

Botulinum Toxin: Lethal Weapon or Magic Bullet?

Botulinum Toxin Primer

Introduction

The first two pages of this background essay are the same as the short student reading found in Activity 1, Assignment 1. The references are the same, but are found at the end of the present document.

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Like penicillin botulinum toxin could become a legend. Until recently, the toxin was known primarily as the cause of botulism, a type of deadly food poisoning produced by improper canning. In the late 1980s, Canada approved use of the toxin to treat strabismus (also known as cross-eyes), and Americans suffering from this ailment began crossing the border for medical relief. In 2001, Canada approved an additional use for the toxin--the removal of facial wrinkles--and in 2002, the FDA in the United States followed suit. (1, 2). Subsequently, the neurotoxin has become a household name as clients line up at local gyms, parties, and spas for their quarterly Botox injections, in order to temporarily rid themselves of wrinkles and sweaty armpits. The toxin now enjoys a type of celebrity caché, for better or worse, and has been featured comically on popular sit-coms and comic strips (3-8).

Profits are expected to hit an all time high as the toxin makes the leap from therapeutic drug to beauty aid, and this financial trend is likely to grow, since a number of new therapeutic applications may be developed and approved for use in the next few years. Dr. Mitchell F. Brin, vice president of Allergan, claims that botulinum toxin type A could treat up to ninety-three human disorders (5, 9). Meanwhile, Elan pharmaceuticals has secured rights to specific clinical applications of botulinum type B (10). Both companies hope to secure a unique financial niche, and scientific investigations of botulinum toxin action suggest that the versatility of the toxin will make this possible. By investigating how the toxin goes about disrupting neurological function, scientists have begun to learn a great deal about the basic properties of neuronal transmitter release and cellular secretion pathways (11-14). These findings may lead to new treatments for neurological and endocrine disorders such as cerebral palsy, Parkinson's Disease, Graves Disease, and Cushing's Disease (9, 15-17). Perhaps most surprising is that the lethal toxin is being engineered as a delivery vehicle for oral vaccines such as those used for cancer (18). So the stakes are very high--both for those who suffer from debilitating diseases and the pharmaceutical companies who will develop and sell the medical applications.

There is also another side to this story, which is perhaps not as well known, but is just as important: namely, the use of the toxin as a lethal agent. *Clostridium botulinum* is the bacterium that produces the world's most deadly toxin. A small dose of the toxin can kill 10 million people within 48 hours. If aerosolized, the bacterial spores linger in the air for up to two days. These spores germinate once they have entered a human host and, if the conditions are right, begin to release the neurotoxin (13, 19, 20).

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With potency 10,000 times stronger than any other biological poison, the botulinum toxin was recruited as a biological weapon many years ago, and it continues to be used today. During WWII, Stanley Lovell, an American officer in the Office of Strategic Services, ordered the manufacture of gelatin capsules containing the toxin. The plan was for Chinese prostitutes to hide these capsules behind their ears and then slip them into the meals of high-level Japanese officials(20). More recently, in 1993 the Iraqi leader Saddam Hussein admitted to manufacturing and stockpiling 19,000 liters of the toxin, despite having signed the 1972 Biological Weapons Convention Treaty which forbids such actions (19, 21-23). And between 1990-1995, the terrorist cult group Aum Shinrikyo unsuccessfully launched three botulinum attacks in Japan (13). State and local health officials are on heightened alert for bioterrorist activities using botulinum toxin, so much so, that in preparing for the millennium celebration, Saint Lukes Hospital in New York stockpiled antitoxin.

Despite the ongoing threat of a bioterrorist attack via botulinum toxin, President Bush has refused to ratify the Biological Weapons Convention Treaty. He argues that the surveillance protocols threaten biotechnology commercial interests. Instead, he has asked for increased federal funding for prevention and treatment of symptoms caused by biological weapons (24, 25).

Unfortunately, little research has been conducted in the way of prevention and antidote for botulism. Presently, the Centers for Disease Control (CDC) has a limited supply of a horse antitoxin that can be shipped via air upon request by a State Health Department (13, 22). Although there is no approved vaccine to prevent botulism, the CDC does possess an investigational toxoid vaccine whose access is restricted to military personnel and researchers. However, some advances are being made. In 2001, the Senate Appropriations Committee for Bioterrorism put forth a bill that called for a \$520 million budget to improve food safety, protect US agriculture, and increase spending for vaccine and treatment development (26). Nevertheless, current methods of prevention are inadequate, and researchers are just beginning to develop high-throughput assays to identify botulism treatments and more effective vaccines (22, 27-29). We clearly have a long way to go.

