Toxinology
Editor-in-Chief: P. Gopalakrishnakone

Mahdi Balali-Mood
Lyndon Llewellyn
Bal Ram Singh *Editors*

Biological Toxins and Bioterrorism

Toxinology

Editor-in-Chief
P. Gopalakrishnakone

In recent years, the field of toxinology has expanded substantially. On the one hand it studies venomous animals, plants and micro organisms in detail to understand their mode of action on targets. While on the other, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their three interaction with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on human body. Given the huge impact of toxin-based deaths globally, and the potential of venom in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that collects all of the important biochemical, biomedical and clinical insights relating to toxinology.
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Biological Toxins and Bioterrorism

With 123 Figures and 30 Tables

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Series Preface

The term TOXIN is derived from the Greek word *Toeikov* and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On the one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology, etc. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally and the potential of venom in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field are imminent.

Springer has taken the bold initiative of producing this series, which is not an easy target of producing about 11 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

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Acknowledgments

I would like to sincerely thank the section editors of this volume, Mahdi Balali-Mood, Lyndon Llewellyn, and Bal Ram Singh, for the invaluable contribution of their expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to Mokshika Gaur, Meghna Singh, and Audrey Wong for their tireless effort in bringing these volumes to reality.

Singapore

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Volume Preface

Biotoxins are an important part of our world, a reality with which we need to cope, so in parallel with understanding their mechanisms of action and thereby improving our fundamental knowledge, there are successful efforts to utilize them as therapeutics against some debilitating human and animal diseases. Over the past several decades, researchers throughout the world have developed techniques and tools to detect these toxins, modify them for specific uses, and develop countermeasures against their effects so that their impacts can be managed.

After the use of chemical warfare agents during World War I, all nations were against any chemical weapon. In spite of the Geneva Protocol in 1925 and further chemical and biological weapon conventions and conferences (the latest one held in 2011) in the last century, both chemical and biological warfare agents were unfortunately applied in war (Iraq-Iran conflict) and terrorism (Matsomoto and Tokyo metro). Due to the application of high technology in biotoxin production, biowar and bioterrorism are now an even bigger threat than chemical war and terrorism. Biotoxins may be abused as bioweapons. As a result, government agencies needed to address it and have developed policies and regulations to allow research on these toxins smoothly.

In view of the complexity of different types of biotoxins and the broad range of toxin structure, physiology, utility, and countermeasures including regulatory issues, it was thus aimed to compile a book on biotoxins and bioweapons.

Some biotoxins are highly potent when administered to victims via a variety of routes and can often be manufactured by means available to even the simplest laboratory, making them ideal candidates for weaponization. But their utility is not solely as weapons. Their exceptional ability to attack physiological mechanisms critical to our survival has allowed us to probe the molecular structures of the enzymes and receptors they attack, improving our understanding of ourselves and the biodiversity important to people everywhere. These natural chemicals can also intrude into our daily lives by contaminating our food and water supplies, being the instrument by which a disease pathogen can sicken us and allowing some animals to defend themselves against predators or subdue their prey.

This volume gathers together knowledge from around the globe about naturally inspired and manufactured bioweapons. The authors describe how they work; how authorities may detect their presence, prevent their use, and diagnose their impacts; and the means by which medical and paramedical professionals may treat victims. Also described are how they have been used to further our knowledge and what insights they have given us into evolutionary and physiological processes. Finally, it is also discussed how these toxins can be used as therapeutics and what the implications of such therapeutics are to their use as biothreat agents.

We have endeavored to provide a reference accessible to scientists, educators, and medical experts alike with an interest in biotoxins, focusing on the major toxins used as bioweapons. Regulatory agencies will also benefit from the information provided in this book. Some in the intended audience may need to understand how they elicit their effects and how we can defend ourselves against them. Others may be interested in the sometimes colorful histories that surround this subset of biotoxins that can be and, in some cases, have been used as weapons.

We are of course deeply grateful to the authors for sharing their expertise with us and contributing to this initiative. We should thank the editor-in-chief, Professor Gopalakrishnakone of the National University of Singapore, whose foresight brought this series to life and allowed us to contribute to the vision. We are also very thankful to Mokshika Gaur, Meghna Singh, and Audrey Wong of Springer who maintained our focus.

We would welcome any comments and feedback from experts in biotoxins and bioweapons to consider for future editions.

January 2015

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Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of *Toxicon*, the society's official journal.

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Dr. Llewellyn also has extensive experience with very large data sets and their management; database design, interrogation, and visualization; as well as technology development, specifically with biosensors. He has led and managed major projects with industry collaborators ranging in size from start-ups to publicly traded companies. Dr. Llewellyn has authored 76 research publications, 12 major technical reports, and an electronic data atlas and is an inventor on international patents. A regular reviewer for national and international granting agencies, he has also been an invited reviewer for almost 30 scientific journals covering a wide variety of disciplines such as analytical chemistry, biochemistry, toxicology, microbiology, and biomedicine.

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Dr. Singh has published over 175 research articles, has edited/coedited 10 books, and has obtained 8 patents. He is the editor of *The Botulinum Journal* and managing editor of *Ayurveda Journal of Health*.

Dr. Singh joined the faculty at the University of Massachusetts Dartmouth in 1990, was tenured in 1995, and rose through ranks to become full professor in 1999. He has held visiting professorships at Georgetown University School of Medicine and Harvard Medical School. He took retirement in June 2014 to set up the Institute of Advanced Sciences to advance his research and educational interests independently.

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Section I

Biotoxins with Potential as Bioweapons

1 Structure, Genetics, and Mode of Disease of Cholera Toxin

Keya Chaudhuri

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Abstract

Cholera is a potentially epidemic severe watery diarrhea which might lead to death, if untreated. The causative agent, *Vibrio cholerae*, secretes a potent enterotoxin called cholera toxin (CT) which is largely responsible for the disease. CT consists of one enzymatically active A-subunit and five immunogenic B-subunits. The B-subunits form a symmetrical pentamer into which A chain sits on the top creating a wedge-shaped structure. Both A- and B-subunits are encoded by the CTX genetic element which corresponds to the genome of an integrated filamentous bacteriophage, CTXφ. The regulation of cholera toxin expression is controlled by a cascade of regulatory proteins typically referred to as the ToxR regulon. The secretion of cholera toxin from the outer membrane is mediated by type II secretion system, popularly known as T2SS pathway. CT has been shown to be associated with outer membrane vesicles which are internalized through CT receptor; whether a fraction of CT can be secreted through these vesicles remains to be determined. For its action in the human intestine, B-subunits of CT secreted extracellularly by *V. cholerae* bind to GM1 ganglioside receptors in the intestinal epithelial cells, and then the A-subunit induces adenylate cyclase activity resulting in cyclic adenosine monophosphate activation and fluid secretion. Various natural ligands have been reported to act as inhibitors of cholera toxin such as those originating from black tea, apple, garlic, and ginger. The knowledge described in the present brief review might be useful for further steps in the studies on CT and its remediation.

Introduction: Historical Perspective

The term cholera originated in the distant past and many ancient cultures were touched by the disease. It existed in the times of Lord Buddha and Hippocrates, possibly earlier. The first recorded instance of the disease was in 1563 in an Indian medical journal (http://www.choleraandthethames.co.uk/cholera-in-london/origins-of-cholera/). The term cholera may be derived from Greek meaning “bilious” or from Hebrew meaning “bad disease.” As early as 1854, Filippo Pacini, an Italian anatomist, first observed the causative agent of cholera under a microscope and named them *Vibrio cholera*e. John Snow, a British physician, described the waterborne nature of the disease through his epidemiological studies. Later on, in 1884, Robert Koch demonstrated in Calcutta (now Kolkata), India, that cholera is caused by some comma-shaped organism which he isolated in pure culture. He named these as “Kommabazillen” or Kommabacillus, and subsequently these were called *Vibrio* comma. Pacini’s work was overshadowed by Koch’s discovery. However, after several decades the work of Pacini was recognized, and the name was changed to *Vibrio cholerae*. In 1817, the first pandemic started in India and spread to several countries dissipating in 1823. Later, seven pandemics of cholera have been reported as summarized earlier (see Chaudhuri and Chatterjee 2009).

The existence of cholera toxin was proposed by Robert Koch. In his original report, Koch described the disease as “intoxication,” which, according to his

opinion, was due to the existence of a "poison" in the causative organism. The discovery of cholera toxin had to wait till the middle of the twentieth century. It was the pioneering research of SN De in Calcutta from 1952 to 1959 which demonstrated the evidence of a toxin in *V. cholerae*. De was able to replicate the disease in a rabbit ileal loop model by injecting *V. cholerae* in the ligated ileal loop of rabbit (De and Chatterje 1953). In a 1959 Nature paper, he reported that cell-free *V. cholerae* culture filtrate injected in rabbit ileal loop was capable of producing massive fluid accumulation, clearly demonstrating that cholera toxin is an exotoxin (De 1959). In the same year NK Dutta and colleagues from Haffkine Institute, Bombay (now Mumbai), India, demonstrated diarrhea in infant rabbits to be caused by a crude protein isolated from the culture filtrate of *V. cholerae* (Dutta et al. 1959) supporting De's observations. De's discovery of cholera toxin and its exotoxin nature became the foundation of modern cholera research of understanding the molecular basis of the disease.

Cholera toxin was subsequently isolated and purified by Richard Finkelstein's group (Finkelstein and LoSpalluto 1969, 1970). His group purified the active ingredient from *V. cholerae* culture supernatants by using gel filtration chromatography and membrane ultrafiltration technique and called it "choleragen." Analysis of cholera toxin by SDS-polyacrylamide gel electrophoresis, which dissociates and separates non-covalently bound protein components, showed two different types of subunits. "Choleragen" was differentiated from "choleragenoid." The latter was a protein of approximately 56,000 Da molecular weight which spontaneously formed from dissociation of choleragen and termed as choleragenoid. It was antigenically identical to the parent toxin but lacked the toxic activity. This was subsequently identified as nontoxic and immunogenic B-subunit pentamer. Finkelstein's group could crystallize choleragen and choleragenoid, recombine them, and reconstitute active toxin (Finkelstein et al. 1974). The active principle "choleragen," which is toxic in nature and identified as A-subunit, and nontoxic B-pentameric "choleragenoid" both contributed to elicit the symptoms of cholera. The heterogeneous subunit structure of CT was subsequently demonstrated by means of biochemical techniques to consist of one A-subunit associated with five B-subunits.

Cholera Toxin Structure

Structurally, cholera toxin belongs to the AB_5 bacterial toxin family named after the characteristic architecture consisting of a catalytically active A-subunit associated with nontoxic pentameric B-subunit (B_5). The A-subunit is of 258 amino acids (aa) in length in strain *V. cholerae* El Tor N16961 (locus number VC1457). This consists of signal peptide (1–18 aa), A1 chain (19–212 aa), and A2 chain (213–258 aa). The A1 chain, alternately called CT alpha chain or nicotinamide adenine dinucleotide (NAD) (+)-diphthamide adenosine diphosphate (ADP)-ribosyl transferase, is responsible for the toxic enzymatic activity. The A2 chain is alternatively called cholera enterotoxin gamma chain. The two chains A1 and A2 are linked by a disulfide bridge between cysteines at 205 and 217 aa positions. The A1 subunit possesses protein

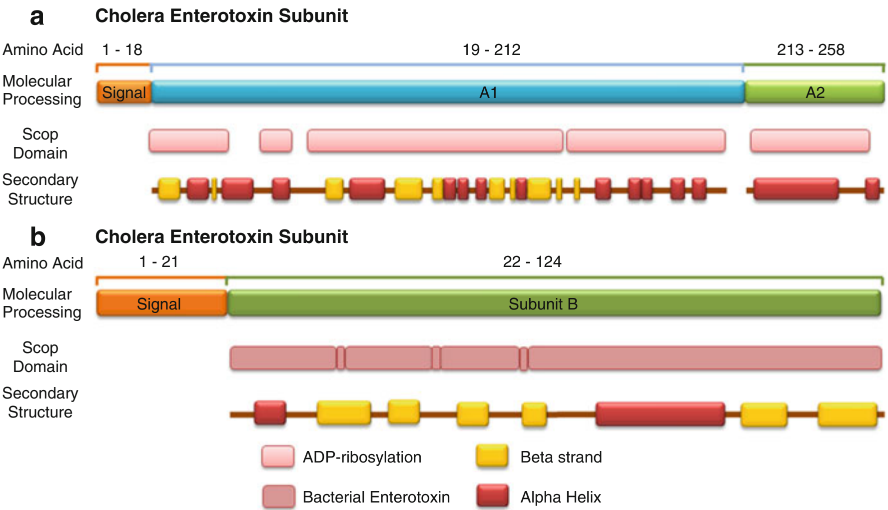

Fig. 1 Domain organization and secondary structure of cholera enterotoxin subunits *A* and *B*. In the secondary structure, the *lines* represent the coiled structure

ADP-ribosylation domain. The B-subunit monomer is 124 aa long. This consists of a signal peptide (1–21 aa) and a mature B chain (22–124 aa). Five B-subunits assemble together to form a pentameric ring.

The secondary structure of subunit A is composed of mixtures of alpha-helix, beta-sheet, and coil structural elements (Fig. 1). The A1 chain (19–212 aa) is mostly alternate alpha and beta chains, while A2 is composed of alpha-helical structure. The secondary structure of cholera toxin B-subunit (Fig. 1) shows that the amino acid residues are distributed among only two alpha-helices and the remainder are beta-strands which enable its membrane association property. It encompasses one disulfide bridge formed between cysteines at 30 and 107 aa residues.

The three-dimensional structure of hexameric AB_5 cholera toxin was solved and refined by X-ray crystallography at 2.5 Å resolution (Zhang et al. 1995). This structure resembles that of heat-labile enterotoxin of *Escherichia coli* with which cholera toxin shares 80 % sequence homology. The X-ray crystallographic studies revealed that the toxic A-subunit remains embedded in the symmetrical pentameric pore-like structure formed by five B-subunits (Fig. 2). The mature A-subunit (240 aa), although synthesized as a single polypeptide chain, is later cleaved into two parts, a wedge-shaped A1 domain and an elongated A2 consisting of an alpha-helical structure and a tail which extends through the pore formed by the B-pentamer.

The B-pentamer is stabilized by at least 20 hydrogen bonds and inter-subunit salt bridges, of which 10 are localized in the central pore. In addition, there is tight interaction of hydrophobic groups at the subunit interface causing extensive packing of subunits against each other. This imparts extensive stability of B-subunits to bile, proteases, etc. in the intestinal milieu. The inner surface of the ring of B-subunits is hydrophobic, and the central wall is lined by a number of positive

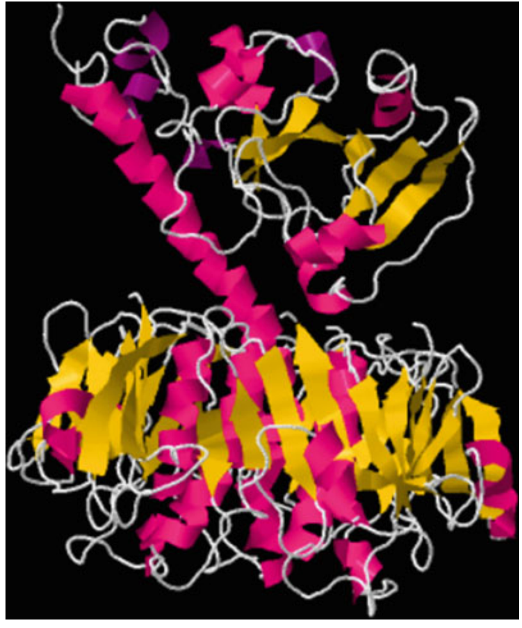

Fig. 2 The 3-D structure of the cholera toxin molecule as derived by X-ray crystallography (Image reproduced from Protein Data Bank (PDB) – PDB ID 1xtc (Zhang et al. 1995))

and negative charges. The monomers making up the pentamer form six-stranded antiparallel β-sheet with a sheet from the next subunit, giving the ring the appearance of a smooth outer surface, while long α-helices form a helical barrel in the center. These helices gently bow inward during the course of their structure formation reducing the effective diameter of the pore from 16 Å (amino terminal) to 11 Å (carboxyl terminal). The pentameric B-subunit (55 kDa) specifically binds five GM1 molecules with high affinity (Fig. 3).

