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Medical aspects of toxin weapons

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Abstract

For centuries, poisons and other biological material have been considered as weapons. However, it has been merely 100 years that the use of biological toxins as weapons has been explored scientifically. Trichothecenes, ricin and botulinum neurotoxins are natural organic toxins with diverse potencies. Their molecular structure, mechanisms of action, detection, clinical diagnosis and therapy are reviewed and their potential as biological weapon is discussed. It is not only the median lethal dose of each toxin that decides on its usability as a biological weapon, but also the availability, scale of production, purity of the isolated material and route of distribution. In general, without a state infrastructure, the use of biological weapons is restricted to assassinations or strictly localised terrorist attacks.

Keywords: Clostridial neurotoxin; Botulinum neurotoxin; Tetanus neurotoxin; Ricin; Trichothecene; Biological weapon; Treatment

1. Introduction

Since the beginning of history, toxins and some biological material have been used for underhand murder and as biological weapons. Due to the lack of knowledge of the mechanisms of action, success was mostly a matter of chance. However, by shrewd observation, deadly materials have been identified such as that described by the Indian poison expert, Schanaqua, 400 years ago: "Fill the gut of a sheep with blood taken from the carotid arteria of a black bull, tighten both ends, and hang it in the shadow of a mulberry tree. When dried grind the content firmly. A pinch of the powder mixed with food will kill the consumers within 3 days." This practical guide to the production of a deadly stuff probably describes a manufacturing process for botulinum neurotoxin (BoNT) which was not recognized as the cause of botulism before the beginning of the 19th century (Kerner, 1817). Its bacterial origin remained in the dark for almost another 100 years (van Ermengem, 1897; Burke, 1919). This was approximately the time when the scientific exploration of the use of biological material for warfare began. Around 1930, experiments to that effect were performed by the Japanese biological warfare group, using humans in place of animals. In occupied Manchuria, prisoners of war were fed *Clostridium botulinum* contaminated food with lethal effects. Fears also arose about the possibility of German BoNT production in World War II. During the cold war, gigantic programs for the development and effective usage of toxins as biological weapons were initiated on both sides of the Iron Curtain. Various other materials of biological origin were considered in addition to BoNT to serve as weapons, e.g. spores of anthrax,

Abbreviations: BoNT, botulinum neurotoxin; CNT, clostridial neurotoxins; ER, endoplasmic reticulum; HC, heavy chain; H_C, 50 kDa C-terminal half of HC; H_N, 50 kDa N-terminal half of HC; LC, light chain; MLD, median lethal dose; RIP, ribosome inactivating protein; RTA, ricin A-chain; RTB, ricin B-chain; scBoNT, single chain BoNT; SNAP-25, synaptosome associated protein of 25 kDa; SNARE, soluble NSF attachment protein receptor; TeNT, tetanus neurotoxin; VAMP, vesicle associated membrane protein or synaptobrevin 2

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ricin or fungal toxins such as trichothecene and aflatoxin. These programs were banned by a United Nations' convention in 1972. However, although nearly all countries signed the treaty, research on BoNT was continued in the former USSR, Iraq and possibly other countries. Currently, biological weapons play only a minor role due to their unpredictable effects, difficulties in usage and, last not least, due to ethical reasons. Nevertheless, it should not be forgotten that terrorist groups might threaten populations with toxins, causing panic and possibly the loss of lives. Although unsuccessful, the Japanese sect, Aum Shinrikyo, actually dispersed aerosols containing BoNT and spores of anthrax at various locations. In autumn 2001 in view of the terroristic attack of 09/11, letters containing spores of a laboratory anthrax strain were sent out to US media personalities and politicians. By the end of the year, there were a total of 22 incidences of anthrax poisoning, of which 19 cases had been confirmed claiming 5 lives and 3 cases had been suspected. This illustrates the omnipresent threat by biological weapons and the need of research to take effective countermeasures. In the following sections, brief descriptions are presented of the modes of action of various toxins, the medical symptoms they cause and treatment of intoxications.

2. Trichothecene mycotoxins

Mycotoxins can frequently cause mild to serious intoxications when spoiled food is consumed. The USSR experimented with mycotoxins during the Cold War, and the Iraq army filled missile warheads with aflatoxins whose distinctive features are extreme hepatotoxicity and carcinogenicity. The trichothecene mycotoxins are markedly cytotoxic (Table 1). Their potency became known from outbreaks of the Akakabi-Byo disease in Japan and an epidemic *Stachybotrys chartarum* intoxication in the southern Ural in 1944. One hundred and fifty sesquiterpenoid compounds with molecular weights of 250–500 Da, which all share a 12,13-epoxytrichothec-9-ene ring system, have been discovered and are subdivided into four chemical groups (Fig. 1). The epoxy function is the reactive part of these toxins. The molecules are heat and hypochlorite resistant and cannot be destroyed by UV light. However, when exposed to sodium hydroxide, the epoxide is hydrolysed and the toxins are rendered inactive. Upon binding to the 60S ribosomal subunit and inactivation of its peptidyltransferase activity (Desjardins et al., 1993), these toxins inhibit the protein biosynthesis of eukaryotic cells.

