 3 per serotype) have recently demonstrated that serotypes C, D, and G are also toxic to rhesus monkeys.³³

DIAGNOSIS

Making a diagnosis of botulism under battlefield conditions might be very difficult during the early stages of a biological warfare attack. A history of simultaneous onset of bulbar and neuromuscular disease in a group of soldiers would alert medical personnel to botulism. The symptoms listed in Exhibit 33-2 are nearly pathognomonic of botulism prior to development of respiratory failure. The absence of convulsions would differentiate botulinum intoxication from chemical nerve agent poisoning. However, a wide variety of natural neurotoxins not related to botulinum toxin could produce roughly the same sequelae. For example, the bite of a snake that produces venom neurotoxins would lead to many, if not all, of the symptoms of botulinum toxin poisoning. However, the patient or soldier or his buddies would probably mention the bite if it had occurred, the telltale fang puncta would be seen on physical examination, and

numerous patients simultaneously exhibiting these signs and symptoms can hardly all have been bitten by snakes.

Medical personnel must remember, however, that with the advances of molecular genetics, it is possible to clone and produce many natural neurotoxins in relatively large quantities. For the present (1996), botulinum poisoning is a much more likely biological warfare agent than snake venom toxins. In future years, other (cloned) neurotoxins should be considered in the diagnosis.

Because of the small quantity of toxin protein needed to kill, botulinum toxin exposure does not typically induce an antibody response after exposure. The most likely means of laboratory diagnosis is through enzyme-linked immunosorbent assay identification of botulinum toxin from swabs taken from the nasal mucosa within 24 hours of inhalational exposure.

MEDICAL MANAGEMENT

Because the incidence of botulinum poisoning is so low in the United States, vaccination of the general public is unwarranted. It is only because of the possible use of the toxin as a biological warfare agent that vaccine and antiserum development have taken place, and this work has been done almost entirely by the U.S. Army. For this reason, nearly all stocks of these products are presently held by the army. There are two basic alternatives for prophylaxis from botulinum poisoning: active immunization using a vaccine, or passive immunotherapy using immunoglobulin.

The vaccine currently available is a toxoid that protects from serotypes A through E. This material is used under Investigational New Drug (IND) status, with a license held by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. The toxoid was developed by scientists at Fort Detrick, Frederick, Maryland, during the 1950s.³ It is a formalin-fixed crude culture supernatant from strains of *C botulinum* that produce the appropriate serotypes. Vaccinations are administered at 0, 2, and 12 weeks, followed by annual booster doses.

Nearly 80% of recipients exhibit protective titers (according to the CDC standard of > 0.25 international units per milliliter) at 14 weeks.³ However, hardly anyone has a measurable titer just prior to receiving the first annual booster dose. The kinetics of this loss are imperfectly understood at the present. A booster dose administered at 1 year leads to a robust response from approximately 90% of the recipients. Although currently an IND vaccine, the botulinum toxoid has been administered to hundreds of humans over many years and is widely regarded as safe. (Approximately 8,000 service members received the toxoid between 23 January and 28 February 1991, as part of the U.S. force deployed to the Persian Gulf War. A significant fraction of recipients experience stinging immediately after injection and a sore arm for 2 to 4 days. However, in the experience of most recipients, including the authors, the short-lived symptoms are not significantly different from those produced by tetanus vaccination. Work is currently underway in U.S. Army laboratories to develop a new generation recombinant botulinum vaccine that would protect from all known serotypes.

Passive protection can be afforded by administration of immunoglobulin products of various types. The earliest developed was a horse antitobu-

linum serum (ie, globulin). Because of the relatively high risk of serum sickness, a despeciated globulin (ie, the species-specific antigenic properties were removed from the equine immunoglobulin) was produced. Thus, the product currently held in quantity is horse antitobulinum toxin immunoglobulin that has been treated with pepsin to produce the fragment F(ab')₂ (ie, the basic immunoglobulin molecule has been altered by removal of the complement fixing [Fc] region to concentrate the antigen binding sites). This material has been tested for efficacy in studies with monkeys. Animals given one human dose, or one tenth of one human dose of the F(ab')₂ antitoxin, and challenged with approximately 10 LD₅₀ of serotype A by inhalation, survived without signs of intoxication.³² Antibody titers ranging from 0.6 to 0.38 international units per milliliter, in those given one human dose, and from 0.02 to not measurable after one tenth of one human dose of F(ab')₂, were fully protective.

When given as therapy after exposure, one tenth of one human dose of the F(ab')₂ product was protective if given before onset of clinical signs of disease. If given after onset of signs, however, even a dose 3-fold greater than the recommended human dose was not protective. We believe that toxin is already inside neurons and producing the symptoms of poisoning, and cannot be reached by circulating antibody. These data demonstrate that, at least in monkeys, titers much lower (< 0.02) than 0.25 international units (as recommended for the vaccine) are protective and suggest that humans may be more easily protected by vaccination than previously believed.

In addition to the recombinant vaccine presently in development, research on cocktails of monoclonal antibodies is being conducted at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland, to replace the despeciated horse serum. The cocktail approach will enhance the safety of the immunotherapy, and recombinant techniques will probably also reduce the cost of therapeutic antibody.

Although some investigators³⁴ have reported limited success in treating human serotype A poisoning with aminopyridines, controlled animal experiments³⁵ performed at USAMRIID showed no effect on non-type A poisoning and only delays in time to death with type A. Obviously, no controlled human trials can be done, so whether the drugs helped

at all remains unclear. Since the drugs have significant toxicity themselves, they have largely been abandoned as a treatment for botulism.

Research is also ongoing (by J.L.M.) to target therapy directly to intoxicated nerves by making a chimera of the receptor binding portion of the botulinum molecule and either monoclonal antibodies

or drugs that neutralize intracellularly. Just as the available toxoid and prototype second-generation vaccines provide solid protection against this most toxic of agents, we hope that second-generation therapeutics will allow us to reverse the lethal effects of this toxin after it has been internalized in the cell.

SUMMARY

The seven serotypes of botulinum toxin produced by *Clostridium botulinum* are the most toxic substances known. They are associated with lethal food poisoning after the consumption of canned foods. This family of toxins was evaluated by the United States as a potential biological weapon in the 1960s and is believed to be an agent that could be used against our troops. Unlike other threat toxins, botulinum neurotoxin appears to cause the same disease after inhalation, oral ingestion, or injection. Death results from skeletal muscle paralysis and resultant ventilatory failure. Because of its extreme toxicity, the toxin typically cannot be identified in body fluids, other than nasal

secretions, after inhalation of a lethal dose. The best diagnostic sample for immunologic identification of the toxin is from swabs taken from the nasal mucosa within 24 hours after inhalational exposure. Because of the small quantity of toxin protein needed to kill, botulinum toxin exposure does not typically induce an antibody response after exposure.

Prophylactic administration of a licensed pentavalent vaccine fully protects laboratory animals from all routes of challenge. Passive immunotherapy with investigational hyperimmune plasma also prevents illness if it is administered before the onset of clinical intoxication.

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