The interactions between A- and B-subunit are non-covalent and involve the A2 domain and B-pentamer. The A1 and A2 domains of the A-subunit are linked by an exposed loop containing a site for proteolytic cleavage (after arginine 192) and a disulfide bond that bridges the cleavage site. Proteolytic cleavage within the exposed loop of the A-subunit generates the enzymatically active A1 peptide, which is responsible for toxic activity. The A2 chain forms the scaffolding that tethers the A- and B-subunits together. The last 4 amino acids, viz., lysine-aspartate-glutamate-leucine or KDEL at the COOH-terminal of A2 protrude from the associated toxin molecule and are not involved in the interactions with pentameric B-subunit.

Genetics: Cholera Toxin Is of Phage Origin

The *ctxAB* genes encoding the holotoxin CT (comprising the subunits A and B) are part of a cluster of genes normally referred to as the CTX genetic element. Subsequently, it was shown to be the genome of the single-stranded DNA phage CTXφ.

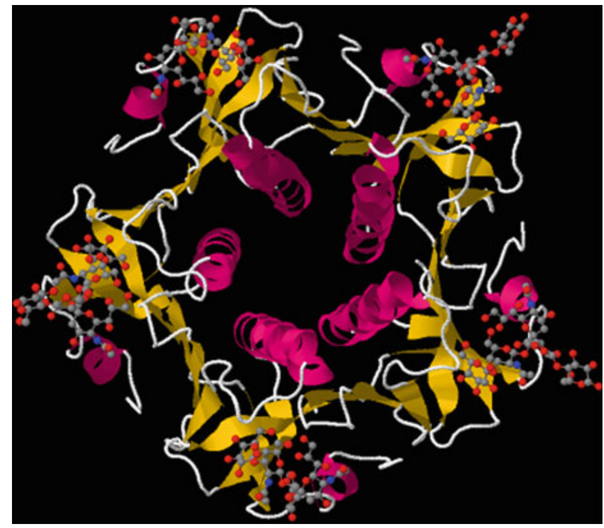

Fig. 3 The structure of cholera toxin B-pentamer complexed with GM1 pentasaccharides as derived by X-ray crystallography (Reproduced from Protein Data Bank (PDB) – PDB ID 2chb (Merritt et al. 1997))

The CTX genetic element was initially identified as the region which was present in toxinogenic *V. cholerae* but was absent in nontoxinogenic strains (Mekalanos 1983). This genetic region consisted of *ctxAB* and its associated 5′ sequences (Pearson et al. 1993). The CTX genetic element comprises of a core region (4.5 kb) flanked by copies of directly repeated sequences initially named as RS1 for the first repeated sequence discovered (Mekalanos 1983). Later on, as divergence was noted between nearly identical repeated sequences, the sequence proximal to the core region is termed as RS2 (~2.4 kb), and the sequence flanking RS2 was referred to as RS1 (~2.7 kb); these RS1 and RS2 together are generically named as "RS" (Pearson et al. 1993). The size of CTX genetic element varies in different strains due to a difference in the copy number of RS. The core region consists of (a) *ctxAB* operon, (b) *zot* gene coding for zonula occludens toxin (ZOT) (Fasano et al. 1991), (c) *ace* gene encoding accessory cholera toxin (Ace) (Trucksis et al. 1993), (d) a core-encoded pilin (Cep) responsible for enhancing colonization (Pearson et al. 1993), and (e) an ORF of unknown function (*orfU*). This CTX genetic element was found to be duplicated in tandem in some strains of the El Tor biotype of *V. cholerae* and to undergo amplification upon growth in the intestine (Mekalanos 1983). The organization of the CTX genetic element is presented in Fig. 4.

In 1996, Waldor and Mekalanos discovered that the genes encoding CT (the *ctxAB* operon) are not integral components of the *V. cholerae* genome, but instead correspond to the genome of a filamentous bacteriophage designated as CTXφ which forms non-lytic particles (Waldor and Mekalanos 1996). CTXφ is a special kind of filamentous bacteriophage as it can either integrate into *V. cholerae* chromosome or unlike other filamentous bacteriophages can replicate as a plasmid. The CTXφ prophage was first demonstrated to yield virions within an El Tor strain of *V. cholerae*. CTXφ is not a plaque-forming phage under normal conditions; virion

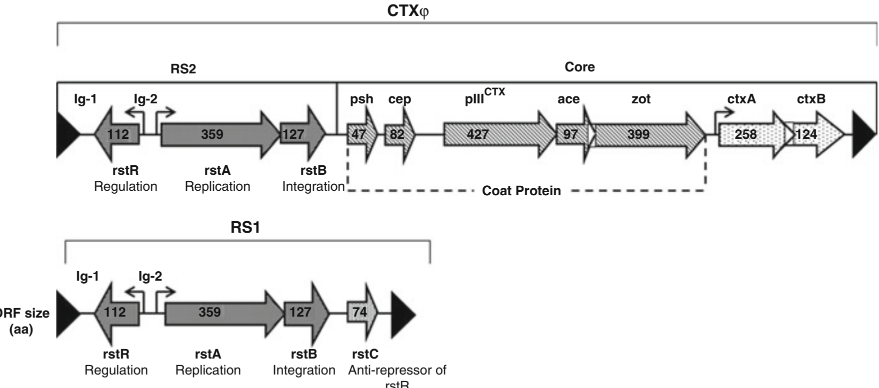

Fig. 4 Genetic organizations of CTXφ (comprising the core and the RS2 regions) and RS1 (shown separately below). The open reading frames are represented by *block arrows* showing the directions of transcription. The numbers inside the *block arrows* represent the numbers of amino acids in the respective ORFs. The *black triangles* represent the repeated sequences flanking the prophages. *Bent arrows* indicate the promoters for rstR, rstA, and ctxAB. The gene names and functions of the ORFs are indicated either below or above the *block arrows*. The genes with similar functions are shaded alike

formation was detected by the ability of supernatants from a strain containing a kanamycin resistance-marked prophage to transduce a recipient strain to kanamycin resistant or Km^R. It has been demonstrated that under appropriate conditions, toxinogenic *V. cholerae* strains can be induced to produce extracellular CTXφ particles. Cultures of *V. cholerae* harboring the replicative form or RF of CTXφ produce high titers of the phage in their supernatants.

The CTXφ genome has two regions, the "core" and RS2. The core region contains the genes encoding CT and functions related to phage morphogenesis, located upstream of *ctxAB*. The gene products of the core region include the putative major coat protein (Cep), three putative minor coat proteins (Psh, OrfU or pIIICTX, and Ace), and ZOT involved in phage morphogenesis. The RS2 region is located just upstream of the core CTXφ and contains three open reading frames designated as *rstR*, *rstA*, and *rstB* and two apparently untranslated regions called intergenic regions Ig-1 and Ig-2 (Waldor and Mekalanos 1996; Waldor et al. 1997). The ORF *rstA* encodes a polypeptide of 359 amino acids, and its functional characterization relates RstA to replication of CTXφ. The *rstB* overlaps *rstA* by 15 bp and is predicted to encode a polypeptide of 126 amino acids. The *rstB* gene function has been shown to be required for the site-specific integration of CTXφ into the *V. cholerae* chromosome. The ORF *rstR* encoding a polypeptide of 112 amino acids plays critical roles in maintaining lysogeny (Waldor et al. 1997).

Like other bacteriophages, the CTXφ receptor on *V. cholerae* for its attachment and transmission was demonstrated to be a toxin coregulated pilus (TCP). TCP is a thin, flexible, filamentous appendage of approximately 1–4 μm long and 8 nm wide found on the surface of *V. cholerae* cells (Davis and Waldor 2003). TCP is composed of homopolymers of the TcpA protein which self-associate to hold

cells together in microcolonies and serve as the phage receptor. The *tcp* operon encoding TCP resides on a horizontally transferred region of *V. cholerae* termed as *V. cholerae* pathogenicity island (VPI) which is essentially a large genetic element (~40 kb) flanked by repetitive region characteristics of pathogenicity islands. Pathogenicity islands designate a group of mobile genetic elements which play an essential role in imparting virulence to human pathogenic organisms.

Regulation of Cholera Toxin Expression

CT expression in *V. cholerae* is a part of a complex regulatory network in which several other virulence factors in addition to CT are expressed and coordinately regulated in response to specific environmental signals. The regulatory cascade is briefly described below, and the readers may consult earlier reviews for details (Chaudhuri and Chatterjee 2009; Childers and Klose 2007; Krukonis and DiRita 2003). ToxT, a soluble transcription factor encoded by the VPI, plays a major role in the regulation of transcription of both *ctxAB* and *tcp* operons in a coordinated fashion. ToxT directly activates the transcription of the ToxT-dependent genes by binding to their promoter regions. ToxT binds to specific sites within the ToxT-activated promoters, characterized as "toxbox" sequences (yrTTTTwwTwAww), a fairly degenerate 13 bp AT-rich motif. ToxT binds to two toxbox sequences in a direct repeat configuration to activate transcription of *ctxAB*, *tcpA*, and *tcpI-2* genes and binds to two sites in inverted repeat orientation for activation of *acfA*, *acfD*, *tagA*, and *tcpI-1* genes, while only a single ToxT binding site is present in *aldA* promoter. The fact that ToxT uses a variety of binding site configurations to activate transcription of different genes raises the question of whether ToxT binds independently to its two sites.

Induction of *toxT* transcription occurs in the intestine, which is an essential prerequisite for cholera toxin expression. The initial induction of ToxT transcription is under the control of two integral membrane regulatory proteins, ToxR and TcpP. Both proteins are homologous to the OmpR family proteins. ToxR binds the *toxT* promoter at −100 to −69 with respect to the transcriptional start site, while TcpP binds the −51 to −32 region. ToxR alone is unable to activate the *toxT* promoter, while TcpP must be overexpressed to activate *toxT* in the absence of ToxR. The evidence suggests that ToxR serves at this promoter as an enhancer for TcpP binding and that TcpP alone makes contact with RNA polymerase but requires interaction with ToxR in order to activate transcription. Membrane localization of ToxR is required for its ability to stimulate TcpP-dependent *toxT* transcription, presumably because this facilitates interaction with membrane-bound TcpP. ToxR interacts with another cotranscribed membrane protein, ToxS for maximal transcriptional activation. TcpP interacts with another cotranscribed membrane protein, TcpH.

The *tcpPH* genes are encoded within the VPI-1, which is only found in pathogenic strains, whereas the *toxRS* genes are found in all *Vibrio* species and therefore part of the ancestral *Vibrio* genome. The *tcpPH* genes are only

transcribed under permissive conditions for virulence factor expression, while *toxRS* appears to be constitutively expressed.

The transcription of *tcpPH* is controlled synergistically by two proteins, AphA and AphB, located on the large chromosome and are not encoded on the VPI-1 or CTX elements. Both AphA and AphB are believed to interact directly with the *tcpPH* promoter. AphA binds to the *tcpPH* promoter from positions −101 to −71 from the start of transcription, while AphB binds from positions −69 to −53, with partial overlap between the two binding sites. Evidence suggests that AphB is the primary activator, while AphA plays a more indirect role in *tcpPH* transcription; it enhances the ability of AphB to activate transcription.

The expression of cholera toxin is often regulated by quorum sensing. Quorum sensing is the coordination of gene expression in response to cell population density. In *V. cholerae* the quorum-sensing machinery represses cholera toxin expression at high cell density, but the expression of cholera toxin is allowed at low cell density.

At low cell densities the regulator LuxO protein is phosphorylated by a relay from the sensor protein LuxU. The activated form of LuxO, via an indirect mechanism involving small regulatory RNAs, destabilizes *hapR* mRNA transcript, thereby preventing HapR from binding to the *aphA* promoter. This, in turn, causes high-level AphA expression with consequent high-level expression of *tcpPH* that activates ToxT expression. Increased ToxT stimulate the transcription of *ctxAB* leading to high-level expression of CT.

At high cell densities, the situation is reverse. LuxO is dephosphorylated and thus inactivated. This leads to diminished LuxO activity and consequently enhances *hapR* expression. HapR binds to a site −85 to −58 from the transcription start in the promoter of *aphA* and represses its expression consequently preventing the activation of the *tcpPH* and the rest of the virulence cascade leading to repression of CT expression.

Besides quorum sensing, the ToxR virulence regulon, controlling CT and other virulence gene expression, is strongly influenced by several other environmental conditions such as temperature, osmolarity, presence of bile, glucose availability, cAMP, and pH. The mechanism for this regulation is not understood completely, especially how the environmental stimuli are sensed. The environmental signals are known to exert their effects at different levels of the regulatory cascade. For example, osmolarity modulates CT expression via two outer membrane proteins OmpU and OmpT, known to respond to osmolarity, and ToxR/ToxS (Fig. 5).

Till the late 1990s, investigations on *V. cholerae* regulatory networks were mostly characterized under in vitro culture conditions. *V. cholerae* cells grown under laboratory conditions contribute greatly to our understanding of the organism and its pathogenicity and are easier to manipulate, but several evidences suggest that they frequently cannot truly represent the pathogens during infection (Chakrabortty et al. 2000; Chiang and Mekalanos 1998). Recently, several studies have attempted to characterize *V. cholerae* under in vivo conditions which include identification of differentially expressed genes under in vivo conditions and

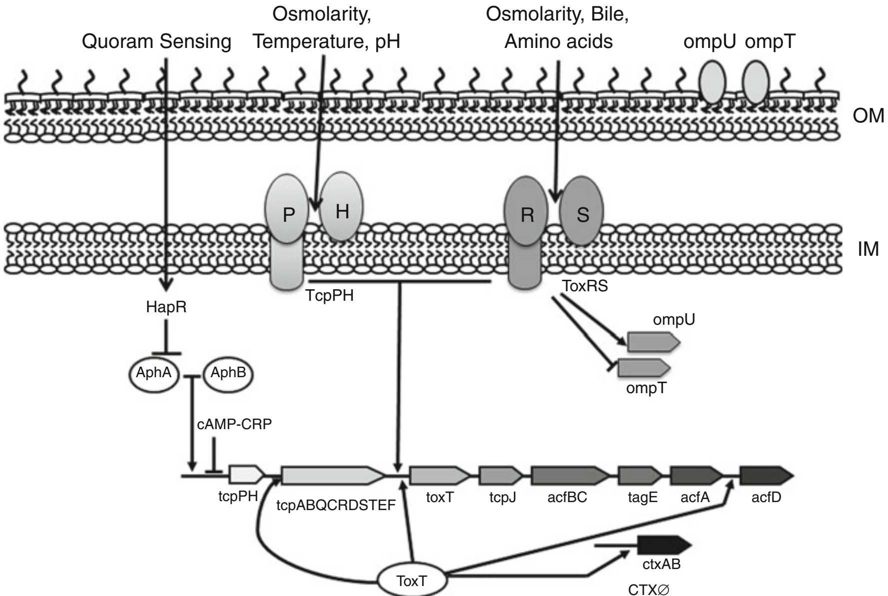

Fig. 5 Schematic diagram illustrating how the various environmental factors influence the regulatory cascade (ToxR regulon) that controls the *ctx* and *tcp* transcription in *V. cholerae*

possible requirements of virulence regulators in vivo (Banerjee et al. 2002; Chakrabortty et al. 2000; Chiang and Mekalanos 1998; Das et al. 2000, 2002; Lee et al. 1999; Nag et al. 2005).