2.1. Clinical manifestation

The median lethal dose (MLD) in mice varies between 0.5 and 50 mg/kg, decreasing in animals of higher body weight. The substances are lipophilic and well absorbed by the gastrointestinal tract. Trichothecene toxins affect mucosal tissue and, upon exposure, can destroy skin as well. Depending on the ingested dose and the location contaminated, symptoms may occur within a few hours. Initial mucosal irritation is followed by emesis, vomiting, bloody diarrhoea, skin inflammation and haemorrhages. Systemic effects cause bone marrow suppression. The symptoms can last for weeks. An effective therapeutic concept is missing, but glucocorticoids lessen the symptoms in animals. Since the lethal dose in humans is relatively high and the manufacturing and purification require sophisticated techniques and skills, these toxins are unlikely to be abused as terroristic tools.

3. Ricin

Ricin is a so-called AB-toxin. The active (A) part is an enzyme and the binding (B) part navigates the

Table 1
Toxicity of selected toxins in mice

Toxin	MLD_{50} [μg/kg; i.p.]	MW [Da]	Source
Botulinum neurotoxin A	0.0003	150000	Bacteria *C. botulinum*
Tetanus neurotoxin	0.001	150000	Bacteria *C. tetani*
Abrin	0.04	65000	Plant *Abrus precatorius*
Diphtheria toxin	0.10	52000	Bacteria *C. diphtheriae*
Iota toxin	0.2	47500	Bacteria *C. perfringens E*
Ricin	3.0	64000	Plant *Ricinus communis*
Tetrodotoxin	8.0	320	Pufferfish/marine bacteria
Saxitoxin	10.0	300	Dinoflagellate
T-2 trichothecene	1210.0	466	Mold *Trichoderma lignorum*

Median lethal dose (MLD) represents the dose that kills 50% of treated mice. Table modified from Marquardt, H., Schäfer, S., 2004. Lehrbuch der Toxikologie, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, p. 886.

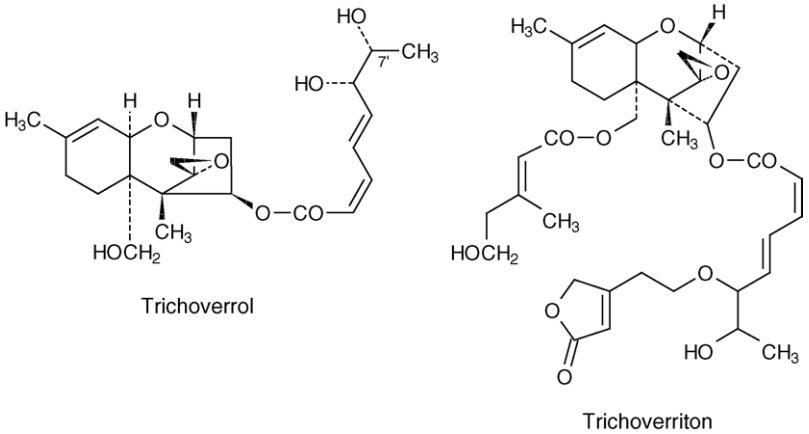

Fig. 1. Trichothecene mycotoxins (examples). All trichothecenes share a 12,13-epoxy-trichothec-9-ene ring system. Trichoverrole and trichoverritone lack an oxo-group at C-8, but possess an unsaturated ester function at C-4.

holotoxin (RTAB) from the extracellular compartment into the cytosol where the substrates are located (Wiley and Oeltmann, 1991). Cholera toxin, e.g. contains one catalytic domain A and five binding domains B and therefore belongs to the class of AB_5 bacterial toxins. Ricin, widely available from the common castor bean plant (*Ricinus communis*), is synthesised as a single polypeptide chain. In plant vacuoles, the single chain is cleaved at a distinct site yielding a dichain protein that remains connected by a disulfide bond (Fig. 2). The ricin B-chain (RTB) is a lectin that binds galactose or *N*-acetylgalactosamine of glycoproteins and glycolipids on the surface of target cells to promote endocytosis and trafficking of ricin from early endosomes to the *trans*-Golgi network (Fig. 3). Subsequent to binding, ricin is transported retrogradely through the Golgi network