No longer just regarded as a deadly food poisoning caused by grandma's pickling or canning, *Clostridium botulinum* and the toxin it produces have jumped to the forefront of military and anti-terrorist campaigns, scientific research, pharmaceutical ventures, and the vane worries of an aging and wrinkling populace. The potential medical benefits of the toxin provide great hopes, but its use as a weapon holds an equal potential for harm. By learning more about both the underlying science of the toxin and the role it may play in our lives, we will hopefully be able to make informed choices about how to maximize the benefits and minimize the destructive potential of the single most poisonous substance currently known to humankind.

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Botulinum Toxin Background

The remainder of this essay contains scientific background intended primarily for instructors.

Molecular Mechanisms of Botulinum Neurotoxin (BoNT)

How can a paralyzing toxin elicit therapeutic effects? A closer look at the toxin and its mechanisms offers some clues. Although the amino acid sequence of botulinum toxin (BoNT) shares a low overall sequence homology of 50% with tetanus toxin (TeNT), the structures of the two toxins are quite similar (30, 31). Both proteins are made by clostridial bacteria and are among the most harmful toxins known. Once inside the neuron, BoNT and TeNT prevent vesicles from docking and fusing with the cell membrane and specifically block the release of neurotransmitters from the presynaptic neuron.

Because each of the clostridial toxins has an affinity for a specific part of the nervous system, these toxins cause different types of paralysis. TeNT blocks neurotransmitter release in GABAergic and glycinergic synapses of the central nervous system, which results in spastic paralysis, whereas BoNT block acetylcholine (Ach) release in the neuromuscular junctions of the peripheral nervous system, which results in flaccid paralysis (32). Botulism, or food poisoning, is the most common result of ingesting food contaminated with botulinum spores or bacteria, and tetanus is a common cause of neonatal death in developing countries. If not treated, paralysis may spread to the respiratory muscles, and the victim will die of respiratory failure (14, 33).

There are seven different serotypes of BoNT (BoNT A-G). Each serotype exhibits toxicity in a highly specific manner through a four-step process consisting of binding, internalization, membrane translocation, and enzymatic cleavage of host proteins in the presynaptic cell (34). The host proteins, called SNAREs (soluble NSF-attachment protein receptors), provide target specificity for vesicle docking and fusion. SNAREs exist as complementary sets of proteins: v-SNAREs, vesicle associated membrane proteins, and t-SNAREs, target membrane associated proteins (12, p.720-26, 35). The cleavage of these target proteins results in a block of Ach release, flaccid paralysis, and if not treated death (14, 33, 35).

Patients who suffer from an excess of Ach release and abnormal frequency of muscle contraction, may benefit from the localized injection of diluted BoNT (10, 15, 17, 36, 37). Likewise, wrinkles that result from overuse of facial muscles "disappear" following dilute injections of the toxin (5-9). The toxin also aids those who suffer from excessive sweating, or hyperhidrosis, and some doctors are now extending these benefits to runway models and savvy New Yorkers (8).

When the toxin is used to harm rather than treat people, it is often weaponized for aerosol dispersal (13). Clostridia spores are released into the air to disable or kill a target population. The spores enter the lungs of unsuspecting victims and, upon finding a

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hospitable environment, germinate and secrete toxin. The toxin then travels through the bloodstream to the neuromuscular junction and binds to the membranes of presynaptic cells and is internalized.

If the bioweapon vehicle happens to be food, the toxin must endure stomach acid, and other environments that would either destroy or induce premature toxin activity. It is believed that a protective armor of auxiliary proteins shields the active portion of the toxin from these harsh conditions. These auxiliary proteins are most commonly hemagglutinins and are part of the 900kDa complex (BoTx) produced by clostridia. In neutral pH, the auxiliary proteins dissociate and do not contribute to host cell toxicity (33). Once the toxin has crossed the intestinal lining via transcytosis it enters the blood stream and is carried to the cells of the neuromuscular junction (38).