Secretion of Cholera Toxin

Cholera toxin is an exotoxin, so the question arises how is the toxin secreted out of the *V. cholerae* organism? The toxin has to cross both the inner and outer membranes to be secreted to the extracellular milieu. The mechanism of secretion has been well studied, and a number of proteins are involved in the process. It is in fact a multistage process (Fig. 6). At first phase, the CT subunits, A and B, are synthesized in the bacterial cytoplasm as unfolded chains containing N-terminal signal peptides (Mekalanos et al. 1983). They are translocated independently across the cytoplasmic or inner membrane via the Sec-dependent pathway to reach the periplasmic space. Upon reaching the periplasm, the A and B chains are freed of the signal peptides, and both A and B chains are folded to form the 3D structures (Hirst et al. 1984). Further, in the periplasmic space, the folded subunits of CT interact with each other to get assembled into the complete AB_5-type of CT holotoxin (Hirst et al. 1984). The fully formed CT holotoxin is then translocated to the extracellular milieu through another secretory pathway known as the type II secretion system (T2SS) spanning the outer membrane and having its base at the inner membrane

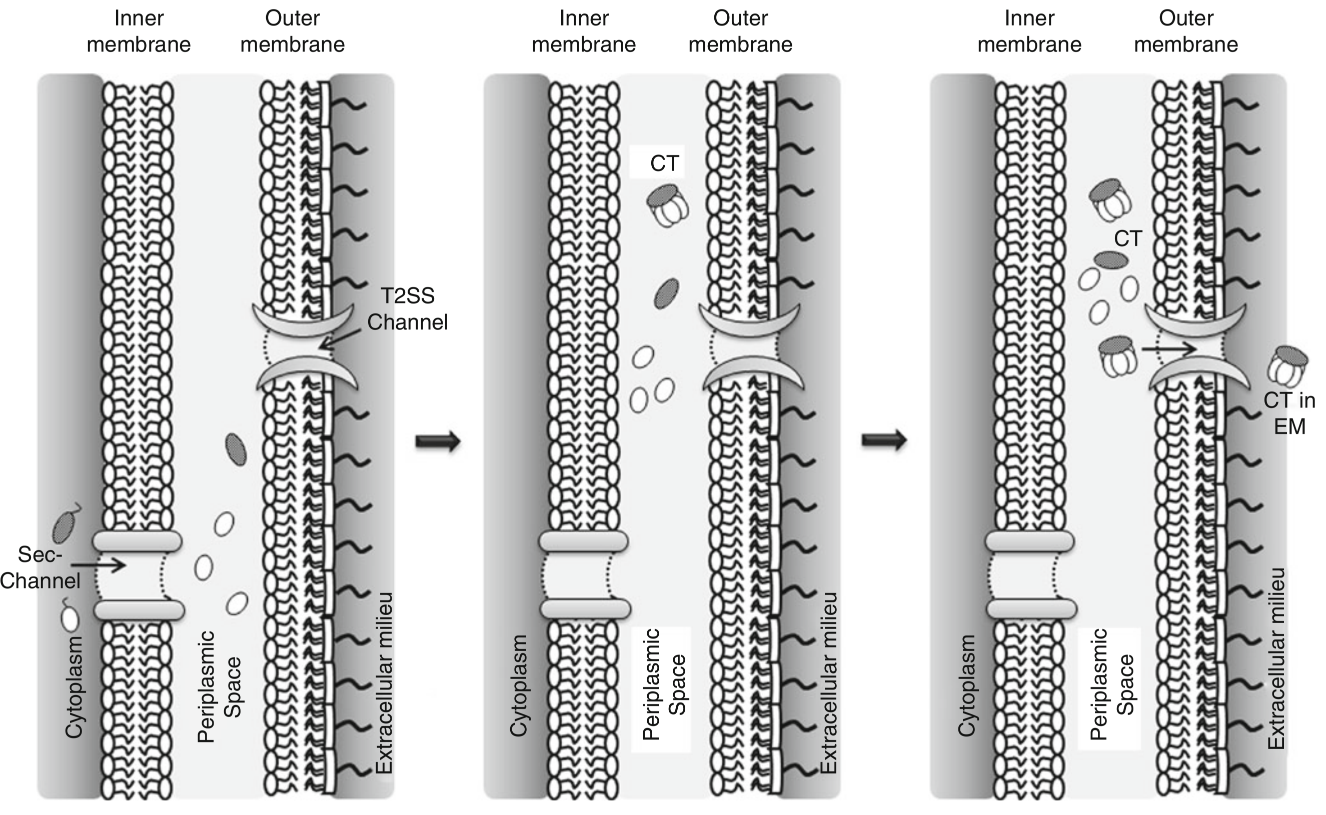

Fig. 6 Schematic diagram showing the different stages of cholera toxin secretion by the organism *V. cholerae*

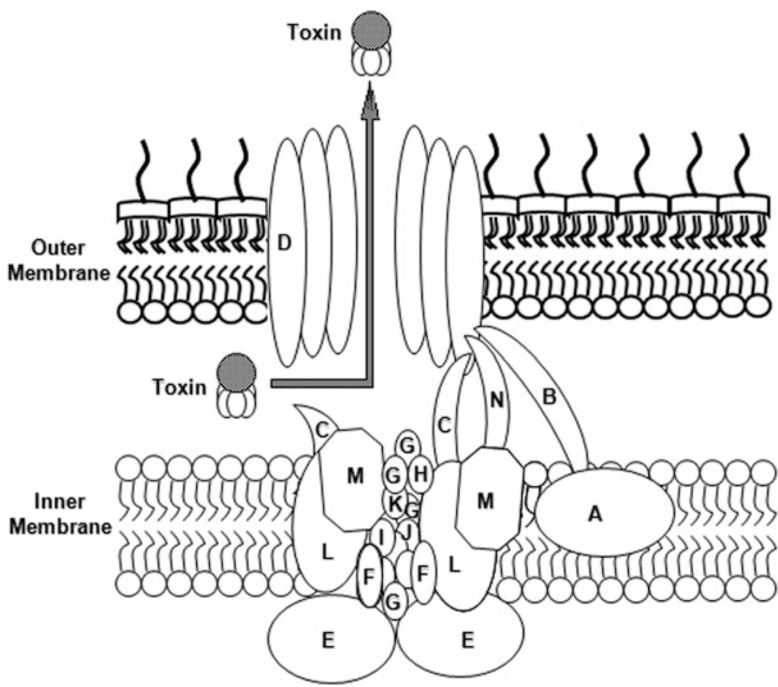

Fig. 7 Schematic representation of the type II secretion system (T2SS) in *V. cholerae* responsible for transport of cholera toxin across the outer membrane to the extracellular environment. A number of proteins located in the inner and outer membrane form a channel-like structure to export CT out of the periplasmic space between the inner and outer membrane of the bacteria. The proteins constituting the T2SS channel are termed as EpsA to EpsM, designated here as *A* to *M*, where Eps stands for extracellular protein secretion. For details on function of the proteins, the readers are referred to Chap. 10 of Chaudhuri and Chatterjee (2009)

(Johnson et al. 2006). This type II pathway is quite specific and can easily distinguish proteins to be secreted from the resident periplasmic proteins (Johnson et al. 2006).

In *V. cholerae*, the extracellular protein secretion process (T2SS) is generally termed as the extracellular protein secretion (Eps) system and the corresponding genes as *eps* genes. Secretion of cholera toxin across the outer membrane requires a large set of accessory proteins, the components of T2SS, designated as EpsA to EpsN and VcpD (PilD) (Johnson et al. 2006). The T2SS can be considered to consist of three subcomplexes: (i) the “inner membrane platform” consisting of the “secretion adenosine triphosphatase (ATPase)” EpsE, and the inner membrane proteins EpsC, EpsF, EpsL, and EpsM; (ii) the “pseudopilus” consisting of the major pseudopilin EpsG and the minor pseudopilins EpsH, EpsI, EpsJ, and EpsK; and (iii) the “outer membrane complex” consisting of large “secretin” EpsD and, in many species, the “pilotin” EpsS. The prepilin peptidase, generally called EpsO,

also belongs to T2SS. A schematic view of the architecture of type II secretion system (T2SS) is presented in Fig. 7.

Action of Cholera Toxin in Mediating Diarrhea

Cholera toxin exerts its action of causing diarrhea by subversion of the normal physiological processes of the polarized epithelial cells that line the intestinal epithelium. The B-subunit binds to the cell surface receptor GM1 on the apical membrane of intestinal epithelial cells and can cluster five GM1 molecules. Clustered GM1 preferentially associates with "lipid rafts." The toxin molecule then enters the cytosol by various endocytic mechanisms and is transported to Trans Golgi Network (TGN) through early and recycling endosomes. Endocytosis, in eukaryotic cells, is the process of internalization of fluids, solutes, and macromolecules by invagination of the plasma membrane through vesicle formation. These vesicles or "bubbles," known as endosomes, degrade or digest the captured extracellular material. Early endosomes refer to the vesicles directly after it is formed and are mildly acidic, and not much degradation is involved. The early endosome migrates deeper into the cell and gives rise to late endosomes and finally forms a lysosome which fully degrades the enclosed material. Sometimes, the endosome recycles back the internalized molecules to the cell surface which can occur directly from early endosome via recycling endosome.

The endocytic mechanisms include (a) non-clathrin-coated caveolar (HeLa cells), (b) clathrin mediated (Caco-2 cells), or (c) non-clathrin/non-caveolar (monkey kidney epithelial BSC-1 cells) mechanisms (Chaudhuri and Chatterjee 2009).

The toxin traffics to endoplasmic reticulum (ER) from TGN bypassing the Golgi cisternae, and often CT may directly be transported from endosome to ER. Here, the A-subunit disulfide bridge is reduced, and the A1 subunit is unfolded by peptide disulfide isomerase enzyme and dissociated from the rest of the assembly of proteins (Chaudhuri and Chatterjee 2009). Then, protein disulfide isomerase (PDI) is oxidized by the membrane-associated ER oxidase ERO1, and this oxidized form of PDI releases the unfolded A1 chain. The next step is the retrotranslocation of A1 through the ER membrane to cytosol which might be mediated through Sec61 protein translocation machinery. In cytosol, A1 refolds to avoid proteasomal degradation and catalyzes the ADP-ribosylation of Gs-alpha, a guanosine nucleotide-binding regulatory protein. The activated guanosine nucleotide-binding protein (G protein) can bind GTP and form active complex with adenylate cyclase and catalyzes the production of the second messenger cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). This leads to the overproduction of cAMP which causes the activation of chloride channels leading to the secretion of large amounts of Cl^-, HCO_3^-, Na^+, and K^+ into the lumen of the intestine. The electrochemical imbalance then creates a large osmotic gradient

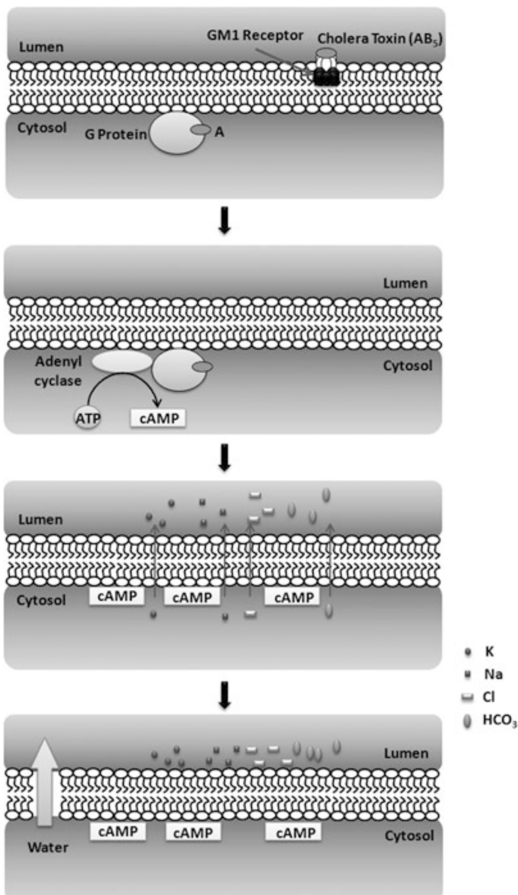

Fig. 8 Mechanism of action of cholera toxin. The steps shown here are the following: The toxin molecule containing one A-subunit (*gray*) associated with five B-subunits (*white*) binds to GM1-ganglioside receptor on the plasma membrane. The A-subunit or the active toxic part of the molecule dissociates from B-subunits and enters cytosol and activates adenylate cyclase. As a result cyclic adenosine-3′,5′-monophosphate (*cAMP*) accumulates along the cytosolic side of the plasma membrane and at the same time results in the secretion of K+, Na+, Cl−, and HCO3– to intestinal lumen. Finally there is excess flow of water along with the ions into the intestinal lumen creating diarrheal symptoms

between the cells and lumen driving enormous amount of water to flow into the lumen (schematically represented in Fig. 8). Thus there is a loss of water from cells to the lumen, resulting in the characteristic cholera stool.

Cholera Toxin Associated with Outer Membrane Vesicles: Alternative Secretion of CT?

Although much work has been done on the secretion of cholera toxin through outer membrane of *V. cholerae* and most of the proteins involved in the process of secretion have been characterized and their 3D structures determined through X-ray crystallography, some early studies attempting to understand the mechanism of toxin secretion by *V. cholerae* cell by electron microscopy remained unnoticed. In 1966–1967, release of surface blebs of dimension 400–1,100 Å by the actively growing cells was reported (Chatterjee and Das 1967). Also some finer particles of

size 40–100 Å were found to be released in association with the blebs. The authors argued that bleb formation represented a novel mechanism by which the actively growing cells secreted extracellularly not only the endotoxins but also materials residing in the periplasmic space of the bacterial cell. It was further observed that the finer particles (40–100 Å) released in association with the blebs were dimensionally similar to the cholera toxin molecule. Subsequently, a correlated biochemical and electron microscopic study (Chatterjee et al. 1974) further revealed that the finer particles were also found to be released independent of the blebs.

The heat-labile enterotoxin (LT), which plays a pivotal role in the severity of the disease caused by enterotoxinogenic *E. coli*, is transported to the periplasmic space of the bacterium like CT of *V. cholerae*. However, unlike CT, LT is not transported out to the extracellular milieu through the general secretory pathway. On the contrary, it was found to be secreted in association with the surface blebs, and it was proposed that vesicles should be considered a specific secretion mechanism for virulence factors (Horstman et al. 2004). A recent study showed that a fraction of CT released by the toxinogenic *V. cholerae* O395 was associated with the surface blebs or outer membrane vesicles (OMVs) (Fig. 9) originating from the same bacteria. Immunoblotting of purified OMVs with polyclonal anti-CT antibody and GM1-ganglioside-dependent ELISA suggested that CT was associated with OMVs (Chatterjee and Chaudhuri 2011). The authors further demonstrated by the CHO cell assay that the OMV-CT complex was physiologically active, was internalized via binding with the CT receptor (Fig. 10) on the surface of the intestinal epithelial cell, and produced increased levels of cAMP inside the epithelial cells. The OMVs thus acted as another vehicle for transport and delivery of CT into the epithelial cells. Presumably the CTs are secreted by the type II general secretory pathway into the extracellular medium, and a fraction of them bind to the surface of the OMVs present there. Whether the CT binds to the 3-deoxy-α-D-mannooctulosonic acid (kdo) or other part of lipopolysaccharide (LPS) of the OMVs remains to be determined, particularly since kdo in *V. cholerae* LPS is phosphorylated.

Natural Products as Inhibitors of CT

As in the case of other diseases, various medicinal plants have been used as healers to the disease cholera. Many studies have also been conducted to realize the potential of natural products as well as their active components. The active substances can operate via different pharmacological mechanisms, such as (a) direct antimicrobial activity against *Vibrio cholerae*, (b) prevention of binding of cholera toxin to GM1 receptor, and (c) direct inhibition of ADP-ribosylation activity of cholera toxin A-subunit.

The extracts of black tea showed antibacterial effects against *V. cholerae* O1. The major active components that are responsible for the protective effect were identified as theaflavin-3, 3′-digallate, and thearubigin (Toda et al. 1991). Another example is garlic extract, which had been in traditional use for the cure of diarrheal diseases like cholera. A galactan polysaccharide has been recognized as a component of garlic effective against cholera (Politi et al. 2006).