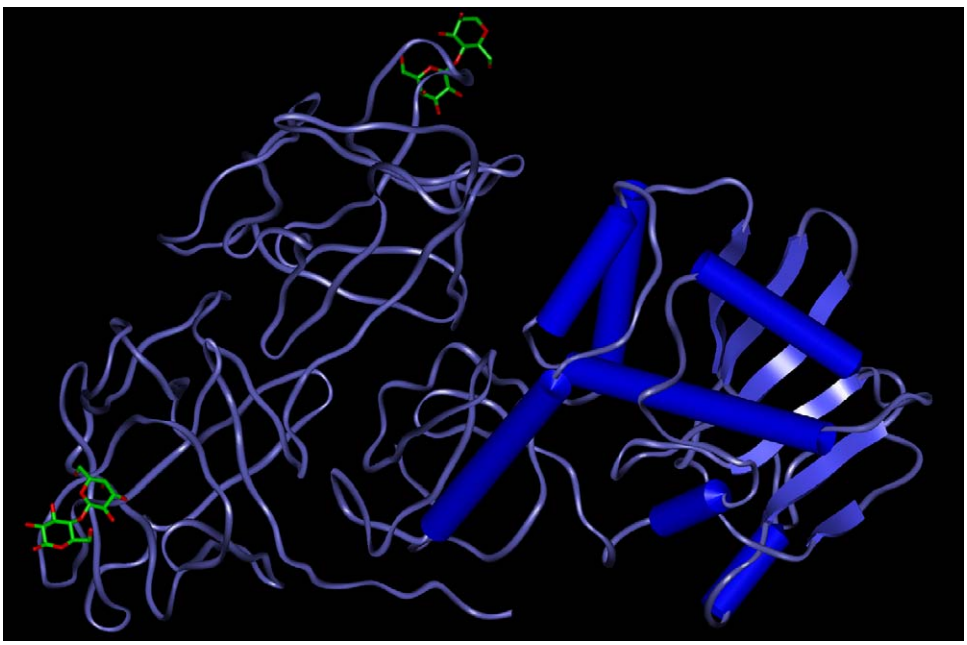

Fig. 2. Cocrystal structure of the AB-toxin ricin. The B-chain (RTB, left domain), responsible for binding, is an *N*-glycosylated homodimer in the shape of a barbell. The 262 amino acids of each monomer form a β-trefoil structure which possesses one saccharide binding pocket each, highlighted by a bound molecule of lactose (stick drawing). The A-chain (RTA, right domain), the catalytically active part, is connected via a disulfide bridge and crystallizes as αβα-sandwich (modified from coordinate file 2AAI.pdb).

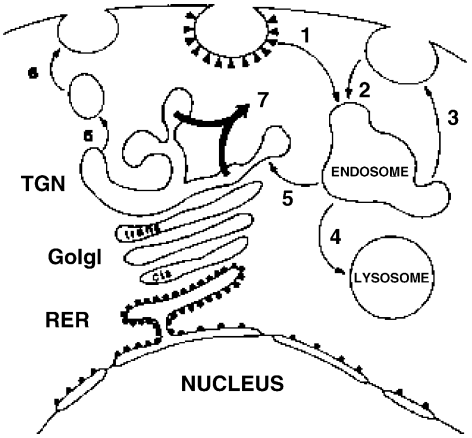

Fig. 3. Pathway for internalization of ricin. Ricin is endocytosed either by coated pits and vesicles (1) or by smooth pits and vesicles (2). The vesicles fuse with an endosome. Many ricin molecules are returned to the cell surface by exocytosis (3), or the vesicles may fuse to lysosomes where the ricin would be destroyed (4). If the ricin-containing vesicles fuse to the *trans*-Golgi network (TGN; 5), there is still a chance they may return to the cell surface (6). Toxic action will occur when RTA, aided by RTB, penetrates the TGN membrane and is liberated into the cytosol (7). Once inside the cytosol, the RTA catalyzes the depurination of the ribosomes, halting protein biosynthesis (EHSO, 2004).

to reach the endoplasmic reticulum (ER), where after reduction of the disulfide bridge, the two chains separate. Then, the ricin A-chain (RTA) is partially unfolded and, thus, enabled to cross the ER membrane via the Sec61p translocon. This happens in a manner similar to that followed by misfolded ER proteins which, when recognized, are targeted to the ER-associated protein degradation (ERAD) machinery. Ubiquitination – the ATP-dependent multiple labelling of a condemned target protein with the 76 residue protein ubiquitin – would lead to rapid degradation of RTA by the 26S cytosolic proteasome immediately after membrane translocation, but can partially be avoided in case of unfolded RTA. Finally, RTA refolds into its protease-resistant, biologically active conformation. The same route from the extracellular plasma membrane into the cytosol is taken by cholera toxin, shiga toxin and shiga like toxins. The RTA is a highly efficient *N*-glycosidase ($k_{cat}/K_m \approx 10^7\ M^{-1}\ s^{-1}$; Endo and Tsurugi, 1988). Once inside the cell, RTA acts as a 'ribosome inactivating protein' (RIP) by removing a specific adenine base of the essential 28S ribosomal RNA and on account of this, aminoacyl-tRNA can no longer bind to the truncated 28S rRNA. Thus, protein biosynthesis is stopped and apoptosis induced (reviewed in Lord et al., 2003). The amino acid sequence of RTA shares large homologies with the enzymatic part of shiga toxin, and the mode of action of both enzymes is the same.

3.1. Detection and clinical manifestation

In the event of contamination with ricin, immunoassays are available for its detection.