The active core of the clostridial protein lies beneath the auxiliary proteins. This 150kDa monomer possesses three functional domains (A-B-C), which are responsible for protease activity and translocation, respectively. The 150kDa monomer is cleaved into two smaller subunits by bacterial or host endopeptidases. The resulting 100kDa and 50kDa subunits remain attached through a disulfide bridge, and this is often referred to as the di-chain (14, 39). The A domain resides in the light chain and is responsible for highly specific protease activity, while the B and C domains lie in the heavy chain and are responsible for cell surface binding and internalization respectively. The heavy chain may also serve as an inhibitor of the light chain enzymatic activity. Structural data indicates that the C domain loop of the heavy chain wraps around the A domain of the light chain, shielding it from potential targets (40, 41). More recently, the amino terminal end of the heavy chain containing the B domain is referred to as H_N while the carboxyl end containing the C domain is referred to as H_C .

In all serotypes, H_C binds to the host cell through a low-affinity ganglioside interaction (GT1b for BoNT/B) (12, p.592-593, 42-44). Because H_C varies among the serotypes, it may allow for serotype host cell specificity. The variable sequences are believed to associate with high-affinity protein co-receptors (synaptotagmin II has been implicated for BoNT/B) necessary for binding and internalization of the toxin (45, 46). There is considerable controversy over the nature of the botulinum toxin host cell receptor (47). A dual receptor hypothesis suggests that the ganglioside brings the toxin in proximity to the host cell membrane and allows for the putative protein receptor to interact with the toxin, promoting membrane fusion (14).

The internalization step is truly a two-step process and is thought to occur via receptor-mediated endocytosis involving coated vesicles followed by membrane translocation (12, p.749-57, 48, 49). Studies have shown that nerve stimulation leads to an increased presence of toxin in small synaptic vesicles (SSVs). As one research group phrased it, the toxins may "use SSVs as Trojan horses" to gain entry to the neuronal cytosol (14). Although the exact mechanism of membrane translocation from the SSV is not known, H_N appears to play an important role in the internalization steps. This region of the heavy chain displays amphipathic properties and increases permeability of lipid bilayers. However, it does not share structural homology with known pore-forming proteins (12,

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p.584-589, 14, 41). Rather, this region shares homology with viral proteins HIV gp41 and influenza hemagglutinin (41). Conformational energy calculations show that a bundle of four amphipathic alpha-helices is a plausible structural motif for H_N, and peptide sequences that mimic this region are capable of forming ion channels in lipid bilayers (50).

To reach its intracellular target, the catalytic domain must enter the cytosol. As the SSV undergoes vesicular recycling the pH of the intracellular compartment drops (12, p.750, 765). Model membrane systems suggest that within this reductive environment H_C undergoes a conformational change that exposes the catalytic A domain of the light chain and disrupts the disulfide bridge (39). The structural change also exposes hydrophobic residues in both the heavy and light chains, allowing them to penetrate the membrane lipid bilayer and form ion channels (50). Some researchers believe that these ion channels serve as a conduit for the light chain to escape into the cytosol as an active endopeptidase, but this is still under dispute. The scenario is in agreement with the finding that the unreduced di-chain has little catalytic activity (14).

The catalytic domain (A) of the light chain possesses zinc-dependent endopeptidase activity and shares homology with thermolysin and leishmanolysin (14, 41, 51). The zinc-binding site, His-Glu-X-X-His, requires a distal glutamatic residue and a water molecule (12, p.386). A distal tyrosine participates with an arginine in the catalysis, presumably by stabilizing the transition state. The endopeptidase targets the individual host SNAREs for cleavage. Once the fusion complex has assembled, however, the SNAREs are protected from cleavage (33). If SNAREs are cleaved prior to assembly, truncated versions will be incorporated during the assembly process, rendering the entire complex non-functional. This activity can be seen *in vitro* using synaptic SNAREs in liposome and reconstituted yeast vesicular assays (52). More recently, *Arabidopsis* and tobacco SNAREs were cleaved by BoNT (53). Since the yeast and plant homologues of the SNARE proteins are cleaved, it seems that BoNT, like other bacterial toxins, target the core membrane fusion apparatus rather than those that are specific to neuronal cells. Although all serotypes cleave either syntaxin, VAMP, or SNAP-25, those that target the same SNARE often have different cleavage sites (34, 54). For example, BoNT/A cleaves nine amino acids off the C-terminal region of SNAP-25, while BoNT/E cleaves 25 amino acids off this same host cell target protein. The difference in the size of cleavage products could explain the longer lasting effects of BoNT/A as compared to BoNT/E (14, 33).