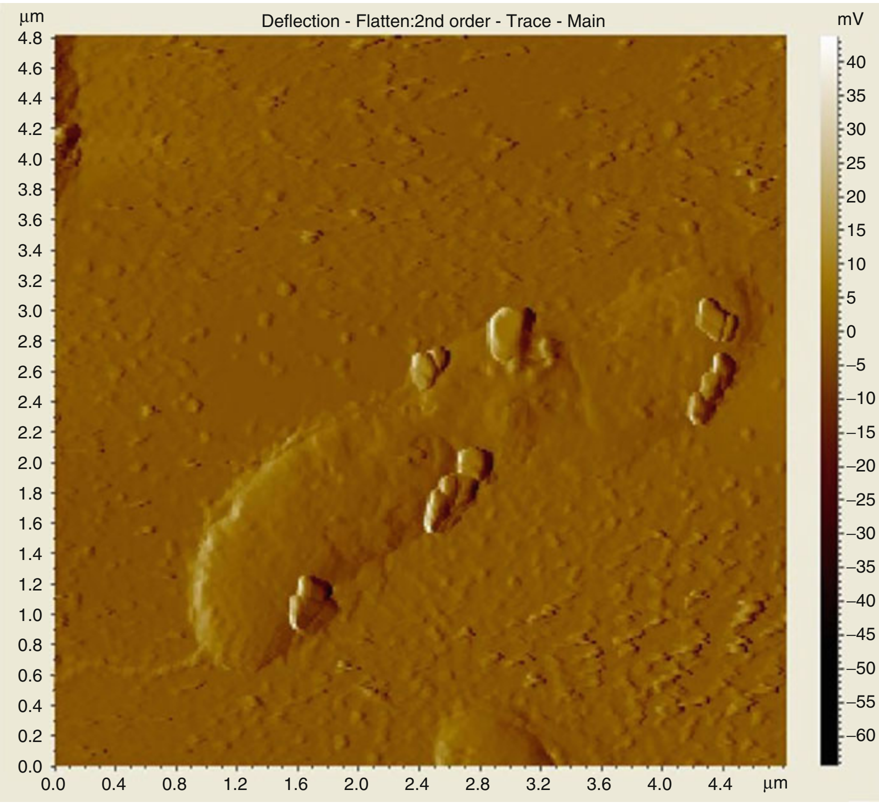

Fig. 9 Atomic force microscopy showing *V. cholerae* cells with formation of surface blebs. The bigger comma-shaped plateau region containing tail-like flagella represents the Vibrio. The distinct small bumpy structures on the plateau region represent the surface blebs

Another interesting study has shown that natural polyphenols extracted from immature apples have anti-choleric activity; the inhibitory effect largely arises due to the inhibition of CT-catalyzed ADP-ribosylation in a dose-dependent manner. The concentration of apple polyphenol extract inhibiting 50 % of enzymatic activity of cholera toxin (15 μg/ml) was about 8.7 μg/ml. Further fractionation of the extract suggested that the highly polymerized catechins, also named procyanidine polymers, are the major inhibitory components of this apple extract (Saito et al. 2002).

Very recently, it has been reported that 6-gingerol, a component of ginger, binds to CT hindering its interaction with the GM1 receptor present on the intestinal epithelial cells. The half maximal inhibitory concentration (IC_{50}) value was determined to be 10 μg/ml. The authors also provided evidence that the cytopathic effect of cholera toxin could be reduced by 6-gingerol both in vitro and in vivo.

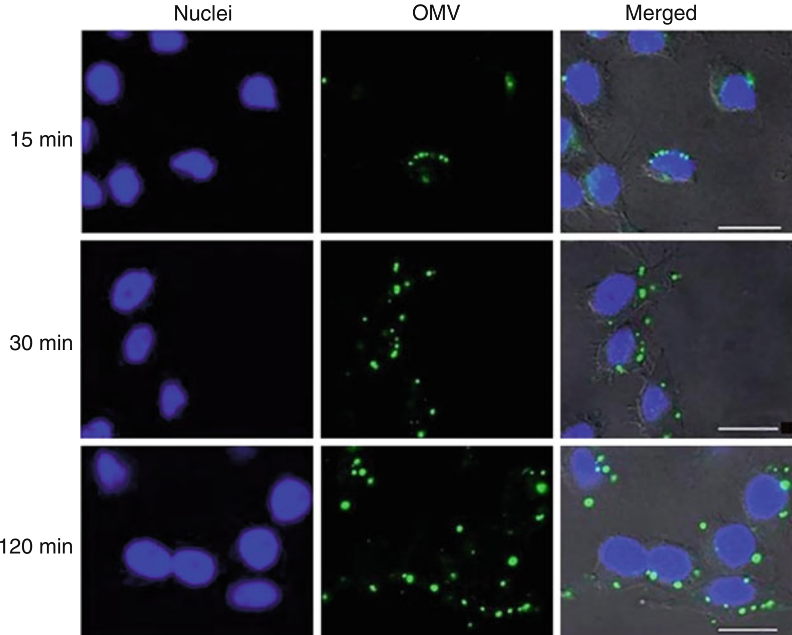

Fig. 10 Internalization of *V. cholerae* OMVs into intestinal epithelial cells (Chatterjee and Chaudhuri 2011). Intestinal epithelial cells (Int407) incubated for 15, 30, and 120 min with fluorescein isothiocyanate (*FITC*)-labeled *V. cholerae* O395 outer membrane vesicles (*OMVs*) staining the OMVs green were counterstained with 4′, 6-diamidino-2-phenylindole (*DAPI*) which stains nuclei as *blue*. Localization of OMVs (*green*) within Int407 cells was examined by confocal microscopy (scale bar = 20 μm)

The in vitro studies included the reduction of toxic effects in CHO, HeLa, and HT-29 cell lines. At the same time, 6-gingerol reduced the intracellular cAMP level in CHO cells, which is also a significant attribute of cholera. Rabbit ileal loop assay showed purified cholera toxin or *V. cholerae* supernatant-induced fluid accumulation to be reduced by the presence of 6-gingerol in a dose-dependent manner suggesting that 6-gingerol could be potentially active in cholera therapeutics by hindering the action of cholera toxin (Saha et al. 2013).

The pharmacological studies on herbal remedies for cholera are largely directed towards examining the anti-*V. cholerae* activity of these extracts. Direct investigations on natural products as potential inhibitors of cholera toxin secretion or adhesion to GM1 or function are scant. Therefore, the field remains open and further studies are needed for establishing the potential implications of the natural products with direct biological action on cholera toxin and other AB_5 toxins in general. Future studies directed towards determining the mechanism of action of the inhibitors found to date as well as searching for more inhibitors with newer technologies will be helpful.

Other Toxins of *Vibrio cholerae*

Cholera toxin or cholera enterotoxin being the major player in the disease cholera has been studied in detail. For vaccination, strains were constructed with deletion of cholera toxin. However, in several human volunteer studies, these CT deleted vaccine strains showed mild to moderate diarrhea and other reactogenic symptoms, mostly inflammatory in nature. Thus, some other toxins and virulence factors might be operative in *V. cholerae* although these may not have the primary effect. Search for other toxins has led to the discovery of a number of novel toxins produced by *V. cholerae*. These include the following: (i) zonula occludens toxin (ZOT), (ii) accessory cholera enterotoxin (Ace), (iii) hemolysin, (iv) repeats in toxin (RTX), (v) Chinese hamster ovary (CHO) cell elongation factor (Cef), (vi) new cholera toxin (NCT), (vii) Shiga-like toxin (SLT), (viii) thermostable direct hemolysin (TDH), (ix) heat-stable enterotoxin of non-agglutinable vibrios (NAG-ST), (x) the toxin WO7, and (xi) RTX-like toxin.

Zonula Occludens Toxin (ZOT)

This toxin reversibly alters the structure of epithelial intracellular tight junctions (zona occludens) and thus increases the intestinal permeability allowing the passage of macromolecules through mucosal barriers (Fasano et al. 1991). ZOT would not only allow luminal contents to diffuse into underlying tissue but would also disrupt the ion balance and thereby causes diarrhea. ZOT is encoded by a 1.3-kb gene located immediately upstream of ctxAB, and its sequence shows some homology to an ATPase. The ZOT protein is a single polypeptide chain of 44.8 kDa, 399 amino acids (aa) in length, with a predicted pI of 8.5, of bacteriophage origin, and present in toxigenic strains of *V. cholerae*.

Accessory Cholera Enterotoxin (Ace)

Ace is a potential enterotoxin of *V. cholerae* toxin which, in animal models, increases transcellular ion transport, which is proposed to contribute to diarrhea in cholera. Ace increases short-circuit current (Isc) in rabbit ileal tissue mounted in Ussing chambers, in intestinal epithelial cell T84 model, and causes fluid secretion in ligated rabbit ileal loops. Like CT, and in contrast to ZOT, Ace increases potential difference (PD) rather than tissue conductivity. The gene encoding Ace is located immediately upstream of ZOT in the core region of the CTX element, and like the *ctxA* and B, genes have an overlap of 4 nucleotides. The sequence of Ace shows significant homology to the eukaryotic ion transporting ATPases and to the δ toxin of *Staphylococcus aureus* (Trucksis et al. 1993). This homology led to the hypothesis that multimers of Ace might insert into eukaryotic membranes and establish an ion channel (Trucksis et al. 1993), but this hypothesis awaits verification.

Hemolysin/Cytolysin

Hemolysin is an exotoxin that attacks blood cell membranes and causes cell rupture with the liberation of hemoglobin. It is the most widely distributed toxin among the pathogenic vibrios. Hemolysin produced by El Tor O1 strains and by most non-O1 strains of *V. cholerae* has been found responsible for agglutination of sheep erythrocytes, an important criterion in biotype differentiation. The *hlyA* gene encodes an 80-kDa protein that is processed to a 65-kDa active cytolysin by a protease. Absence of hemolytic activity (Hly^{-}) of classical strains was due to an 11 bp deletion of the *hlyA* gene resulting in a truncated product. Previous reports suggest that hemolysin damages cells by acting as a pore-forming toxin and a number of animal studies have shown that purified hemolysin is enterotoxic. Phospholipase activity related to enterotoxigenicity and cytotoxic cell vacuolating activity of hemolysin have also been recently identified (Chaudhuri and Chatterjee 2009).

RTX (Repeats in Toxin) Toxin Family

The RTX exotoxin encoded by the gene *rtxA* was found responsible for epithelial cell rounding and affected cytoskeletal structure in Hep-2 cells (Fullner and Mekalanos 2000). The common toxic mechanism that leads to rounding of a broad range of cell types without directly affecting the cell viability is alteration of the polymerization state of the actin cytoskeleton. The *rtx* gene is absent in classical biotype strains of *V. cholerae* and present only in El Tor biotype strains. The toxin, RtxA, resembles members of the RTX (repeats in toxin) toxin family in that it contains a GD-rich repeated motif. Like other RTX toxins, its activity depends on an activator, RtxC, an acyltransferase, and an associated ABC transporter system, RtxB and RtxD. The RTX gene cluster physically linked to the CTX element on the *V. cholerae* genome is 693 bp downstream from CTXΦ insertion site. Presence of intact RTX gene cluster was causal for cell-associated cytotoxic activity causing detachment and rounding of mammalian epithelial Hep-2 cells (Fullner and Mekalanos 2000). A deletion within the gene cluster eliminating *rtxC* in classical strains of *V. cholerae* removes cytotoxic activity. The residual adverse properties of certain live attenuated cholera vaccines may be attributed to the Rtx toxin of *V. cholerae*.

Secreted CHO Cell Elongation Protein

Cytotoxic activity of culture supernatants from ctxA-negative *V. cholerae* El Tor strains was demonstrated on cultured CHO cells (Sathyamoorthy et al. 2000). The CHO cell elongation activity was partially purified and was found to cause fluid accumulation in sealed infant mouse model suggesting its enterotoxic nature and was named as CHO elongation factor (Cef). The Cef protein was around 79 kDa.

New Cholera Toxin (NCT)

In 1983, it was reported from India that some nontoxinogenic environmental strains of *V. cholerae* O1, which failed to hybridize with CT or LT probes due to the absence of genes encoding cholera toxin, could cause fluid accumulation in rabbit ileal loop and diarrhea in infant rabbits (Sanyal et al. 1983). Culture filtrates of these strains were able to cause fluid accumulation. The filtrates also increased the capillary permeability of rabbit skin, but unlike CT, caused bluing reaction accompanied by blanching or necrosis (Sanyal et al. 1983). In laboratory animals, toxic response is often measured by injecting trypan blue dye that produces a zone of bluing at the intracutaneous inoculation of toxin.

The toxin in the culture filtrate could not be neutralized in rabbit skin or ileal loop assays by antisera against CT or its A- and B-subunits. The loop and skin toxic activities were lost when the culture filtrate was heated to 1,000 °C for 10 min. The toxin was named as new cholera toxin and proposed to be the cause of diarrhea in CT− *V. cholerae* strain (Sanyal et al. 1983). Later studies on CT+ and CT− *V. cholerae* O1 classical and El Tor biotype strains of clinical and environmental sources revealed that NCT is produced by O1, O139, non-O1, and non-O139 strains.

NCT has been partially purified. This partially purified NCT also showed enterotoxic activity in a rabbit ileal loop model suggesting some similarity in secretory response between NCT and CT. No antigenic relationship between partially purified NCT and CT could be demonstrated in gel-diffusion tests. On the other hand, similarities of the NCT produced by all strains have been demonstrated in immunodiffusion studies. However, additional genetic and biochemical characterization of NCT is lacking, as a result of which the identity of the protein in the complete genome could not be established.

Shiga-Like Toxin (SLT)

This toxin has been identified in *V. cholerae* on the basis of cytotoxicity in HeLa cells, which can be neutralized by antibody raised against Shiga toxin. The investigators detected moderate to high levels of heat-stable (56 °C, 15 min) cell-associated cytotoxin in cell lysates of *V. cholerae* O1, non-O1, and *V. parahaemolyticus* strains. It is suspected that the residual diarrhea in volunteers challenged with recombinant live oral vaccine strains might be due to a Shiga-like toxin. But all the attempts to clone the gene encoding this toxin have been unsuccessful so far (O'Brien et al. 1984). *V. cholerae* genomic fragments hybridized with *slt-1* of *E. coli* under low stringency; DNA sequence analysis of these cloned fragments did not show significant homology to *slt-1*. No homologues of SLT could be found in *V. cholerae* complete genome sequence.

Thermostable Direct Hemolysin (TDH)

Thermostable direct hemolysin is a putative toxin that has been epidemiologically associated with cases of gastroenteritis in humans caused by *V. parahaemolyticus* (Nishibuchi et al. 1992). Production of TDH is routinely tested by β-type hemolysis of erythrocytes incorporated into a special medium called Wagatsuma agar. This hemolytic reaction is known as the Kanagawa phenomenon (KP). This hemolysin was named as thermostable direct hemolysin (TDH), as it is stable upon heating (100 °C, 10 min), and the hemolytic activity did not enhance by addition of lecithin indicating its direct action on erythrocytes. The biological activities of TDH include hemolysis of various species of erythrocytes, cytotoxicity, lethal toxicity towards small experimental animals, and increased vascular permeability in rabbit skin.

The TDH type toxin has not been reported in V. cholerae O1, but is found in plasmids as well as in chromosomal locations in a few non-O1 V. cholerae and named as NAG-rTDH as it was produced by non-O1 or non-agglutinating vibrios and related to TDH. NAG-rTDH has been purified, which like TDH migrated with a molecular mass of about 18.5 kDa on SDS-PAGE and showed lytic activities on erythrocytes which were stable at 100 °C for 10 min and cross-reacted with *Vibrio parahaemolyticus* Vp-TDH in both Ouchterlony and the neutralization test (Yoh et al. 1986).

Heat-Stable Enterotoxin of Non-Agglutinable Vibrios (NAG-ST)

A new type of heat-stable enterotoxin (ST) which shares 50 % amino acid sequence homology to ST of enterotoxigenic *E. coli* (ETEC) was described in *V. cholerae* non-O1 (Arita et al. 1986). This was designated as non-agglutinable vibrio ST or NAG-ST. Like the ST of *E. coli*, NAG-ST of *V. cholerae* causes rapid fluid accumulation in a suckling mouse assay (Arita et al. 1986). Occurrence of NAG-ST in non-O1 isolates is quite low and only rarely found in *V. cholerae* O1 (Takeda et al. 1991).

Like *E. coli* STa, NAG-ST has been demonstrated to raise the intracellular calcium level in rat enterocytes in a dose-dependent manner and was associated with activation of guanylate cyclase thereby increasing the intracellular level of cyclic guanosine-3′,5′-monophosphate (cGMP) in rat intestinal epithelial cells (Chaudhuri et al. 1998). From subsequent studies the authors proposed that NAG-ST caused inositol trisphosphate (IP3)-mediated calcium release from intracellular calcium stores, which then stimulates nitric oxide production by activating nitric oxide synthase, and the nitric oxide through cGMP activates calcium influx. Enhanced intracellular calcium potentiates translocation of protein kinase C-alpha (PKCα) from cytosol to membrane which phosphorylates some membrane protein, one of which is guanylate cyclase which subsequently leads to the accumulation of cyclic GMP resulting in fluid loss and subsequent diarrhea (Hoque et al. 2004).

WO7 Toxin

An extracellular toxin has been identified in an Inaba El Tor *V. cholerae* strain, which was isolated from an outbreak of cholera in Warangal city in Andhra Pradesh in southern India and was found to be devoid of the *ctx*, *ace*, or *zot* genes (Walia et al. 1999). The culture supernatant of this strain caused fluid accumulation in the rabbit ileal loop assay, elongation of Chinese hamster ovary (CHO) cells, and rounding of Vero cells. Immunological study and N-terminal sequence analysis show this toxin to be distinct from any previously described cholera toxin and was named as WO7 toxin according to the nomenclature of the parent strain. This WO7 toxin consists of two subunits with molecular masses of 58 and 40 kDa and binds to GM1 gangliosides and causes fluid accumulation in ligated loops. The precise role of this toxin in the disease of cholera is yet to be established.