Ricin is destroyed by acid but resists heat and the pancreatic enzymes. Its toxicity is approximately 3–5 μg/kg body weight (Table 1). Ricin has no selectivity for specific cells. Since all types of cells are affected, symptoms depend on the locus of entry of the toxin into the body. Depending on the dose, first symptoms occur after a latent period of 2–24 h.

After inhaling significant amounts of ricin, the victim may suffer from breathing difficulties, fever, cough, nausea and tightness in the chest. Heavy sweating may follow as well as pulmonary oedema. This makes breathing even more difficult, and the victim becomes cyanotic. Finally, low blood pressure and respiratory failure may occur, leading to death. After oral ingestion, victims develop vomiting and diarrhoea that may become bloody. Severe dehydration and low blood pressure are the consequence. In severe cases, victims suffer from hallucinations and seizures. Within a few days, the victim's liver, spleen and kidneys may stop working. Fatalities from ricin poisoning may ensue within 36–72 h after exposure, depending on the dose and route of administration.

3.2. Therapy

Previous attempts to produce a vaccine for ricin have been hampered by safety concerns arising from residual toxicity of native material after formaldehyde treatment. The use of recombinant isolated RTA carrying mutations that render it inactive lead to undesirable aggregation or precipitation caused by hydrophobic sites that were exposed on the RTA in the absence of its natural RTB-chain partner. With the help of protein engineering, an immune serum is presently under development (Olson et al., 2004).

After exposure to ricin, possible antidotes include sugar analogues that prevent binding of ricin to its target and the AIDS drug azidothymidine (AZT) that may inhibit the catalytic subunit but antitoxins are lacking. The most important point is cleaning the affected body parts as soon as possible. Supportive medical care should be supplied to minimize the effects of poisoning. The extent of supportive medical care depends on several factors, such as the route of poisoning. Care could include artificial respiration and replacements of electrolytes and fluids. Medications against seizures and low blood pressure should be given if required. In case of ricin ingestion, the toxin is absorbed onto generous

quantities of superactivated charcoal and the digestive tract is then cleared out with a cathartic. If death does not occur within 3–5 days, the victims will usually recover.

3.3. Bioterroristic hazards

Castor beans are the fruits of the castor plant which grows in Asia, the Middle East and southern Europe. In northern countries, it serves as an ornamental plant. A few beans may contain a dose lethal for an adult. Manufacturing of a crude ricin preparation is easy and sufficient for terroristic purposes. Since it affects the mucous membranes of the mouth and the upper airways, the eyes and even the unprotected skin, an aerosol of any size of particle would harm people. Employing affinity chromatography, it is possible to isolate in a single step 180 mg of pure ricin from 100 g of castor beans (Woo et al., 1998). This procedure, however, necessitates a higher technical standard than is required for the preparation of a crude extract from harvested beans. The production of recombinant ricin holotoxin has been limited to the expression of RTA or RTB moieties alone, or the expression of incompletely processed toxin in *Xenopus laevis oocytes* due to extensive hydrophobic areas on the ricin molecule surface. Thus, since the raw material is readily available and can be easily processed to yield a crude extract of high potency, ricin represents a serious terroristic threat.

Abrin, a toxin from the pea *Abrus precatorius* with similar characteristics as ricin, however, with an even higher toxicity (Table 1), must be assessed similarly.

4. Botulinum neurotoxins

BoNT, which cause three varieties of naturally occurring human botulism (food-borne, wound and intestinal botulism in infants and adults), are the most potent agents known (Bigalke and Shoer, 2000). The calculated MLD of BoNT/A for humans is approximately 0.3 ng/kg after intravenous application (Table 1), 20 ng/(min m^3) after inhalation and 1 μg/kg following ingestion. These figures, however, give only a rough estimate and were partly deduced from experiments with rodents (Cardella, 1964) and accidental intoxications in man (Naumann and Moore, 2003). Experiments with primates showed a higher toxicity, when BoNT entered the organism via the respiratory route (Franz et al., 1993). The lethal dose depends dramatically on the grade of purity. The cleaner the neurotoxin is, the less toxic it is, if administered by the oral route. For example, this is true for pure BoNT/A, which is almost a factor 100,000 less toxic than the BoNT/A complex by the oral route. The reason for this contradictory behaviour rests on the sophisticated composition of the neurotoxin complex, as assembled by the anaerobic, spore forming Gram-positive bacillus, *C. botulinum*. The neurotoxin complex is released into the environment and, in case food is contaminated, potential consumers may ingest the BoNT complex together with the food. The BoNT, which is a protein, has to resist the low pH of the stomach as well as the attack of pancreatic enzymes in the upper small intestine before it is absorbed in the lower intestine. The safe passage through the hostile environment of the gastrointestinal tract is ensured by accompanying proteins, consisting of various haemagglutinins (HA) and a non-toxic non-haemagglutinating (NTNH) protein of 120 kDa (Mutoh et al., 2003). Together with the BoNT, they form different complexes, also-called progenitor toxins, which are resistant to proteases and acids but decompose immediately at physiological pH. HA acts as an adhesin, allowing the progenitor toxin to bind to intestinal epithelial cells and erythrocytes (Fujinaga et al., 2004). When applied by the oral route, however, the pure neurotoxin loses most of its toxicity, because it is almost completely destroyed before absorption. The same might be true if the neurotoxin is inhaled, because the surface of the mucous membrane in the lung is rich in proteolytic activity. The pure neurotoxin, however, does not lose its toxicity when applied parenterally, e.g. intraperitoneally, because a protection against proteases is not required in that case.