BoNT recognize their protein targets because each has a SNARE motif. This motif is nine amino acids in length and is characterized by alternating hydrophilic and hydrophobic residues. The number of motifs in each target protein varies: SNAP-25 contains four motifs; and VAMP and syntaxin each contain only two. The presence of this motif in all these proteins suggests that the BoNT recognize their targets via a three-dimensional structural motif. The table below lists the serotypes, indicating host toxicity, host cell targets, and cleavage recognition sequences (14, 33).

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Table of botulinum serotype target cleavage sites

BoNT serotype	host target	intracellular target	cleavage site
BoNT/A	human (common in western U.S.)	SNAP-25	EANQ-RATK
BoNT/B	human (common in eastern U.S.)	VAMP/synaptobrevin	GASQ-FETS
BoNT/C	Primates	SNAP-25 syntaxin	ANQR-ATKM DTKK-AVKF
BoNT/D	Primates	VAMP/synaptobrevin	RDQK--LSEL
BoNT/E	Human (common in Alaska)	SNAP-25	QIDR--IMEK
BoNT/F	human (rare)	VAMP/synaptobrevin	ERDQ-KLSE
BoNT/G	Primates	VAMP/synaptobrevin	ETSA-AKLLK

In summary, BoNT have permitted scientists to better understand vesicle trafficking, vesicle fusion, and secretion while providing clinicians with useful tools to treat disease. The toxin has been instrumental in revealing the identity of a collection of proteins necessary for vesicular traffic, the v-SNAREs and t-SNAREs. BoNT will continue to be used in creative ways. Currently the toxin is being harnessed to deliver a variety of molecules in a neuron-specific manner and engineered to deliver molecules to a variety of other cell types through domain swapping (16, 18, 55).

BoNT Host Cell Receptors: Controversial Beginnings

Most pathogens gain entry to their hosts by binding to modified proteins or sugars on the cell surface. Not surprisingly, individuals who have aberrant sugar modification (glycosylation) pathways display hypersusceptibility or resistance to a subset of pathogens. An example of hypersusceptibility comes from cystic fibrosis patients. The most common cause of death in these patients is *Pseudomonas aeruginosa* infection. Genetic mutations on the CFTR gene lead to altered sugar modification of proteins on the surface of these patients' respiratory cells, permitting *Pseudomonas* to colonize and invade the respiratory tract (56). This altered glycosylation pathway also disrupts ion channels and leads to a thickening of the mucus, making it virtually impossible to clear bacteria from the bronchial airways. Sugar modifications also play a role in blood typing. The ABO blood groups are assigned based on the identification cell surface molecules that carry different sugar modifications. Individuals with AB blood type synthesize a functional glycosylation enzyme and exhibit resistance to cholera infection, while individuals who have O blood type carry a frameshift mutation (D258) that results in a non-functional enzyme, less sugar modification, and susceptibility to cholera infection. The additional sugar modification on the surface of intestinal cells in AB individuals may prevent *Cholera vibrio* toxin from binding and entering the intestinal mucosa. By passing the toxin in their stool, these individuals escape dehydration caused by diarrhea (57, 58 p 136-146).

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BoNT also use sugar to gain access to their intracellular targets. There is consensus among researchers that gangliosides, a complex sugar commonly found on the surface of host cells, play a role in BoNT cell surface binding, but there is dispute about whether the gangliosides are *sufficient* in this regard (43, 44, 46, 59). Studies have shown that BoNT bind to gangliosides residing on synaptosomes, reconstituted lipid vesicles, and neuronal membranes via a low affinity interaction (43). In addition, gangliosides expressed in non-neuronal cell lines are sufficient for BoNT binding. However, other reports indicate that gangliosides are necessary, but not sufficient, in binding BoNT and that protein receptors play an important role in toxin binding and internalization (59-61).

Here a second level of dispute arises. Do the seven different BoNT serotypes bind to neuronal cells via specific protein receptors, or do they share a common protein receptor? Preliminary data suggest that researchers are not far from answering this question.

Early on, researchers determined that GT_{1b} and Gd_{1a} serve as receptors for BoNT (34). Although initial progress in identifying a component of the binding complex was helpful, researchers recognized that gangliosides are present on many neuronal cell types, while BoNT specifically attack cholinergic neurons. This knowledge propelled researchers to seek out components of the binding complex that would confer this binding specificity. This approach is in line with a dual receptor model (60). This model involves a loose ganglioside binding that brings the toxin in close proximity with a specific protein receptor that binds the toxin more tightly. The mechanism is similar to the one proposed for HIV binding to CD4 cells in which low affinity binding of the CD4 receptor allows the virus to interact with the chemokine receptor for which it has much higher affinity (12 p.724-26).