RTX-Like Toxin

The search for new toxins in *V. cholerae* through computational analysis predicted the presence of two open reading frames (ORFs) in the completely sequenced genome of *V. cholerae* El Tor N16961 which were functionally annotated as hypothetical proteins. Later on, these ORFs named as *rtxL1* and *rtxL2* were experimentally demonstrated to be associated with virulence in *V. cholerae* and code for new RTX toxin like proteins (Chatterjee et al. 2008). Mutational studies characterized both RtxL1 and RtxL2 by (a) the presence of more than one cadherin domain, (b) cytotoxic activity towards Hep-2 cells, (c) hemolytic activity, and (d) modulation of virulence like CT production, motility, adherence to Int407, and in vivo intestinal colonization. Although it is clear that both RtxL1 and RtxL2 have similar characteristics, the insertional mutant RTXL2N showed impairment in all the above functional characterization to a greater extent compared to RTXL1N. Both *rtxL1* and *rtxL2* are in the same operon, and *rtxL2* is located upstream of *rtxL1*. Thus insertion in rtxL2 might downregulate *rtxL1* by polar effect. Further studies are needed to define the precise roles of these two new toxins.

Conclusion and Future Directions

Cholera toxins are important virulence factors responsible for the pathogenicity of *V. cholerae*, the causative agent of cholera. Application of molecular approaches has led to the understanding of these virulence factors. The present review provides an up-to-date knowledge on the structure of CT, genetic organization and phage origin, secretion by type II mechanism and its association with outer membrane vesicles, and mechanism of action at the cellular level of the host. Other toxins of *V. cholerae* are also important for developing the disease. Based on the knowledge at the molecular level, appropriate antitoxin strategies can be designed in the future

for the prevention and cure of the disease. For example, novel antitoxin agents like small molecules or peptides can be designed to prevent binding of cholera toxin to the small intestine, at the same time ensuring that the toxin is harmlessly ejected from the body. Thus future efforts will require (i) identification of new virulence factors, (ii) identification of new therapeutic targets and their validation in animal models, and (iii) accurate assessment of the role of non-CT virulence factors for therapeutic targets.

Cross-References

► Challenges in Developing Biotoxin Inhibitors
► Biotoxins and Food Safety

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Botulinum Toxin: Present Knowledge and Threats

2

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Abstract

Botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* is the most toxic substance known to humans that causes the clinical condition known as botulism. The classic manifestation involves skeletal muscle paralysis as a result of a presynaptic blockade to the release of acetylcholine. Since the discovery of the toxin about 100 years ago, various clinical forms of botulism have been described comprising classic or foodborne botulism, wound botulism, infant botulism, and inadvertent botulism. Almost all human cases of botulism are caused by one of four serotypes (A, B, E, or F). Inadvertent

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botulism is the most recent form to be described. It occurs in patients who have been treated with injections of botulinum toxin for dystonic and other movement disorders.

Laboratory proof of botulism is established with the detection of toxin in the patient's serum, stool, or wound. The most sensitive and widely accepted diagnostic method for BoNTs is the mouse bioassay, which takes 4 days to complete. This clearly cannot meet the immediate need for clinical diagnosis of botulism, botulinum detection in field conditions, and screening of large scale samples. Consequently, the clinical diagnosis of botulism relies on the clinical symptom development, thus limiting the effectiveness of antitoxin treatment. In response to this critical need, many in vitro methods for BoNT detection have been developed. This review is focused on the toxin structure, mechanism of action, detection methods, clinical diagnosis, and therapy including the second-generation vaccines using cell-free expression system as alternate strategy.

Introduction

Botulinum neurotoxins (BoNTs) are the most potent and lethal toxins produced primarily by the gram-positive spore-forming anaerobic bacteria *Clostridium botulinum*. BoNTs cause the clinical condition botulism, which is a serious and fatal neuroparalytic disease of humans and animals. The name botulism was derived from the Latin word "botulus" meaning sausage as it was frequently associated with consumption of blood sausage. There are seven botulinum neurotoxin types, which are immunologically different and are denoted by letters from A to G. The most common serotypes affecting humans include toxin types A, B, E, and F. The species was divided into four physiological groups, with group I organisms producing the neurotoxins A, B, and F; group II organisms producing B, E, and F; group III organisms producing C and D forms; and group IV producing G type.

BoNTs are the most poisonous toxin known to humans. Extrapolated data from primates to humans indicate that a lethal dose of crystalline type A toxin for a 70 kg human is approximately 90–150 nanograms (ng) by intravenous (i.v.) or intramuscular (i.m.), 70–90 ng by aerosolization, and 70 microgram (μg) by oral route (Arnon et al. 2001; Scarlatos et al. 2005). Because of their extreme toxicity, ease of production, and robust stability under adverse environmental conditions, BoNTs are on the top of the list of biological warfare threats and have been classified as a Category A bioterrorism agent by the Center for Disease Control (CDC) and National Institute of Allergy and Infectious Diseases (NIAID) and grouped under Schedule V of the biological agents and toxins act (BATA) that are to be handled in a protected place for any type of research work.

Pathogenesis, Clinical Manifestation, Diagnosis, and Intervention Measures

Apart from being a dangerous biohazard agent and a potential biological weapon, BoNTs also have important therapeutic value and have been shown to be effective in the treatment of a variety of chronic conditions. For example, in the United States, onabotulinumtoxinA is approved for the treatment of cervical dystonia (CD), poststroke spasticity of the upper limb, blepharospasm, strabismus, hyperhidrosis, chronic migraine, glabellar lines, and neurogenic detrusor overactivity. The most well-known application of botulinum neurotoxin serotype A (BoNT/A) is its use in the cosmetic industry as an anti-wrinkle agent, under the commercial name BOTOX® (Allergan Inc, Irvine). Other BoNT products include abobotulinumtoxinA (DYSPORT®, Ipsen Biopharm Ltd, UK), incobotulinumtoxinA (XEOMIN®, Merz Pharmaceuticals LLC, Germany) and rimabotulinumtoxinB (MYOBLOC®, Solstice Neuroscience Inc, San Francisco, CA) (Naumann et al. 2013).

Structure and Mode of Action

BoNTs belong to the A-B-type toxins wherein they contain two polypeptide chains referred to as A and B chains that play different roles. The molecular size is approximately 150 kDa proteins, comprising of a 100 kDa heavy (H) chain (aka B chain) and a 50 kDa light (L) chain (aka A chain) linked through a disulfide bond. BoNTs are folded into three distinct domains (Fig. 1), viz., catalytic domain (LC), transmembrane domain (HN), and a binding domain (HC), which are functionally related to the cell intoxication mechanism. The binding domain is composed of two unique sub-domains (HCN and HCC). The catalytic domain (LC) comprises both α-helices and β-strands and is a metalloprotease with a zinc atom in the center of the active site coordinated via the two histidines and a glutamic acid residue of the highly conserved HEXXH zinc binding motif and by Glu262 in BoNT/A, a residue conserved among clostridial neurotoxins.

BoNTs exert their toxic effect primarily by binding and entering peripheral cholinergic nerves and blocking acetylcholine release at neuromuscular junctions. This eventually leads to long-lasting descending paralysis. The intoxication mechanism is a multistep process involving toxin-cell binding, internalization, translocation into the cytosol, and enzymatic modification of the cytosolic target. The C-terminal domain of the H chain (HCC) plays a major role in the neurospecific binding that is mediated through the gangliosides on the neural membrane and facilitates the internalization of the toxin by initiating the endocytotic pathway. This is succeeded by acidification of the vesicle by an ATPase pump leading to conformational changes which mediates translocation of L-chain (LC) domain into the cytosol. Once internalized LC acts as a zinc-dependent endoprotease enzyme that selectively cleaves one of the three synaptic vesicle fusion proteins known as SNARE (soluble

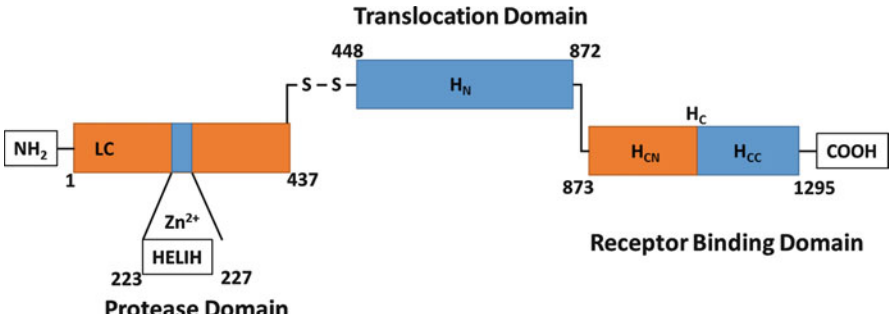

Fig. 1 Schematic diagram of the structure of a botulinum neurotoxin A (BoNT/A) showing the different domains

N-ethylmaleimide-sensitive factor attachment protein receptor) proteins (Fig. 2). SNAREs are small conserved membrane proteins that include vesicle-associated membrane protein (VAMP/synaptobrevin), syntaxin, and synaptosomal associated protein of 25 kDa (SNAP-25). The LC domain has unique substrate specificity though it requires anywhere from 16 to 50 aminoacids to act upon depending on the serotypes of toxin. All serotypes have unique sites on the SNARE target. When any of the SNARE proteins in presynaptic nerve terminals are cleaved by the LC domain, the neurotransmitter release is inhibited and is Ca^{2+} dependent.

Clinical Manifestation and Diagnosis

The natural forms of human botulism comprise of wound, infant, or food botulism with the less common ones being inadvertent and intentional botulism.

Wound botulism occurs as a consequence of toxin produced in wounds contaminated with the clostridial bacterium. Wound botulism is rare in humans and mostly associated with toxin types A or B, found among injecting drug abusers (Cooper et al. 2005). Infant botulism is caused by the ingestion of spores that then germinate and produce toxin in the infant's gastrointestinal tract. It is often infectious to infants as the gut microflora of infants are poorly developed. Most common toxin types involved are A, B, E, and F.

Foodborne botulism is an intoxication resulting from the consumption of contaminated food containing preformed BoNTs, with as little as 30 ng sufficient to cause illness and even death. The ingested toxin through contaminated food is absorbed across the gut to reach the blood circulation and then redistributed into the interstitial fluid. The toxin then gains entry by diffusion into the synaptic clefts of neuromuscular junctions and specifically binds to the nerve terminal via the toxin's HC (receptor-binding) domain of the heavy (H) chain. Most of the foodborne botulism are reported to be associated with BoNT type A, followed by B and E types, and rarely by F type. In a recent study food botulism was reported involving consumption of intoxicated korma sauce (Browning et al. 2011).

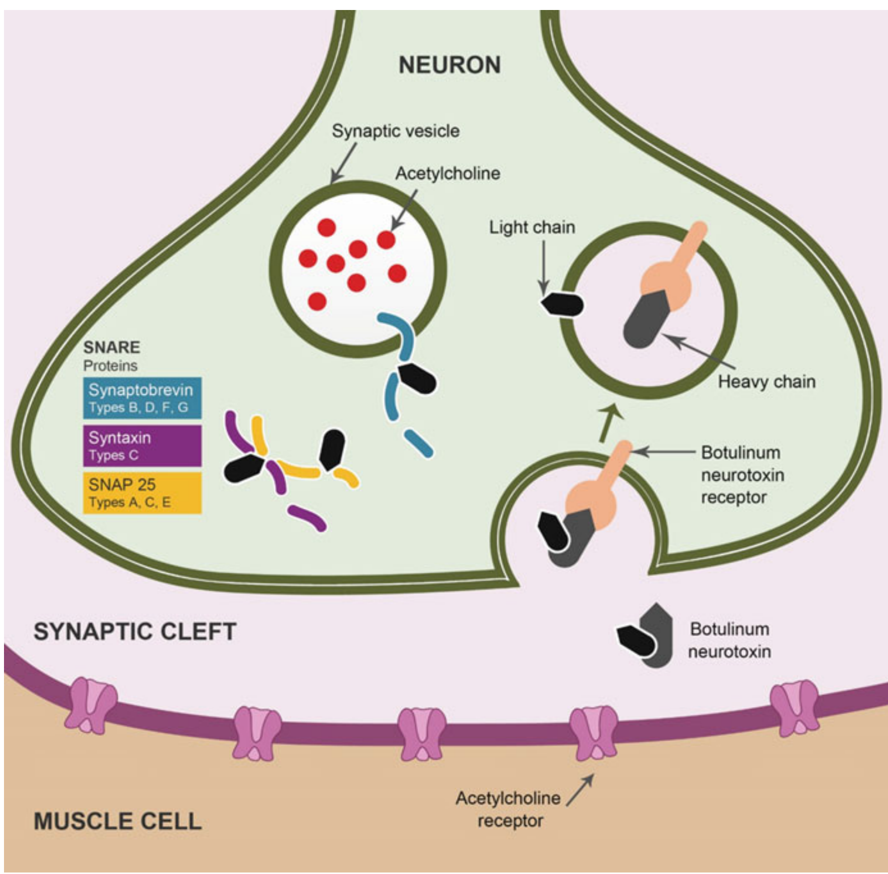

Fig. 2 Schematic model depicting the mechanism of botulinum neurotoxin (BoNT) action: toxin binds to presynaptic membrane through gangliosides and gets internalized through endocytosis and its LC translocation across the membrane. Post internalization the LC cleaves specific sites on the SNARE complex protein. Serotypes B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C also cleaves syntaxin. The cleavage inhibits the release of neurotransmitter acetylcholine causing paralysis of muscle activity

Inadvertent botulism is an unintended effect due to its exploitation as a therapeutic agent in the treatment of neuromuscular disorders and in cosmetics for the removal of facial wrinkles. On the contrary, intentional botulism arises from contamination of air or food by terrorist acts (Bigalke and Rummel 2005).

All forms of human botulism present mostly identical neurological signs because all BoNTs mediate similar physiological abnormalities in the peripheral nervous system. However, the neurological signs in foodborne botulism may include abdominal cramps, nausea, and vomiting. The neurological form is characterized by symmetrical, descending, and flaccid paralysis of parasympathetic nerves. The early signs include cranial nerve (face) palsies, including drooping of the upper eyelids (ptosis), double vision (diplopia), blurred vision, difficulty in articulating

words (dysarthria), difficulty in speaking (dysphonia), and difficulty in swallowing (dysphagia). The paralysis then develops to general weakness of several muscles such as arms, legs, and diaphragm which may lead to fatality if unattended. The extent and pace of paralysis may vary considerably depending on the serotype and the dose ingested. Individual susceptibility may also play a role in the extent of manifestations. A trivalent (A, B, and E) botulism antitoxin is given rapidly at the earliest indication to prevent any further progression and speedy recovery.

Clinical diagnosis of botulism is often confused with other neuromuscular disorders, such as Guillain-Barr’e syndrome, myasthenia gravis, strokes, or other central nervous system diseases. Definitive diagnosis requires identification of the toxin in serum, vomitus, gastric aspirate, and stool.

Laboratory Diagnosis

Mouse bioassay (MBA) remains the gold standard diagnostic assay for clinical and food specimens which is extremely sensitive, with a detection limit of 1 LD_{50} (50 % Lethal dose). It is generally estimated that 1 mouse LD_{50}/mL is equivalent to 10–20 picogram (pg) for the most active serotypes A, B, E, and F (Wictome et al. 1999; Schantz and Kautter 1978). In a MBA, the presence of toxin is detected by intraperitoneal injection of mice with the test sample, which are then observed for characteristic symptoms of botulism and ultimately death over a two-day period. The sensitivity of the mouse bioassay is measured as MLD (minimum lethal dose), which is the minimum amount of toxin (or the highest dilution of the sample) that kills all the inoculated mice. The sensitivity of the mouse bioassay is 1 MLD, in terms of toxin amount, and 2 MLD/ml (injection volume is 0.5 ml) in terms of sample concentration. For BoNT/A, 1 MLD is equivalent to 10 pg toxin (Ferreira 2001). Nevertheless, it has limitations, such as the use of a large number of animals and expensive, laborious, and time-consuming procedure (up to 4 days to complete).