4.1. Mechanism of action

The seven serologically different BoNT, named A–G, belong to the clostridial neurotoxins (CNT) and are produced by various strains of *C. botulinum*, *C. barati* and *C. butyricum*. These toxins and the closely related tetanus neurotoxin (TeNT) synthesised by *C. tetani* are produced as ∼150 kDa single chain (sc) proteins and subsequently cleaved by endogenous proteases into a ∼100 kDa heavy chain (HC) and a ∼50 kDa light chain (LC), which remain linked together by a single disulfide bridge and non-covalent interactions. Like ricin, the CNT therefore belong to the AB class of toxins. The recent elucidation of the crystal structures of BoNT/A and B (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000) illustrates that all CNT are composed of three functionally independent domains that perform individual tasks in the multi-step intoxication process (Fig. 4). Intoxication at the neuromuscular junction starts with specific binding to peripheral nerve endings. It is well established that gangliosides, a class of complex glycosphingolipids

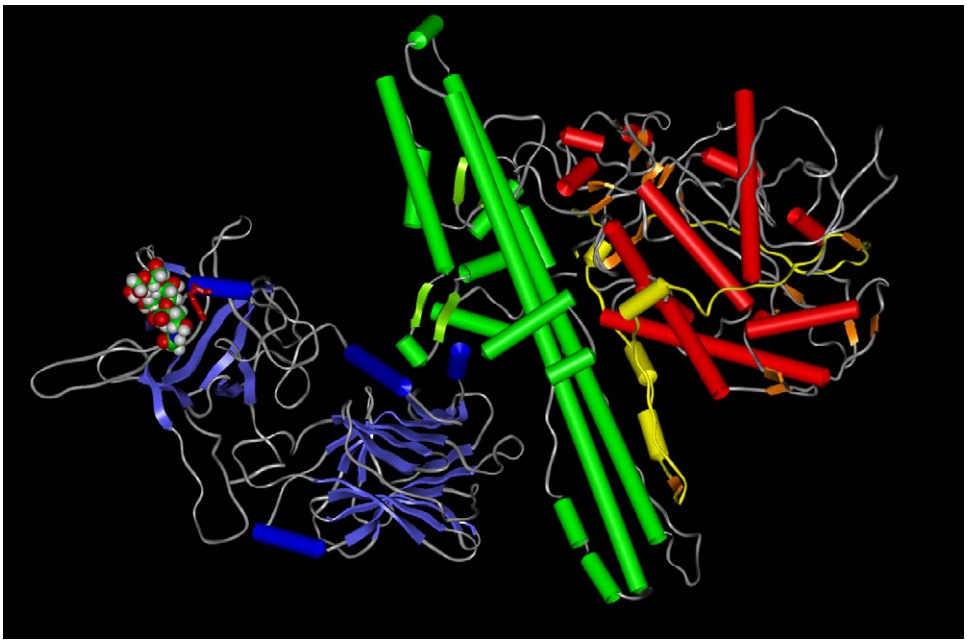

Fig. 4. Cocrystal structure of BoNT/B and sialyllactose. The 50 kDa H_C-fragment is responsible for neurospecific binding (far left domain). It contains two subdomains of which the C-terminal half possesses the ganglioside binding pocket indicated by the binding of one molecule of sialyllactose (spacefilling drawing). The 50 kDa translocation domain (H_N, domain in the middle) is highly α-helical and holds the 50 kDa light chain (LC, far right domain), acting as Zn^{2+}-dependent endoprotease, connected via a disulfide bridge and a polypeptide chain (modified from coordinate file 1F31.pdb).