In 1999, Lalli et al. investigated the dual receptor model for BoNT binding of neuronal cells. In a series of experiments using recombinant proteins and recombinant protein fragments, they demonstrated that the heavy chains of BoNT/A, B and E bind to GT_{1b} (although BoNT/A had much lower affinity) and are necessary for binding and internalization of the toxins in spinal cord cells in culture. They also demonstrated that there is some specificity in serotype binding because heavy chain fragments from BoNT/B were unable to block heavy chain binding by BoNT/E, suggesting that another receptor might be involved (42).

Meanwhile, in 1999, Lacy et al. produced the crystal structure of BoNT/A and Ginalski et al., in 2000, used structure based sequence alignments to illustrate that BoNT/A and TeNT have two potential host binding sites. One binding site contains a relatively conserved domain that binds to gangliosides in a low-affinity interaction, while the second binding site involves a more variant domain that could bind host protein receptors in a high-affinity manner (30, 44). More recently, the crystal structures of intact BoNT/B and its complex with sialyllactose were solved, providing more information about sugar binding and suggesting that there are some differences between BoNT/A and BoNT/B (62).

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The structural data added support for Nishiki's work, and his group set out to identify the protein receptor for BoNT/B. Using a biochemical approach that involved column chromatography of rat synaptosome preparations and cross-linking studies, they identified synaptotagmin I as the receptor. Since synaptotagmins are found on small synaptic vesicles (SSV), they proposed that synaptotagmins could serve as protein receptors for all BoNT via vesicle recycling. During recycling, the luminal side of the protein would be exposed to the extracellular surface of the cell, allowing for BoNT binding and host cell entry (12 p.764-65, 47).

Because synaptotagmins exist in multiple isoforms and are differentially distributed, one could imagine a variety of permutations that would lead to substantially different results. Perhaps each BoNT serotype is capable of binding multiple synaptotagmin isoforms *in vitro*, but selectively binds to one isoform *in vivo*. For instance, in older parts of the brain, such as the cerebellum, synaptotagmin II is present and synaptotagmin I is virtually absent (63). Thus, in experiments using rat cerebellar membranes, BoNT/B would bind the cell surface via the synaptotagmin II protein.

To investigate this idea, Nishiki's group extended their biochemical analysis. They labeled recombinant synaptotagmin I and II with I^{125} and mixed these proteins with BoNT/B in the presence of gangliosides. Scatchard plot analyses revealed a single class of binding site with dissociation constants of 0.23 and 2.3 nM for synaptotagmin II and synaptotagmin I, respectively, demonstrating that BoNT/B preferentially binds synaptotagmin II *in vitro* (46).

Although progress on the BoNT/B protein receptor was made, conflicting reports emerged with respect to other BoNT protein receptors. In support of a common protein receptor, Li and Singh reported that synaptotagmin I was identified as a protein receptor for both BoNT/A and BoNT/E using toxin affinity column chromatography and microtiter plate competition assays (45). But other researchers believe, on the basis of the lack of competition observed between different BoNT, that each BoNT serotype binds to a different host cell protein receptor, explaining the differences in length and potency of toxin action (42).

The idea of a common receptor for BoNT/A and B was further weakened by the fact that antibodies against synaptotagmin I had no effect on BoNT/ B binding (64). This research group demonstrated that antibodies against synaptotagmin did not block BoNT/B binding to rat cerebellar brain membranes, nor did they protect the mouse phrenic nerve-hemidiaphragm from toxin-induced neuromuscular blockade.

What is interesting about this last report, is the choice of antibody. Although the antibody used in this study was raised against synaptotagmin I, it cross-reacts with synaptotagmin II and, therefore, the study challenged the finding that either protein could serve as the protein receptor for BoNT/B *in vivo*. As the researchers themselves note in the discussion of this paper, perhaps the inability of the synaptotagmin I antibody to block BoNT/B binding, or action, was due to the nature of the assay used. They preincubated rat cerebellar membranes with antibody, washed them, and added BoNT/B.

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Since the cerebellar membranes are actively recycling, BoNT/B could bind to newly exposed synaptotagmin II (64).

Presently, the scientific and medical communities are waiting to see which protein receptors will be characterized from the study of putative binding sites artificially expressed in *Xenopus* oocytes using mRNA from rat cerebellar brains (64). The results will be valuable in understanding BoNT specificity for cholinergic neurons as well as provide some clues into botulism treatment and vaccination.