Several immunodiagnostic assays such as enzyme-linked immunosorbent assay (ELISA) have emerged as sensitive alternatives with reduced total analysis time. There are several modifications in this technique like amplified ELISA, single-domain antibody ELISA, peptide ELISA, and liposome ELISA resulting in improved detection (Singh et al. 2013). Moreover, new assays tapping on the enzymatic properties of the BoNTs have emerged with more sensitive detection limits than achieved by the MBA (Table 1). Despite the fact that many of these technologies claim to be rapid, sensitive, and reliable, they have constraints of laboratory dependence and/or use of expensive instrumentation. Moreover, the sensitivities of the commercially available smart tickets were variable and were ineffective to discriminate between the four serotypes (A, B, E, and F) (Gessler et al. 2007).

Gopalakrishnakone and team have recently used an optical immunoassay (OIA) platform for the detection and characterization of venom toxin. In a collaborative research with Defence Medical Environmental Research Institute (DMERI), Defence Science Organization (DSO) National Laboratories, this platform has

Table 1 Summation of the various detection methods for botulinum neurotoxins (BoNTs)

Method	Assay time	Sample	Detection limit LOD[a]	Reference
Passive hemagglutination	5–6 h	Serum BoNT/A/B/E	1.3–1.6 mLD_{50}	Johnson et al. (1966)
Radio immunoassay	8 h	BoNT/A purified	100 mLD_{50}	Boroff and Shu-Chen (1973)
Mouse bioassay	3–4 days	BoNT/A	1–2 mLD_{50} (10–20 pg/mL)	Schantz and Kautter (1978)
Enzyme-linked coagulation assay (ELCA)	>18 h	Culture supernates	<1 mLD_{50}	Doellgast et al. (1993)
Protease activity assay-fluorescence	3 h		4 pM	Schmidt et al. (2001)
Time-resolved fluorescence	>2 h	BoNT A/B in spiked buffer	4–20 pg/ml	Peruski et al. (2002)
Amplified ELISA	8 h	BoNT/A/B/E/F in food samples (chilli, potato)	0.2–1 ng/ml	Ferreira et al. (2004)
Ganglioside-liposome immunoassay	20 min	Buffer	100 pg/ml (visual), 15 pg/ml (densitometry) = 3 mLD_{50}	Ahn-Yoon et al. (2004)
Immuno-PCR	6–9 h		1–5 pg/mL	Chao et al. (2004)
Protease activity assay-MS	4 h	BoNT/A/B/E/F	0.0.7 pM, 1.2 MLD_{50}/ml (A/B) 6.2 MLD/ml (E)	Barr et al. (2005)
Electrochemiluminescence (ECL)	<6 h	BoNT/A in milk	40 ng/mL	Sachdeva et al. (2014) and Rivera et al. (2006)
Digoxigenin (DIG)-ELISA	4 h 15 min	Casein buffer and Food sample	60.3 pg/ml (A),176 pg/ml (B), 163 pg/ml (E), 117 pg/ml (F), 2 ng/ml	Sharma et al. (2006)
Plastic ELISA-on-a-chip (EOC) biosensor	20 min	Purified BoNT/A in spiked buffer	2 ng/ml	Han et al. (2007)
Disposable immunoaffinity column (IAC)	40 min	BoNT/A complex assay buffer milk serum	0.4.5 pM (5 MLD_{50}/ml) 1.8.2 pM 0.0.9 pM	Attrée et al. (2007)
Chemiluminescence enzyme immunoassay (CLEIA)	3 h	BoNT/A in milk and beef extract	0.45 pg mL − 1	Singh et al. (2013)
ELISA-based protein antibody microarray	<1 day	BoNT serotypes A, B, C, D, E, and F in serum and milk	Ranging from 0.2 pg/mL (1.3 fM) to 2.2 pg/ml (14.7 fM)	Cheng and Stanker (2013) and Zhang et al. (2012)
SPR assay	10 min	BoNT/B holotoxin	2 pM	Ferracci et al. (2011)

(*continued*)

Table 1 (continued)

Method	Assay time	Sample	Detection limit LOD[a]	Reference
Endopep-MS assay	?	BoNT/A/B/E/F in spiked buffer	1 mLD50 for BoNT/A, 0.05 mLD50 for BoNT/B, 0.1 mLD50 for BoNT/E, and 0.05 mLD50 for BoNT/F	Kalb et al. (2010) and Raphael et al. (2012)
Neuromuscular bioassay	4–5 h	BoNT/A/B/E	<20 pg/mL, <1 MLD	Keller (2006)
ELISA	5–6 h	BoNT/B in milk	5 pg–2 ng/mL	Rasetti-Escargueil et al. (2009)
Immunochromatography assay (ICA)	30 min	BoNT/A/B	1–50 ng/mL	Scotcher et al. (2010)
Flow cytometry assay	4 h	BoNT/A/B	50 pg/mL–20 ng/mL	Ching et al. (2012)
Immuno-PCR	<9 h	BoNT/A	1–5 pg/mL	Pauly et al. (2009)
Liposome-PCR	<9 h	BoNT/A water samples	0.02 fg/mL	Chao et al. (2004)
Bidiffractive grating (BDG) biosensor assay	<30 min	BoNT/A/B	100 ng/mL	Mason et al. (2006)
Array biosensor assay	<30 min	BoNT/A	40–200 ng/mL	O'Brien et al. (2000)
ELISA-on-a-chip (EOC)	<30 min	BoNT/A	2 ng/mL	Sapsford et al. (2005)
Aptamer-electrochemical assay	<30 min	Botulinum neurotoxin	40 pg/mL	Han et al. (2007)
Protein chip membrane capture assay – SPR	3–5 h	BoNT/A/B	20 pg; 2 ng/mL	Wei and Ho (2009)
Lab-on-a-chip (LOC) assay	<20 min	BoNT/A	1 pg	Marconi et al. (2008)
Peptide monolayers arrayed microfluidic assay	<3 h	BoNT/A	3 pg/mL	Lillehoj et al. (2010)
ALISSA	2.5 h	BoNT/A	0.5 fg	Frisk et al. (2009)
Cell-based assays	2–3 days	BoNT/A	1–10 ng/mL	Pellett et al. (2010)
Functional dual coating (FDC) assay	1 day	BoNT/A	0.03 mLD50 or 0.13 pg/mL serum, 1–2 mLD50/mL serum	Jones and Marks (2013)

[a] 1 mouse LD50/ml is ~ equivalent to 10–20 pg/ml or ~70–140 fM

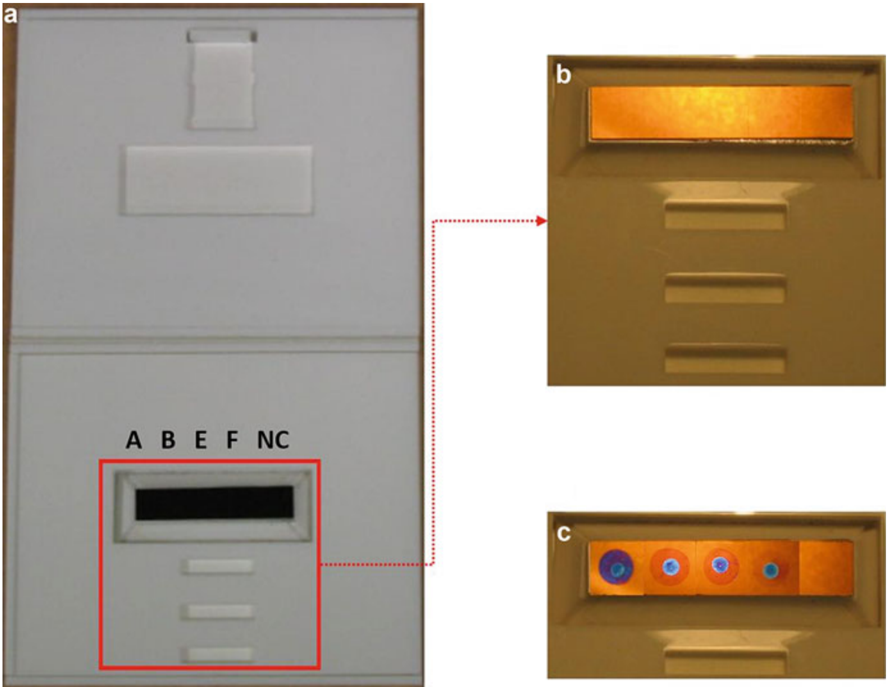

Fig. 3 Optical immunoassay test device designed for the detection of botulinum neurotoxins (BoNTs) A, B, E, and F. (**a**) Test cassette interior with mounted OIA test strip from *left* to *right* showing specific antitoxin coated for BoNTs A, B, E, and F. NC being negative control. (**b**) OIA unreacted test strip under light reflection. (**c**) Reacted test strip showing positivity for BoNT/A

now been improved and modified to detect and serotype BoNTs A, B, E, and F (Fig. 3) in a single test (Rajaseger et al. 2008). The detection sensitivity achieved with liquid, semisolid, and solid food matrices ranged from 3.125 to 7.8 ng/mL. The assay duration was determined to be <35 min. The OIA was less sensitive when compared to MBA (Rajaseger et al. 2010) but, nonetheless, has several operational advantages as a fieldable device, which include less logistic load, capability of semiquantitative determination, visual readout, and non-powered making it a potential on-site screening tool.

Liu et al. (2012) have standardized a simple new method utilizing dual-coated microtiter plates (monoclonal antibody to the toxin's HC domain plus SNAP251–206 protein substrate) to specifically quantify biologically active toxin molecules in commercially available therapeutic botulinum type A toxin preparations. The FDC (functional dual coating) assay showed high sensitivity down to 40 fg/mL [or 130 aM limit of detection (LoD)] and could replace the conventional mouse LD50 test. Very recently, a modification of the FDC assay has been described that allowed the sensitive, specific detection and quantification of BoNT/A in serum, lysed serum, and food samples in an animal-free assay (Jones and Marks 2013).

Prophylaxis and Therapeutics

Therapy for botulism consists of supportive care with special attention to respiratory status (artificial respiration) and passive administration of human- and equine-derived anti-BoNT immune globulin (antitoxin). Antitoxins cannot reverse the existent paralysis, since the antitoxin cannot cross the nerve membrane to neutralize the toxin inside the poisoned cells. The time of convalescence in severe cases may take from weeks to months as it requires the regeneration of new end plates.

Initial attempts to produce a vaccine against botulism utilized formalin-treated crude *C. botulinum* extracts and liquid filtrates treated with formalin. The human botulism vaccines (bivalent toxoids, A and B) are produced by formaldehyde inactivation of botulinum toxin (toxoid) and have several disadvantages including being laborious, time-consuming, and presented with severe adverse reactions. Later, a purified pentavalent botulinum toxoid incorporating serotypes A–E was developed for investigational use in the US Department of Defense (DoD). This vaccine was found to be effective against the homologous serotypes with minimal reactivity following immunization; however, the supply of this vaccine is limited. Nevertheless, CDC has withdrawn the pentavalent (ABCDE) botulinum toxoid (PBT) for vaccination of workers at risk for occupational exposure effective from November 30, 2011. This decision was made after the observance of decline in immunogenicity of some of the toxin serotypes and occurrence of moderate to severe local reactions related to annual booster doses (CDC 2011).

Recently recombinant H_{CC} heavy chain sub-domain of BoNT/A which is a nontoxic fragment has been proposed as a potential candidate in the development of a subunit vaccine for prophylaxis against BoNT/A based on its strong immunogenicity and specific reactivity (Yu et al. 2011).

An alternative strategy for the expression of such unique recombinant proteins relies on the use of cell-free expression systems (CFES). These systems include all the regulatory elements required for in vitro transcription and translation of proteins from template DNAs encoding the Hc fragments of botulinum toxins A, B, and E that were expressed using a continuous exchange cell-free system. As the cell wall barrier is not present in cell-free systems, the reaction environment can be readily controlled, and different components can be directly added to promote protein folding and solubility (Zichel et al. 2010).

Conclusion and Future Directions

Tremendous progress has been made in the development of BoNT diagnostics and the developed assays to date may not yet be fully validated. While there is no single method that fulfills all the requirements of an ideal assay, individual methods have been developed that can address varying aspects of these requirements, allowing one to find the right assay for a given scenario.

Multiplexed identification of several toxins simultaneously with their serotypes and full automation of the assay is a further desirable extension beyond this. Additionally, an assay that can distinguish active and inactive forms of the toxin and quantify both would be advantageous since depending on the circumstances, detection of one form over the other may be beneficial. Point-of-care testing is a priority for field deployment as in the scenario of a response to a bioterrorist attack; thus, technologies targeting a handheld type of device, or at least portable device, will be highly beneficial.

For prevention and therapeutic interventions, research efforts have been directed at the development of both toxoid and recombinant/subunit vaccine candidates. Newer toxoid candidates have addressed modifications in manufacturing procedures and routes of administration to increase immunogenicity and to decrease local reactogenicity (as observed with the PBT). In the context of biosafety requirement for a dedicated high-containment laboratory as needed to produce formalin-inactivated toxoids, subunit vaccines can be a preferable alternative. Furthermore, a humanized single-domain monoclonal transbody (cell-penetrating VHH) that binds and blocks the intracellular BoNT specifically and directly, and does not cause the undesirable side effects, should be a novel and better immunotherapeutic remedy for human botulism. And the search for an effective cure or treatment of botulism beyond antitoxin, which has to be administered within the first 24–48 h after toxin exposure, continues.

Cross-References

- ▶ Antidotes to Botulinum Neurotoxin
- ▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
- ▶ Cell-Based Assays for Neurotoxins
- ▶ Counterfeit Botulinum Medical Products and the Risk of Bioterrorism
- ▶ The Biosecurity Threat Posed by Biological Toxins

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The Biowarfare Agent Ricin

3

Mohammad Moshiri, Leila Etemad, and Mahdi Balali-Mood

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Abstract

Ricin toxin (RT) is an abundant glycoprotein component of Castor beans (CB) that is highly toxic to mammalian cells. RT is water soluble and does not partition into the oil extract, and there are different isoforms with varied toxicities and antigenicities. RT belongs to the B group of biological warfare agents (BWA). It is made up of two functionally different parts, an A polypeptide chain (RTA: 32 kDa) and a B polypeptide chain (RTB: 34KDa). RTB attaches onto the cell surface, while RTA enters the cell, attaching to ribosomes and inhibiting protein synthesis. A single RTA biomolecule is able to inactivate 1,500–2,000 ribosomes per minute, ultimately leading to cell death. As a BWA, RT is most likely to be deployed as an aerosol; however, it could be administered by other means: ingestion, or possibly even by injection, for assassination. The effectiveness of modified RT for immunochemotherapy has been evaluated. Toxicity and severity of clinical manifestations vary between different routes of administration. Inhalation is the most potent route of entry, and oral ingestion is less toxic and may be nonlethal. RT is detected in tissue sections, body fluids, and nasal swabs of poisoned animals by enzyme-linked immunosorbent assay (ELISA). Treatment of intoxicated patients is very difficult, because RT acts rapidly and irreversibly. The main parts of treatments are decontamination and supportive therapy. Some methods of active and passive immunization have also been investigated.

Introduction

Ricin toxin (RT) is a highly toxic compound found in the seeds of the castor oil plant. *Ricinus communis* L. known as the castor bean (CB) and castor oil in the USA is a species of flowering plant in the Euphorbiaceae (spurge family). This evergreen herbaceous is native to the northeastern Africa and the Middle East but today is widespread all over the tropical regions. The CB was grown, over the centuries, for the nonyellowing oil that it produces, especially by its seeds (Ferraz et al. 1999).

CB oil has been used in ancient Greece, Egypt, and Roman Empire, primarily as an unguent and aperient. Abu Mansur Muvaffak Harawi, a tenth-century physician in Persia, explained the use of this plant for medical (on an empty stomach) and industrial (inexpensive fuel for oil lamps) purposes (Scarpa and Guerci 1982). Also, in India, the leaf, root, and seed oil of this plant have been used for the treatment of inflammation, liver disorders, hypoglycemia, and as a laxative (Rana et al. 2012). Castor seeds have an approximately 50 % oil content. The oil is composed mostly of ricinoleic acid and small amounts of other glycerides including oleic, linoleic, stearic, and dihydroxystearic acids (Scarpa and Guerci 1982). Due to the high proportion of the fatty acid ricinoleic acid, today *Ricinus communis* is a valuable industrial raw material for lubricants, paints, coats, cosmetic products, and several other usages (Worbs et al. 2011).