that are found particularly in membranes of neuronal cells, and the carboxy-terminal half of the HC, the 50 kDa H_C-fragment, are involved in this (Halpern and Neale, 1995; Rummel et al., 2003, 2004a). To account for greatly differing affinities depending on whether CNT binding takes place in vitro or in vivo, a two-receptor model has been postulated. According to this model, gangliosides accumulate CNT on the plasma membrane surface, while protein receptors mediate specific endocytosis (Montecucco, 1986; Niemann, 1991). Synaptotagmin I and II were shown to be involved in the binding of BoNT/B (Nishiki et al., 1994) and BoNT/G (Rummel et al., 2004b). Following cell attachment, internalization via receptor-mediated endocytosis directs the neurotoxins into the endosomal compartment. The acidic environment of this organelle triggers a structural rearrangement and concomitant insertion of the amino-terminal half of the HC, the 50 kDa H_N domain, into the membrane. This route differs from that of ricin which travels via the Golgi network retrogradely to the ER. At the same time, the LC is partially unfolded (Koriazova and Montal, 2003) (Fig. 5). Upon reduction of the disulfide bond, the LC acts as a zinc-dependent endopeptidase in the cytosol. LC hydrolyses specifically one of the three neuronal soluble NSF attachment protein receptor (SNARE) proteins, which form the SNARE-complex thereby fusing synaptic vesicles with the presynaptic membrane. The vesicle associated membrane protein (VAMP) or synaptobrevin 2 is one of the three SNARE proteins and constitutes the substrate for BoNT/B, D, F, G and TeNT, whereas the synaptosome associated protein of 25 kDa (SNAP-25) is cleaved by BoNT/A, C and E. Except for BoNT/B and TeNT, which share the same cleavage site, hydrolysis occurs in unique positions. In addition, BoNT/C is capable of hydrolysing the third SNARE protein, syntaxin 1A. Cleavage of any of the neuronal SNARE proteins results in inhibition of the fusion of synaptic vesicles with the presynaptic membrane, and, consequently, neurotransmitter release is blocked (Bigalke and Shoer, 2000) (Fig. 6).

The symptoms caused by BoNT and TeNT are diametrically opposite: BoNT cause flaccid paralysis, while TeNT provokes spastic paralysis. BoNT act at their site of entry in synapses of the neuromuscular junction, whereas TeNT embarks on a journey to the spinal cord, using the axons of motoneurons for transportation. On arrival, it is discharged into the synaptic cleft, binds to inhibitory interneurons and undergoes endocytosis and translocation into presynaptic terminals, where the LC finally inhibits neurotransmitter release. Why the CNT

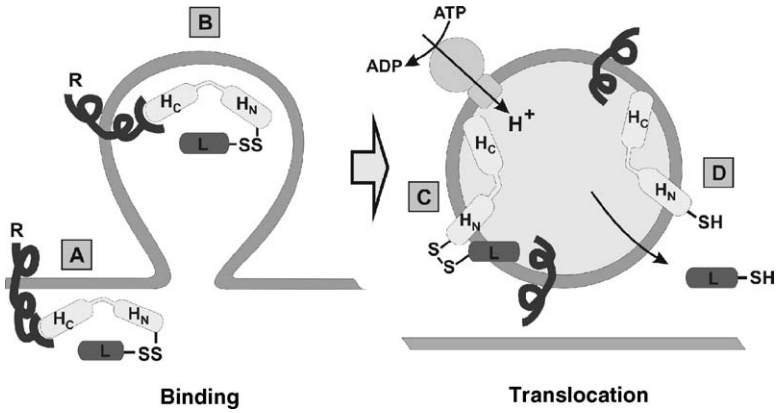

Fig. 5. Binding, translocation and reduction of BoNT. BoNT binds to two membrane located receptors via its H_C-fragment (A), is endocytosed (B) and arrives via vesicle maturing in the endosomal compartment where an ATPase acidifies the environment. Thereupon, the H_N domain incorporates into the membrane (C) and the disulfide bridge gains access to the cytosol where a thioredoxine reductase cleaves the bond liberating the LC (D). The catalytically active LC hydrolyses one of the three SNARE proteins to inhibit SNARE complex formation and subsequent exocytosis.

are sorted differently in peripheral neurons has not yet been established.

4.2. Clinical manifestation

Four clinical forms of botulism occur in humans: food-borne botulism, wound botulism, infant botulism (infant intestinal colonization) and, rarely, adult infectious botulism (adult intestinal colonization). From 1973 to 1996 in the US, 724 cases of food-borne botulism (median, 24 cases annually [range, 8–86 cases]), 103 cases of wound botulism (median, 3 cases annually [range, 0–25 cases]), 1444 cases of infant botulism (median, 71 cases annually [range, 0–99 cases]) and 39 cases of botulism of undetermined type were reported to the Centers for Disease Control and Prevention (CDC, 1998). In the US, approximately half of the cases of food-borne botulism are caused by BoNT/A; the remaining food-borne cases are almost equally divided between BoNT/B and E. Among cases of infant botulism, approximately half are caused by BoNT/A and B, respectively; among cases of wound botulism, approximately 80% are caused by BoNT/A and 20% by BoNT/B (CDC, 1998). BoNT/C_1 and D cause botulism in wildlife and domestic animals but have no part in human food-borne disease. However, humans are thought to be susceptible to these serotypes because they have caused botulism in primates (Gunnison and Meyer, 1930). Similar to BoNT/A and B with respect to dose, mode and duration of action, BoNT/C_1 has recently been shown to affect humans (Eleopra et al., 2004). BoNT/G, produced by a bacteria species discovered in South American soil in 1969 (Giménez and Ciccarelli, 1970), has never been described to cause recognized food-borne botulism. The differences in BoNT toxicity may be due to the individual compositions of progenitor toxins, varying degrees of nicking between LC and HC, or different binding affinities and distributions of surface receptors. Despite these disagreements, the symptoms of intoxications with the various serotypes of BoNT closely resemble each other, because the modes of action of the neurotoxins are the same. However, there might be differences in the degree peripheral organs are affected. For example, BoNT/B mainly blocks acetylcholine release from vegetative nerve endings (Merz et al., 2003) while BoNT/A acts on somatomotoric and vegetative nerve endings. Nevertheless, first symptoms appear 12–72 h after ingestion. In