BoNT as a BioWeapon

Attacks

During the 20th century, some countries and terrorist groups chose to develop botulinum toxin as a bioweapon, and many more countries responded by launching military biodefense programs. To the surprise of many Americans, the U.S. launched a biowarfare program in the early 1940's at Camp Detrick in Maryland. Some believe that this program was prompted by the discovery that Japanese soldiers had fed cultures of *Clostridium botulinum* to prisoners of war during the occupation of Manchuria; the U.S. not only desired defense from such a weapon, but to have equal military capacity as well. Starting in 1943, scientists at Camp Detrick weaponized botulinum toxin and produced anthrax spores. Dr. Edward J. Schantz led a Camp Detrick research program to design effective biodefense agents necessary for WWII. For fear that Germany may have weaponized botulinum spores, Allied troops who landed in Normandy were immunized against botulinum toxin by a vaccine produced at Camp Detrick, although many were unaware of the procedure (13, 20).

During the 1990's, Iran, Iraq, Korea, and Russia were believed to be including botulinum toxin in their bioweapons arsenals (21). Defectors of the former Soviet Union claimed that the BoNT genes were being engineered into other microorganisms in an effort to create lethal chimeras that could be easily dispersed in populations or by missile attack (23). In 1995, shortly after the United Nations Special Commission (UNSCOM) investigation of Iraqi facilities, Iraqi bio-weapons specialist General Husayn al-Kamal defected to Jordan and made claims that the al-Hakam facility was not used as claimed to produce animal feed, but rather to produce biological weapons. Iraq finally conceded, and it was discovered that 19,000 liters of the neurotoxin had been manufactured and stock-piled. This amount is enough to kill the entire world population three times over. The UN destroyed the al-Hakam facility in 1996 (13, 21).

A number of botched attempts to release botulinum toxin by terrorists have occurred in the last decade as well. In April 1990, the Japanese cult group Aum Shinrikyo sent trucks through central Tokyo spraying botulinum toxin into populated areas. Targets included the US Navy installation at Yokohama and the base at Yokosuka, as well as Narita International Airport. Fortunately, these attacks and several other attempts in subsequent years were unsuccessful (19).

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In an effort to curb access to biowarfare reagents and to reduce the frequency of attacks, all biowarfare agents produced by the U.S. for offensive purposes were destroyed following the Biological and Toxin Weapons Convention. On April 10, 1972, a treaty was signed by 108 countries that entered into force on March 26, 1975. Since 1972, fifty-four more countries have signed on (13, 19). However, early in 2002 President Bush rebuked the treaty saying that surveillance was inadequate and would compromise commercial interests in the biotechnology sector.

Treatments

Currently, the CDC holds a horse antitoxin as the only treatment for any form of botulism (13). This antitoxin must be requested by State Health Departments and may be shipped air express in order to reach the patient within the critical period of 24-36 hours of onset of symptoms. Patients who are treated, usually recover and muscle movement is completely restored within six months. Of those cases that remain untreated, 80% result in death. Despite knowing that the botulinum toxins bind host cells via a ganglioside interaction, few researchers have investigated the possibility of treating the disease with sugar. Excess sugar could sequester the toxin and prevent host cell binding. This avenue has been explored as an improved method of treating microbial diseases that normally become resistant when antibiotics are administered.

Vaccines

Vaccination for botulism is a risky and costly endeavor. A pentavalent crude toxoid vaccine (A-E) and a singular F toxoid are investigational drugs distributed by the CDC to military and research workers that might come into contact with toxin (13, 19). Since they have not acquired FDA approval, these toxoid vaccines are not licensed for general distribution. The impetus to meet FDA requirements is low, because these vaccines require frequent boosters and are toxic due to the formaldehyde used to inactivate the toxins.

A new vaccine, synthesized in yeast, is in the works based on non-toxic recombinant protein components of five of the botulinum serotypes. However, clinical trials for this vaccine are not in progress, and it is unclear why (65). An oral vaccine based on a non-toxic variant of BoNT/C developed by site-directed mutagenesis provided immunity to mice, and an injectable vaccine based on the BoNT/A heavy chain provided immunity to mice as well (66, 67). Although these results are encouraging, the newer vaccines based on recombinant DNA techniques have only been tested in animals.