RT is an abundant glycoprotein component of CB that is strongly toxic to mammalian cells. RT is water soluble and does not partition into the oil extract;

therefore, castor oil does not contain RT. The toxicity of this plant has been known since ancient times. In the late nineteenth century, Peter Hermann Stillmark (1860–1923) at the University in Dorpat extracted a toxic protein from the beans, which he named ricin. He revealed that the ricin induces agglutination of erythrocytes and serum protein precipitation. However, subsequent studies showed that Stillmark's ricin compound is a mixture of RT and hemagglutinin (*Ricinus communis* agglutinin) (Lord et al. 1994).

Historical Use of RT

The US Department of War considered RT as a potential weapon as early as 1918. Due to high toxicity and easy production, RT was code-named compound W by the US military. US and British collaboration during the Second World War developed the W bomb which was tested but never used (Stewart 2005).

One documented case of RT poisoning was the 1978 assassination of a Bulgarian defector, Georgi Markov. Markov was a BBC journalist with life-threatening information about the Bulgarian communist leadership. He was shot by a ricin-loaded platinum pellet from an umbrella dying after RT release from the pellet which melted at body temperature within 3 days (Crompton and Gall 1980).

At least six other terrorist attacks have been attributed to the same technique. The first case of prosecution under the Anti-Terrorism Act was two tax protestors convicted in 1995 of possessing RT as a biological weapon in Brooten of the USA. Inspectors believed that Iraq began research into biological warfare agents (BWA) including RT between 1985 and 1991 and it may have been used in the Iran-Iraq war, but there is no evidence to confirm it. Iraq stated that all of the RT was used in field trials but not in the war, though this claim was not verified (Zilinskas 1997). In 2003 and 2004, an RT contaminated letter addressed to the White House was found in a Senate mail room that served the Bill Frist office (Anonymus 2003). More recently, RT-laced letters were sent to the President of the United States, Barack Obama, and New York Mayor Michael Bloomberg for which a Texas actress, Shannon Richardson, has been indicted (Saeidnia and Abdollahi 2013).

Castor seed extracts have also been documented in suicide attempts. Five suicide cases have been reported (four men and one woman) from Poland, Belgium, and the USA by intravenous, intramuscular, or subcutaneous injection of a self-made seed extract. Reflecting the lesser potency of RT via oral ingestion, other cases of attempted suicide by ingestion of CB have had a low fatality rate (Worbs et al. 2011).

Ricin Structure and Mechanism of Toxicity

RT and related toxic lectins belong to the A–B family of toxins which also includes several bacterial toxins, such as cholera and pertussis toxin. Toxins in this group comprise two functionally different parts, an A (RTA, ricin toxin A; 32 kDa) and B

polypeptide chain (RTB, ricin toxin B; 34KDa). The two chains are linked by a disulfide bond. RTB has a binding site for galactose at each end with the two sites forming hydrogen bonds with cell surface carbohydrates. RTB is composed of 262 amino acids and attaches to terminal galactose residues in glycoproteins on cell membrane of eukaryotic cells or to mannose if galactose is absent. RT then enters the cells by endocytosis mechanisms including clathrin-dependent and clathrin-independent pathways. Uptake of cell-bound RT is slow, being about 10 % of bound toxin per hour. The majority of the toxin is internalized by non-clathrin-coated pits pathways (Olsnes 2004). RT molecules transported to endosomes may be recycled back to the cell surface or degraded in lysosomes. Only a small fraction of endocytosed RT reaches the trans-Golgi network, but once in the Golgi, the RT follows retrograde transport to the endoplasmic reticulum (ER) by as yet unknown pathways. In the ER, the RT disulfide-bond is broken by protein disulfide isomerase, separating the A and B subunits releasing a steric block of the RTA active site. Free RTA in the ER lumen then moderately unfolds and partially integrates into the ER membrane, where it is thought to mimic a misfolded membrane-associated protein. Only a portion of RTA can escape degradation by proteasomes in the cytosol and reach any ribosomes.

The A-chain catalyzes the removal of adenine from the sarcin–ricin loop of the eukaryotic 28S ribosomal RNA in the 60S ribosome subunit. Sarcin–ricin loop is important in binding elongation factors during protein synthesis. This inhibits protein synthesis by protein elongation factors binding to depurinated ribosomal RNA (rRNA). A single RTA molecule is able to inactivate 1,500–2,000 ribosomes per minute, ultimately killing the cell.

RT may also mediate apoptosis by mechanisms not yet fully understood. Other toxic manifestations such as magnesium and calcium imbalance, cytokine release, acute phase reactions, and oxidative stress in the liver are also attributed to RT toxicity (Sehgal et al. 2010).

RTA or holoricin has been used to construct immunotoxins for cancer therapy (see below). RTA is applied as antigens to produce antibody or vaccine, and RTB is used as a cell binding subunit for various purposes.

Due to multigenic expression of RT in different types of CB plant species and also within the same plant, there are some different kinds of RT with variable degree of glycosylation. RT typing cannot be done by the appearance of CB. However, the type of RT is related to bean variety and CB maturity, and cultivation conditions may also influence the RT type produced. There are three RT isoforms: ricin D (RTD), ricin E (RTE), and *R. communis* agglutinin (RCA). They are encoded by eight different multigene families. RTE and RTD differ in their amino acid sequences, affinity to Sepharose 4B, isoelectric points, and cytotoxicity. RCA differs substantially by being a tetramer formed by two RT molecular bound by non-covalent forces. RCA is not directly cytotoxic. It is able to bind to red blood cells and induces agglutination and hemolysis (Sehgal et al. 2010).

Sehgal et al. (2010) recognized three RTD isoforms by chromatography, I, II, and III, whose molecular weights lie between 60 and 65 kDa and differ in their relative electrophoretic mobility. RTD III is 4–8 times more cytotoxic to Vero cell

Table 1 Median lethal dose (LD_{50}) of ricin toxin in different animals and by different routes and times to death

Route	Animal	LD_{50}	Time to death (hours)
Intravenous	Mice	5 μg/kg	90
Intraperitoneal	Mice	22 μg/kg	100
Subcutaneous	Mice	24 μg/kg	100
Intramuscular	Rat	33–50 μg/kg	35
Inhalation	Mice	3–5 μg/kg	60
Inhalation	Rat	3.7 μg/kg	–
Oral	Mice	20 mg/kg	85

lines than RTD I and II. RTD III is also more toxic to mice than the two other RTD isoforms being lethal in acute intraperitoneal administration at 5–20 ng/kg body weight, whereas isoforms I and II were not lethal at 20 ng/kg body weight. Also RTD I and II made no weight change after 7-day period treatment of mice at mentioned dose. RTD III elicits toxicity via hemagglutination. The RTD III also has a different antigenic structure from other RTD. Anti-RTD III only binds to this isoform; however, anti-RTD I and II antibodies are cross-reactive with all the RT variants.

Toxicity

RT is one of the most potent plant-derived proteins with ribosome-inactivation ability (Audi et al. 2005). Various vertebrate species have different RT sensitivity, such as horses which are more sensitive than frogs or chickens (Poli et al. 2008). RT is most potent by inhalation and least toxic when administered orally (Poli et al. 2008). The oral median lethal dose (LD_{50}) in rodents is more than 1,000 times that by inhalation, specifically 20 mg/kg versus 3–5 μg/kg, respectively (Table 1). The lower oral toxicity of RT might be related to gastric degradation and poor absorption in the gastrointestinal (GI) tract due to its large size. A large amount of ingested RT has been found in the rats' large bowel after 24 h post ingestion (Poli et al. 2008).

Clinical Manifestations

Manifestations of RT poisoning in animals depend upon the route of administration. Oral ingestion of RT causes GI hemorrhage, liver, spleen, and kidney necrosis. Intramuscular injection induces severe localized pain and necrosis of regional lymph nodes and muscles with moderate systemic signs. Respiratory distress with pulmonary and airway lesions presents secondary to RT inhalation. In humans, transient leukocytosis, 2–5 fold above normal, presents after oral ingestion or intramuscular injection (Olsnes and Kozlov 2001; Audi et al. 2005;

Rana et al. 2012). The effectiveness of oral dosing with RT varies with purity of toxin, administration of RT orally or gavage feeding, and volume of gastric content (Pincus et al. 2011).

The majority of cases due to oral RT ingestion are related to eating of CB, and more than 1,000 cases of toxic bean consumption have been reported with a mortality rate of 1.9–6 %. The highest rate of death occurred when there was little or insufficient supportive care. Challoner and McCarron (1990) reviewed 424 cases of CB ingestion and reported 14 deaths with a mortality rate of 8.1 % when victims were untreated versus 0.4 % for treated patents.

Release of RT from CB requires digestion and delipidation of the bean matrix, without which a swallowed CB might pass intact and harmless through the GI tract intact because of its solid shell-like coating. Therefore, chewed, damaged, or immature CBs are much more toxic than the intact mature CBs which has the most robust shell (Pincus et al. 2011; Bradberry 2012). One reviewer reported that severity of clinical manifestations is related to the degree of mastication of the beans (Challoner and McCarron 1990). It has been suggested that orally administrated RT is less toxic than gavaged RT, possibly because carbohydrate structures with terminal galactose residues, expressed by GI microbial flora, compete with glycoproteins and glycolipids on tissue surfaces of GI tract to RT binding. Saccharide secreted by saliva or other GI goblet glands may also compete with RT binding (Pincus et al. 2011). Size, weight, and moisture content of CB, as well as region, season, and period of plant growth at the time of harvest, also influence the morbidity of CB intoxication (Audi et al. 2005).

Ingested RT is absorbed within 2 h via blood and lymphatic vessels, accumulating in the liver and spleen. In experimental mice, RT is detectable in feces after 2 h of oral gavage, but after 72 h, about 20–45 % of ingested RT is excreted via feces unchanged (He et al. 2010a).

RT toxicity is usually apparent 4–6 h after ingestion but may take as long as 10 h. Abdominal pain, oropharyngeal irritation, vomiting, and diarrhea are the primary clinical presentations of patients with CB intoxication. Different types of GI bleeding such as hematemesis, melena, or hematochezia may occur because of local necrosis of the GI tract. The volume loss causes dehydration, tachycardia, hypotension, and cyanosis. Hypovolemic shock and renal failure are induced by excessive volume loss. Hypoglycemia and hemolysis are other common manifestations. Cytotoxic effects may occur up to 5 days after exposure, even in asymptomatic individuals. Most studies of oral RT intoxication employed rodents which have highly cornified layer of stratified epithelia on the luminal surfaces of their GI tract, whereas the surface of the human GI tract is minimally keratinized (Pincus et al. 2011).

Macrophages of the reticuloendothelial system, such as Kupffer cells, have mannose receptors on the membrane surface predisposing them to RT toxicity. The resulting damage might persist for a long time and can progress to hepatic failure if a sufficient dose of RT is ingested (Poli et al. 2008).

High doses of RT injected intramuscularly or subcutaneously in humans cause local necrosis at the injection site, severe local lymphoid necrosis, liver necrosis,

GI hemorrhage, diffuse nephritis, and diffuse splenitis are other important manifestations of RT injection. The majority of the injected RT is excreted through urine after 24 h, and less than 2 % can be found in feces (Audi et al. 2005).

Intravenous administration of RT in rat causes hepatocellular, Kupffer cells, and renal lesions within 4 h and leads to endothelial cell damage, liver vascular thrombus, and hepatocellular necrosis. Disseminated intravascular coagulation has also been reported in these animal models (Lord et al. 2003; Audi et al. 2005; Coopman et al. 2009). RT has elicited no direct cardiotoxicity or arrhythmias (Christiansen et al. 1994).

A sepsis-like syndrome including nausea, anorexia, headache, fever, hypotension, and dizziness is an initial clinical presentation of intramuscular RT administration, lagging by as much as 10–12 h from injection. The injection site usually exhibits local tissue damage. In mice models, intramuscular RT increases liver transaminases, creatinine kinase, amylase, bilirubin, and renal insufficiency accompanied by myoglobinuria, lethal hypoglycemia, and metabolic abnormality (Poli et al. 2008). One to two days after intraperitoneal RT injection in mice, moderate to severe lymphoid necrosis in the lymph nodes, spleen, and thymus were observed, but other tissues were not affected (Audi et al. 2005; He et al. 2010a).

Human fatalities give us an insight into the value of animal studies with RT. A 20-year-old man, who committed suicide by injection of CB extract, was admitted 36 h post-injection. He presented with headache; abdominal, chest, and back pain; nausea; severe weakness; and dizziness. He also had metabolic acidosis, anuria, and hematochezia. His manifestations progressed to low blood pressure and renal and hepatic failure with bleeding diathesis and did not respond to vasopressors and supportive care. Bleeding led to cardiac arrest, resistant to full resuscitative efforts. Hemorrhagic foci of pleura, brain, and myocardium were found on autopsy. Georgi Markov, a Bulgarian journalist, was assassinated by injecting of as much as 500 μg RT. He immediately suffered localized pain escalating to general weakness over the ensuing 5 h. On admission, he had fever, nausea, vomiting, and tachycardia but normal blood pressure. There was an induration on his thigh with a 6 cm diameter which was likely to be the injection site. Regional lymph nodes of the effected limb were swollen. On the second day, he became tachycardic (heart rate = 160 beats per minute) and hypotensive and had leucocytosis (26,300/mm^3). On the following day, he was anuric and had hematemesis and complete atrioventricular conduction block that lead to death (Crompton and Gall 1980; Audi et al. 2005; Papaloucas et al. 2008; Balali-Mood et al. 2013).

To date, there is no report of fatal human RT intoxication by aerosol, but there have been reports of typical allergic syndrome including nose and throat congestion, itching eyes, urticaria, and chest tightness in CB dust-exposed workers.

Animal studies demonstrated that inhaled RT particles deposit in the lung and trachea. The RT particle concentration gradually decreases in the lung, increasing in the trachea due to mucociliary movement. The particle size of RT is an important factor that affects pulmonary deposition and lethality in animal models (Poli et al. 2008). Therefore, weaponizing RT by manufacturing a suitable aerosol to target the lung of sufficient concentration and for it to remain there is more difficult

than contaminating water or food (Pincus et al. 2011). Aerosolized RT binds to ciliated bronchial cells, alveolar macrophages, and lining cells (type I and II pneumocytes). Within 8 h, there is no detectable pulmonary injury in animals. The total protein and inflammatory cell counts of bronchoalveolar fluid increase within 12 h, due to cytotoxicity and enhancement of permeability of the air–blood barrier. It progresses to alveolar flooding and non-cardiogenic pulmonary edema up to 30 h after exposure. At this time, the animal has hypoxia and acidosis, followed by respiratory failure causing death. Systemic absorption of RT from the respiratory system upregulates inflammatory genes, releasing various cytokines and chemokines, leading to systemic inflammation, arthralgias, and fever (Pincus et al. 2011). However, monkeys intoxicated with aerosol RT had no sign of systemic absorption. Pathologic evaluation revealed a diffuse necrotizing pneumonia, interstitial and alveolar inflammation, edema, and alveolar flooding (Wilhelmsen and Pitt 1996).

The cause of death depends upon the route of intoxication. However, as RT is a relatively indiscriminant cellular toxin, all organs and systems are affected through RT intoxication. Oral ingestion of RT induces necrotic and hemorrhagic lesions in GI tract associated with hepatic and renal failure which lead to hypotension and vascular collapse. RT injection also causes GI hemorrhage, hepatic necrosis, and renal failure. Hypoxia secondary to pulmonary damage might be the main cause of death after RT inhalation.

Urticaria and allergic reaction induced by handling of CB or its products has also been reported. Pseudomembranous conjunctivitis or conjunctival irritation has also been observed in animal models due to ocular exposure to 1:1,000– 1:10,000 RT solutions (Audi et al. 2005).

Diagnosis

Application of RT as a BWA in the battlefield can be indicated by pulmonary distress in a large number of healthy soldiers if exposed to RT contaminated air; or GI hemorrhage with hypotension by multiple victims who ingested the same food. Equally, RT injection should be considered when a person at high assassination risk or terrorism threat and experiences rapid onset of one of the vascular leak syndromes (VLS) such as edema and hypotension.