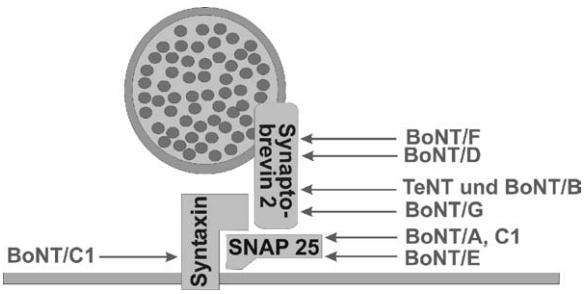

Fig. 6. Substrates of CNT. The plasma membrane- and vesicle membrane-located SNARE proteins syntaxin 1A, SNAP-25 and synaptobrevin 2 build the stable SNARE complex that is being formed upon an increase of the cytosolic Ca^{2+}-concentration thereby pulling the vesicle and the plasma membranes together. Eventually, both membranes fuse and the neurotransmitters are released into the synaptic cleft. The SNARE proteins are the substrates of the CNT. After hydrolysis of the SNARE proteins, their complex cannot be formed anymore and exocytosis is blocked.

Table 2
Symptoms of food-borne botulism by BoNT/A and B

Symptoms of botulism
Fatigue
Dizziness
Double vision
Blurred vision
Dysphagia
Dry mouth
Dysarthria
Sore throat
Dyspnea
Weakness of extremities
Paresthesia
Ptosis
Gaze paralysis
Pupils dilated or fixed
Nystagmus
Diminished reflex
Tongue weakness
Hyporeflexia or areflexia
Ataxia

the case of BoNT/A, the inhibition at the neuromuscular junction is prominent. Tightly controlled muscles, such as those in charge of eye movements, are paralysed first. In severe cases, all striated muscles are enervated to some extent so that respiratory failure may develop. With BoNT/B autonomic disturbances, such as mydriasis, dryness of the mouth and eyes, tachyarrhythmia, atony of the intestine and adiaphoresis, are prominent. At higher doses, motor disturbances resemble those provoked by BoNT/A. An almost complete list of symptoms is given in Table 2. Depending on the serotype and the doses ingested, the disease may last from a few days to several months. Intoxication with BoNT/A is of the longest duration, whereas a patient poisoned with BoNT/E, even if it is a high dose, will recover within a few days. Recovery occurs when the LC of the neurotoxin inside the nerve ending is degraded to inactive fragments. Then, newly synthesised SNARE proteins will remain intact and membrane fusion will be reactivated (Erdal et al., 1995; Adler et al., 2001; Fernandez-Salas et al., 2004).

4.3. Detection

Spores or bacteria of *C. botulinum* can be highly sensitively detected and characterised by PCR methods like pulsed-field gel electrophoresis (PFGE). Recent advances employing mass spectroscopy either allow the direct detection of the protein BoNT or use an enzymatic assay to analyze peptides that are specific cleavage products of the substrates of BoNT.

In the event of intoxication with BoNT, immunoassays for the detection of the neurotoxin in body fluids are available. The neurotoxicity of the BoNT source can be either determined using the extensive mouse bioassay or the highly sensitive, quick and reliable mice phrenic nerve assay. The latter one also provides a method to detect neutralizing antibodies in infected or medically treated human beings (Göschel et al., 1997).

4.4. Therapy

A pentavalent toxoid, already in use inside the US army, is available for special workforces to prevent intoxication. A mixture of seven recombinant H_C-fragments should replace the toxoid as a vaccine in the future (Smith et al., 2004). Research is conducted to identify inhibitors of the toxins that may inhibit the catalytic active site in the LC. These drugs will only be available in an emergency and may circumvent the adverse effects of active immunization.

Therapy consists mainly of supportive care because there is no specific treatment. Passive immunization with heptavalent equine antiserum may inactivate the BoNT that is still present in the blood stream. Neurotoxin taken up by nerve endings cannot be neutralized, and manifest symptoms will not be reversed by antiserum. Therefore, the earlier antiserum is applied, the more effective the protection is. In mild cases, when respiration is not affected, clinical observation of the patient and symptomatic treatment of unpleasant symptoms may be sufficient. More severe progressive forms may require feeding by gavage or parenteral nutrition and prophylactic treatment with antibiotics to prevent secondary infections. Since the pharyngeal muscles are paralysed, patients have difficulties in swallowing. Danger of aspiration is a constant threat. Therefore, the airways have to be kept open and, in severe cases, artificial respiration with a pharyngeal tube is indicated. The longer the disease takes, the greater is the risk of a secondary infection. If a patient dies from the disease, it is usually such a badly controlled infection that it is the cause of death.