Toxin Inactivation

Inactivation of a toxin spill can be executed using 0.5% sodium hypochlorite. Spores can be inactivated using ionizing radiation of D10. Inactivation of toxin in food requires heating to 80 degrees Celsius for 10 minutes, while inactivation of spores in food requires harsher treatment involving heating to 100 degrees Celsius for 10 minutes or ionizing radiation of D10 (13).

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Molecular Inhibitors of toxin *in vivo*

Most molecular inhibitors of the neurotoxin target the zinc-endopeptidase activity of the light chain and are therefore zinc-protease inhibitors such as thiorphan, phosphoramidan, and captopril. Unfortunately, none of these have shown substantial inhibition of toxin activity *in vivo*, and many require environments that are not physiologically sustainable (phosphoramidan and captopril show minimal inhibition of BoNT in cell free culture) (28, 68).

Rational drug design has also been approached. A natural peptide found in the stomach tissue of toads and bullfrogs, Bufenin I, has also been shown to inhibit BoNT/B *in vitro* (69). Bufenin I was chosen as a candidate inhibitor based on a similarity of 18% for conserved amino acids around the neurotoxin cleavage site. One synthetic compound, ICD 1578, was selected for study based on the fact that it is a phosphoramidan analog (70). ICD 1578 has been shown to inhibit BoNT/B light chain *in vitro* using a fluorescence assay that measures cleavage of a peptide that mimics the human synaptobrevin protein cleavage site. ICD 1578 was also identified using a similar assay based on indirect ELISA. Recently, scientists have begun to use these assays in high-throughput screens to identify more inhibitors of the toxin (71).

Chimeric BoNT Therapeutics

During the early 1970s, the U.S. was recovering from the fallout of the Vietnam War. Many wounded soldiers became addicted to morphine administered for pain relief and others suffered from chemical weapon exposure. These veterans brought ugly souvenirs home from the war: heroin abuse and Agent Orange-related illnesses, such as cancer. Meanwhile, the aging homeland population was also confronting cancer (72, 73). The rising rates of cancer had reached epidemic proportions and many Americans demanded a solution.

In an effort to drum up support for his re-election, President Nixon attempted to reassure the public by tackling these societal ills. The maladies associated with Agent Orange and talk of cult groups accessing bioweapons stockpiled in the U.S. prompted him to enforce a shut down of all biological and chemical warfare programs. In 1969, he ordered the destruction of all remaining biological weapons at the Pine Bluff Arsenal, Rocky Mountain Arsenal, and Fort Detrick arsenal. Between 1971 and 1973 these orders were carried out and efforts were redirected towards defense programs (19). In 1971, just one year before the election, he launched the “War on Cancer,” the same year he began the never-ending “War on Drugs.”

Some thirty years later our country is still battling these wars. The worldwide development and use of biological and chemical weapons escalated during the 1980’s and 1990’s, despite the 1972 Biological and Chemical Weapons Convention Treaty that banned such actions (13, 19). Alongside these offensive programs, research into defense against these agents yielded some ironic results. The same molecules that could cause mass human destruction could be engineered to deliver drugs to cancer patients in a

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localized fashion, eliminating many of the painful side effects and short comings of conventional chemotherapeutic agents and thus reducing the need for heavy doses of narcotics for pain relief.

Chimeric Proteins

In the last few years, researchers have turned their attention to chimeric proteins designed to target cancer cells for destruction. Originally, these chimeras were comprised of an antibody specific for a cancer antigen, fused to a protein that would signal cell death (74-76). These cell death proteins were derived from human apoptotic proteins or microbial and plant toxins.

Microbial toxins are particularly suited to chimera production, because they contain discrete protein domains, making domain swapping a feasible task (77). Many of these toxin genes evolved through splicing events that brought together a collection of ancient common sequences that code for distinct protein domains that are structurally self-sufficient. Most toxins have a binding domain, a translocation domain, and a catalytic domain. The binding and translocation domains can be swapped for other protein domains that bind to specific cell types (78).

More recently, a variety of chimeras have been developed using growth factor and toxin domains. These fusion proteins target cell surface receptors on tumor cells. In these cases, the growth factor domain of the chimera binds to growth factor receptors, allowing the chimera to be taken up by the cell via receptor-mediated endocytosis. From here, the toxin domain is translocated across the membrane, enters the cytosol, and triggers cell death. These fusions have been made with components of anthrax and diphtheria toxin fused to epidermal growth factor (EGF) or vascular endothelial growth factor (VEGF) (79-83). The chimeras have been shown to reduce growth in tumor cell lines, and some have entered Phase III human clinical trials.