4.5. Bioterroristic hazards

Because of the extreme toxic nature of BoNT, growing concerns have arisen about the possibility of abusing these agents as terroristic weapons. In contrast to other biological organisms, e.g. virulent and hazardous viruses or anthrax spores, clostridia occur everywhere in our environment, and it is easy to produce a crude neurotoxin preparation (see method

provided by Shanaqua). However, material produced by less sophisticated methods would probably be very voluminous and its distribution not very efficient. Since pure neurotoxin is only toxic by a parenteral route, a potential manufacturer would have to try to produce the intact complex and purge it, to a great extent, of concomitant impurities. This purification process requires a well equipped microbiological laboratory and skilled techniques. From a terrorist point of view, to distribute the neurotoxin complex efficiently, it would have to be processed so as to allow contamination over a large area without loss of toxicity. Moreover, circumstances should ensure that potential victims come into contact and be poisoned with the neurotoxin. As discussed earlier, absorption could occur via the lungs or the intestine. When choosing the respiratory route, one has to be aware that the neurotoxin cannot easily penetrate membranes. In contrast to ricin, BoNT is absorbed in the lower part of the lungs, the alveoli. Thus, it has to be aerosolised in a particle size that allows it to invade this part of the lung. The manufacturing of aerosols with these characteristics requires also sophisticated technical skills that can only be provided by laboratories with a high technical standard. The Aum cult probably had no sufficient knowledge of these difficulties and so they did not succeed in administering neurotoxin in an aerosol. The different toxicities observed by Franz et al. (1993) and Cardella (1964) also illustrate the problem. The technical progress over a period of 30 years enables us nowadays to manufacture aerosols of very small sized particles, which was technically not feasible in 1964. Therefore, Cardella found a much lower toxicity than Franz. A government can provide these sophisticated techniques, but can terrorists match it? An easier way to terrorise people is the contamination of food and drinking water. In general, the sources of drinking water are not freely accessible to the public. However, large reservoirs are a potential target. Taken into consideration that average people drink less than 1 l of fresh water per day (water used for coffee or tea would be decontaminated by boiling) and that at least approximately 15 μg BoNT has to cross the intestine to reach the terrorist's goal, one can calculate how much BoNT stock would be required to contaminate a lake containing billions of litres of water. It is obvious that such a scheme would not be a success. Thus, contamination of food would appear to be the easiest way left to terrorise the population, and there have been cases of blackmailers who have poisoned packed food in supermarkets. However, the risk to be caught is substantial because, generally, supermarkets are kept in under supervision with closed-circuit television.

Another aspect is the mass production of recombinant BoNT. With the help of affinity tags one-step purifications can be applied to large amounts of material. But again, this procedure would hardly be a success since recombinant BoNT lacks the protection by accompanying proteins which are physiologically provided to native BoNT. Therefore, it would be nearly inactive, if given by the oral route and the same holds probably true for the respiratory route as well. These considerations may not apply when state terrorism is involved, because governments could provide skilled techniques, lots of money to buy the necessary equipment and keep the production a secret. Iraq offers the best example of state terrorism. After the 1991 Persian Gulf War, Iraq admitted to the United Nations' inspection team to having produced 19,000 l of concentrated BoNT, of which approximately 10,000 l were loaded into military weapons. These 19,000 l of concentrated neurotoxin constitute approximately three times the theoretical amount needed to kill the entire current human population by inhalation. In 1990, Iraq deployed specially designed missiles with a 600-km range; the warheads of 13 of these were charged with BoNT, 10 were charged with aflatoxin and another 2 with anthrax spores. Iraq also deployed special 180 kg bombs for immediate use; 100 bombs contained BoNT, 50 contained anthrax spores and 7 contained aflatoxin (United Nations Security Council, 1995). It is noteworthy that Iraq chose to weaponise more BoNT than any other of its known biological agents.

5. Summary

History teaches that biological toxins can always be employed as weapons. Detailed scientific knowledge is the basis for effective countermeasures. Despite the lack of effective therapeutic concepts against trichothecenes intoxications, the use of these mycotoxins as biological weapon is insignificant because their isolation requires sophisticated techniques and their MLD of 5–50 mg/kg is relatively high compared with that of ricin and BoNT. The most toxic biological substance, BoNT, is easily processed as a crude mixture but its extensive delivery requires very sophisticated procedures. Furthermore, sensitive detection and diagnostic assays as well as various therapeutic options devalue the high potency of BoNT. Although ricin is less toxic than BoNT/A by a factor of about 10,000, it is ubiquitously available in big quantities and an intoxication cannot be effectively treated. Thus, ricin represents a higher threat to the public than the other discussed agents which was evidenced by reports in January 2003 about the arrest of several Arabs with connections to Al-Qa'ida trying to produce

ricin in a north London apartment. In general, without a state infrastructure, the use of biological toxins is limited to assassinations or strictly localised terrorist attacks.

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