Growth Factors and Cancer

The success of these chimeras is a result of a rational drug design approach. Because human carcinomas frequently express high levels of growth factor receptors, the chimera's effects are localized to cancer cells. The EGF receptor family of tyrosine kinase receptors has been implicated in the majority of endothelial cancers and some breast cancers (12, p.1358). Overexpression of at least two of these receptors, the EGF receptor (EGFR) and closely related ErbB2 has been associated with a more aggressive clinical behavior. Furthermore, increased expression of these two receptors in nonmalignant cell lines can lead to a transformed phenotype (74, 80, 81).

For these reasons, initiating cell death in cells which overexpress these receptors may be a more effective cancer therapy than conventional therapeutic agents which usually induce systemic debilitating side effects or result in resistance. The latter group of drugs target actively dividing cells and so, along with cancer cells, hair follicle cells, intestinal cells, and white blood cells are destroyed leading to hair loss, loss of appetite, and

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immunosuppression. Conversely, a growth factor toxin chimera would elicit death only in cancer cells (12, p.1313-1362).

Regardless of the type of chimera used to treat cancer (growth factor toxin fusions or immunotoxins), the beauty of this therapy lies in the ease of production and versatility of administration. The fusion proteins can be synthesized in yeast or bacteria, purified, and locally administered via injection. Alternatively, the recombinant DNA (r DNA) encoding the chimeric protein can be injected into a patient using a DNA gun or a viral vector (78).

Botulinum Toxin Chimera Advantages

Botulinum toxin's unique characteristics hold new promise for the development of chimeras designed to treat or prevent disease. The toxin appears to be extremely immunogenic, capable of translocating from the gut to the blood, and cleaves proteins involved in the core machinery of the secretory pathway.

Immunogenicity is an important aspect of chimeras designed to work as vaccines. Recent work by Simpson has shown that a non-toxic variant of botulinum toxin, containing a mutation that renders the light chain inactive, elicits antibody production against the toxin. This response was seen in both oral and subcutaneous administration of the variant toxin in mice, suggesting that the toxin is highly immunogenic (67). Other studies demonstrated that a catalytically active form of the toxin used to treat muscle disorders also led to toxin immunity in a small percentage of patients (84). Simpson plans to exploit the immunogenicity of botulinum toxin domains for development of composite oral vaccines for the prevention of infectious diseases (18).

Botulinum toxin also provides an alternative form of drug and vaccine delivery. Currently, immunotoxins and vaccines are administered via injection, a costly and often risky procedure. But because botulinum toxin is capable of binding epithelial cells of the gut via the light chain, a non-toxic variant of BoNT could be engineered for oral delivery of vaccines targeting infectious diseases or cancer. Simpson has proposed that the EGF-anthrax chimera be linked up to a botulinum toxin variant (27). Currently, the EGF-anthrax chimera is injected to treat breast cancer, whereas the higher order chimera linked to the non-toxic botulinum variant would have the advantage of being orally administered.

The catalytic domain of BoNT is also proving to be useful in chimeras designed to treat endocrine disorders. Since the catalytic domain blocks the secretory pathway of all cells, this domain can be spliced to proteins that specifically bind to pancreatic or adrenal cells and correct faulty secretion in these cells (37).

Other researchers are not so eager to engineer botulinum toxin for therapeutic use, citing the dangers associated with highly immunogenic properties of the neurotoxin. If the toxin subunits elicit too much of an immune response, the drug could negate its own effects or worse, lead to an exaggerated immune response.

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To steer clear from these potential hazards, anti-tumor chimeras made entirely of human proteins have been developed. Yoon et al. have exploited the ability of human angiogenin to inhibit protein synthesis and cause cell death. This inhibition only occurs if angiogenin is present in the cytosol, as high concentrations of angiogenin in plasma have no cytotoxic effect. Therefore, these researchers constructed four chimeras fusing human angiogenin to a recombinant human EGF domain. When the chimera binds to the EGFR receptor, the entire complex is internalized thus delivering angiogenin to the cytosol. These constructs inhibited the growth of human EGFR-positive target cells in culture (85).

Engineering a lethal toxin for therapeutic purposes is not simple. Research in this area has been slow and methodical, but with the advent of new technologies it may not be long before chronic diseases such as movement disorders, cancer, and infectious disease are maladies of the past.

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