Diagnosis of RT intoxication is difficult at first, especially if the physician is naïve to RT intoxication. Some epidemiological clues can lead physicians to diagnose RT intoxication, especially in a bioweapon attack scenario. They include:

(a) An unusual number of patients presenting with similar toxic symptoms
(b) Unexplained death or illness of healthy individuals
(c) People with a common characteristic, such as a workplace and source of drinking water, showing similar clinical manifestations
(d) Rapid onset of symptoms

Table 2 Differential diagnosis for ricin poisoning based on route of exposure

Route of exposure	Source of exposure	Infectious
Oral	Abrin (abrusprecatorius)	Enterotoxins of *Staphylococcus aureus*
	Detergents	*Clostridium perfringens*
	Simple hydrocarbons	Anisakiasis
	Pharmaceuticals (e.g., colchicines, salicylates, digoxin, antimetabolite cancer drugs)	*Escherichia coli*, *Bacillus cereus*(type I)
	Caustic agents (corrosives, acids, bases) diarrheic shellfish poisoning (okadaic acid)	Enterocolitica
	Mushroom species (boletes species, lactarius species, amanita phalloides)	Yersinia
	Plant species (e.g., pokeweed; phytolacca species, solanine-containing plants)	Salmonella
	Metals (arsenic, mercury, copper, lead, cadmium)	Cholera
		Shigella
		Campylobacter jejuni
		Any infectious agent that cause sepsis
Inhalation	Abrin (abrusprecatorius)	Influenza
	Paraquat	Q fever (biowarfare agent or natural infection)
	Irritant gases (ozone, chlorine, phosphine, phosgene, oxides of nitrogen)	Anthrax (biowarfare agent or natural infection)
	Metal fume fever	Inhalational tularemia
	Polymer fume fever (by-products of organofluorine polymers pyrolyzing such as Teflon and Kevlar)	Any bacterial or viral agents that cause a diffuse, localized
	Particulate irritants (smoke, fumes, acid mists, dusts)	Pneumonic process
Injection	Snake bite (vipers)	Pneumonic plague
	Scorpion sting (hemiscorpius lepturus)	Cellulitis

(e) Unusual death in animal, plants, or fish for which there is no reasonable explanation
(f) A toxidrome that matches with RT intoxication in one or more patients

As the initial manifestations of RT intoxication are nonspecific, there is a long list of differential diagnoses (Table 2). The majority of pulmonary disease such as exacerbation of chronic obstructive pulmonary disease and asthma is also in Table 2.

Manifestations of ingested RT are dose-dependent, from localized to systemic poisoning such as hepatic and renal dysfunctions. The infectious agents able to cause GI symptoms within 1–6 h are more likely to consider in the differential diagnosis list of oral RT intoxication (Audi et al. 2005).

Detection

RT is detected in tissue sections, body fluids, and nasal swabs of animals by immunologically based methods like enzyme-linked immunosorbent assay (ELISA). This method is also applied for human specimens. Its lower limit of detection is about 0.1 ng/mL (1.54 pmol/L) and RT can be detected up to 24 h post exposure. However, there are few commercially available assays. Detection can be compromised by RT binding to proteins and its metabolizing before excretion. Ricinine, which is extracted from urine up to 48 h after exposure to RT, may prove a good marker for RT exposure. As RT is extremely immunogenic, human survivors of RT poisoning have detectable antibodies for up to 2 weeks (Bradberry 2012).

CDC and reference laboratories also prefer screen for RT in environmental samples using time-resolved immunofluorescence assays and polymerase chain reaction (PCR) tests. Cell-based bioassays can also be used to confirm the presence in environmental specimens of intact RT with killing activity. Handheld assays, or "smart tickets," based on immunoassays, are used by military services for testing RT contamination in the field (Audi et al. 2005; Poli et al. 2008).

A monoclonal antibody-based electrochemical luminescence method was reported by Brandon et al. (2012) to detect and quantify RT in food, such as liquid egg, and has a limit of detection of 0.2 ng/mL. Another novel method is immuno-PCR (IPCR) where RT is directly adsorbed onto wells of a microtiter plate or indirectly immobilized via a capture antibody (sandwich IPCR). This method can detect amounts of RT as low as 1 pg/mL with its limit of detection in milk and egg samples being 10 pg/ml and 100 pg/ml in beef. This is 100–1,000-fold lower than results obtained by ELISA (He et al. 2010a, b). Matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) is also a method for detection of RT in biological specimens (Audi et al. 2005).

Medical Management

Treatment of RT intoxicated patients is very difficult, because the toxin acts rapidly and irreversibly. Therefore, preventative measure such as vaccination of high-risk groups, such as military personnel or diplomatic persons at risk, is an important strategy.

Decontamination

Since most cases of RT intoxication usually occur as bioterrorism or as a biological warfare attack, all protocols and guidelines of BWA decontamination and protection should be employed.

On dermal contamination, remove all clothes, jewelry, and dressings; place the clothing in a labeled durable 6-mil polyethylene bag; and obliterate them as other CWA. Wash the victim's skin with sufficient water and soap (Audi et al. 2005).

On oral ingestion of RT or CB, gastric lavage and administration of activated charcoal should be given, in the absence of contraindications. However, the adsorption rate of RT by activated charcoal is not yet clarified (Pincus et al. 2011) and so its effectiveness is not yet quantitated. After inhalational exposure, decontamination of the GI tract is not necessary.

It is recommended that surfaces and clothing be cleaned with a 0.1 % sodium hypochlorite solution for at least 30 min. The solution may completely inactivate RT (Audi et al. 2005).

Personal Protective Equipment and Decontamination

Personnel who work in areas contaminated with RT aerosols should protect themselves with B level personal protective equipment (PPE) such as a self-contained breathing apparatus. However, as at the first encounter the exact contaminator agents may not be known, it would be prudent to protect personnel using the highest protection level [see http://www.osha.gov/dts/osta/bestpractices/html/hospital_firstreceivers.htm or http://cdc.gov/niosh/unp-intrecppe.htm]. The workers should have a full-face respirator with P-100 filter, and a disposable suit chemically coated to prevent penetration. Patients should be decontaminated away from the site of exposure and before being transferred to hospital in designated hazardous materials (HAZMAT) decontamination areas. When victim decontamination is completed, all PPE should also be decontaminated by water before removing. This will reduce the risk of secondary contamination of rescuers. Clothes should be removed and soaked in a 0.1 % sodium hypochlorite solution for 30 min and then rinsed with water and dried in air. Rescuers should also take a shower to decontaminate the exterior of their protective suits or apparatus (Audi et al. 2005).

Supportive and Specific Therapy

Supportive care is the predominant form of RT poisoning therapy and differs depending on the route of RT exposure with the most important elements being water and electrolyte replacement and corrections of liver and renal functions. As cytotoxic effects of RT may occur up to 5 days after intoxication, biochemical monitoring is recommended through this period, even in asymptomatic patients.

Intravenous or intramuscular RT intoxication needs more careful monitoring for cardiovascular and lung functions and prompt treatment of pulmonary edema and hypotension. Correction of coagulopathies, respiratory support, and monitoring of liver and renal functions are also important to parental RT intoxication. Supportive therapy of acute pulmonary edema and respiratory distress such as fluid and electrolyte replacement, administration of anti-inflammatory agents, analgesics, positive-pressure breathing, and artificial ventilation are indicated in inhalational RT intoxication (Griffiths et al. 1995, 2007; Bradberry et al. 2003; Doan 2004; Audi et al. 2005).

Victims that remain asymptomatic 12 h after exposure (oral or inhalational) are at low risk of developing toxicity and can be discharged but with some precautions (Audi et al. 2005). It should be remembered that animal studies revealed that clinical manifestations may not appear in RT inhaled animals for 20–24 h after intoxication, and cytotoxic effects of RT may not occur for up to 5 days. Thus, biochemical monitoring is recommended throughout this 1–5-day period, even in asymptomatic patients. Asymptomatic patients with isolated dermal contact can be discharged after complete decontamination, unless they have abraded or burned skin through which the toxin may enter the blood or lymphatic system. Victims usually recover if they do not expire within 3–5 days after exposure (Poli et al. 2008).

Two compounds (difluoromethylornithine and dexamethasone) have been recommended for treatment of RT poisoning and have induced longer survival times in intoxicated mice (Poli et al. 2008). No specific RT antidotes have been approved for human use. Therapeutic inhibition of RT intoxication includes blocking the binding of RTB to cell-surface glycans, hindering N-glycosidase activity and inhibiting retrograde transport via the protein synthesis pathway. During recent decades, researchers have attempted to develop RT inhibitors by reducing its N-glycosidase activity, but the more recent focus has been on molecules that disrupt intracellular trafficking. This approach not only protects cells but also sometimes protects animals against RT toxicity (Barbier et al. 2012).

RT passes retrogradely from the plasma membrane to the endoplasmic reticulum through the Golgi apparatus and endosomes. The effectiveness of molecules that protect the Golgi apparatus against RT toxicity has been evaluated by a cell-based high-throughput screening test. These molecules protected cells against RT toxicity (Stechmann et al. 2010; Barbier et al. 2012).

Antibody therapy also seems effective in treating toxin-mediated diseases. Anti-RT antibodies (anti-RTA, anti-RTB, and anti-RT) prevent binding, internalization, or routing of RTA to the endosomal compartment, changing intracellular trafficking, and neutralizing the RT inside the cell. However, it is suggested by most authors that anti-RTA antibodies are more protective than anti-RTB antibodies based on in vitro *studies*. It is reported that some antibodies protect cells even when added 8 h after cellular exposure. Other studies have demonstrated that animals can be protected against RT by immunotherapy with monoclonal antibody (MAb) RAC18 being effective even when it was administrated 12 h after exposure (Pincus et al. 2011).

MAbs with high specificity and defined properties have been developed by hybridoma technology. However, the majority of the developed mAbs against RT have shown indifferent properties, binding the toxin without any effect on toxicity and or host damage (Chow and Casadevall 2012).

3′-azido-3′-deoxythymidine is another therapeutic lead evaluated for treatment of RT toxicity in an in vitro model (Vero cells). Its inhibition of RT cytotoxicity does not involve perturbations of RT cell-surface binding, internalization, or enzymatic activity but might result from an alteration in RT translocation. (Hassoun and Stohs 1996).

Vaccination and Passive Protection

Different high-risk groups need tailored approaches for protection against RT. Active vaccination suits military personnel, very important or diplomatic individuals at high assassination risk, emergency first responders, and laboratory personnel investigating RT. For civilians however that are at low risk for exposure, a superior approach is post exposure vaccination or antibody therapy. The general public should be vaccinated if there is the real risk of repeated terrorist attacks. Postexposure treatment, however, needs fast diagnosis, rapid exposure confirmation, and easy access to a suitable vaccine (Pincus et al. 2011). The resemblance of early manifestations caused by RT intoxication to many more common diseases like influenza can confuse a rapid diagnosis (see differential diagnosis in Table 2).

The ideal RT vaccine would protect victims against all routes of RT intoxication and for large emergency immunizations, should have a suitable half-life and provoke long-lived immunization with only 1 or 2 doses. Different types of ricin toxoid, generated by heating or adding chemical substances, were evaluated in rodent models subcutaneously or as aerosol. While they reduce RT-induced mortality, they fail to protect against lung damage following intratracheal administration of RT. Oral administration of ricin toxoids provide no protection against aerosol RT exposure. Another problem of the toxoid is residual risk of toxicity from inefficient inactivation (Pincus et al. 2011). Inhalational exposure is best countered with active immunization (Poli et al. 2008).

Deglycosylated RTA chain may induce local or systemic VLS; however, deglycosylation prevents liver uptake and therefore prevents liver damage. These vaccines induce suitable mucosal response (protection against a mucosal challenge) by IgA production; but the various forms, with or without mucosal adjuvants, do not elicit higher titers of antibodies or protection when comparing mucosal administered toxoid.

Another vaccine is formalin-inactivated toxoid, although formalin cannot fully inactivate RT. This type of vaccine is effective against aerosolized RT. Recombinant ricin A chain vaccines are also applied to reduce adverse effects of vaccine and increase the stability. The US Army has developed RTA 1–33/44–198, a structurally modified ribosome-inactivating protein, which has afforded 100 % protection of animals against supralethal doses of RT aerosol (Audi et al. 2005; Carra et al. 2007; Legler et al. 2011).

Another vaccine, developed by a research group in Texas (Smallshaw et al. 2007), has been developed using a recombinant ricin A chain including the enzymatic and VLS-inducing sites. Now known as RiVax™ (DOR BioPharma, Inc, Miami, Fla), its ability to inhibit protein synthesis is at least 10,000-fold less effective than wild-type RT A chain and does not induce VLS thus providing protection without potential RT-induced side effects. This vaccine could protect animals against exposures of 10 times of the intraperitoneal LD_{50} (Smallshaw et al. 2007). RiVax™ is highly soluble and stable in a variety of formulations. Intramuscular RiVax™ administration into mice protected their lung functionality and tissue integrity against aerosol RT in a dose-dependent manner. RiVax™ has

already passed some clinical trials where it was safe and elicited ricin-neutralizing Abs in all volunteers in the high-dose group (Vitetta et al. 2006; Legler et al. 2011).

Inhalation of anti-ricin immunoglobulin (IgG) within the first hour of exposure could protect animals against lung lesions and reduce mortality rate. The anti-ricin immunoglobulin may also keep animals safe up to 2–3 days after administration, based on observed clearance rates of IgG from the rabbits' airways. Applying anti-ricin IgG with a portable nebulizer immediately before an exposure is likely to provide some protection for nonimmune individuals or reduce their toxicity manifestations (Pincus et al. 2011).

Beneficial Uses of RT

RT consists of two parts with different roles in its cytotoxicity. Therefore, researchers have suggested the possible replacement of one part with another ligand such as antibodies against cancer-associated antigens on the cell surface, growth factors, and hormones direct the toxins to the targeted cells. Although the new conjugated compounds often have lesser cytoactivity than the native toxin, there are several successful reports of in vivo tests (Yoshida et al. 1997; Shah et al. 1993). To counter this limitation, compounds have been conjugated to the whole RT instead of the A-chain alone. However, the conjugates containing the B chain of RT possess much lower specificity for the target cells, increasing adverse effects and toxicity to other cells. However, there are few reports of highly active conjugate compounds without the B chain. The majority of ligands and antibodies linked to the RT A chain, replacing the B chain, are unable to deliver to the trans-Golgi network to the endoplasmic reticulum (Olsnes and Kozlov 2001).

Conclusion

RT is a potent cell toxin from the bean of the castor plant *Ricinus communis*, and a potential BWA and assassination tool because of ease of access, simple extraction, and stability. Toxicity results from protein synthesis inhibition, but other contributing mechanisms have been noted including apoptosis pathways, magnesium and calcium imbalance, cytokines release, acute phase reactions, and oxidative stress in the liver. Use of ricin as a terrorist weapon highlights the need for clinicians and public health officials to be aware of ricin-associated illness. Diagnosis of RT intoxication is difficult, especially in the first encounter, and ricin poisoning manifestations will vary depending on the route of exposure. Oral ingestion of RT causes GI hemorrhage and liver, spleen, and kidney necrosis. Intramuscular injection induces severe localized pain, necrosis of regional lymph nodes, and muscles with moderate systemic signs. Respiratory distress with pulmonary and airway lesions present secondary to RT inhalation. Several currently available methods such as ELISA, PCR, and immunofluorescence assays can detect the presence of ricin in tissue sections, body fluids, environmental

samples, and food. Treatment of RT intoxicated patients is very difficult, because the toxin acts rapidly and irreversibly. Therefore, preventative measures such as vaccination of high-risk groups are an important strategy. However, because of its cytotoxic effect, modified ricin which retains a potency for the selective killing of unwanted cells (immunotherapy of cancer) may be used in future therapies.

Cross-References

▶ Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment

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Current Insights into Mycotoxins

4

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Abstract

Mycotoxins are secondary metabolites that are produced by different fungi and can occur more frequently in areas with a hot and humid weather. Several foods derived from plants can be infected by fungi during growing of the plants, harvest, and storage of the food; therefore, exposure to mycotoxins can easily occur in human populations. Researchers have special focus on mycotoxins because of their toxic effects in both animals and humans. Although these agents have toxic effects in several organs, the liver, kidneys, lungs, endocrine, nervous, and immune systems have been considered as more notable target organs. The mechanisms of mycotoxin-induced toxicity have been studied using different in vivo and in vitro models. Disruption of protein, DNA and RNA synthesis, depletion of GSH (glutathione) content, generation of reactive oxygen species, and induction of apoptosis have been reported as cellular signaling. Further studies are needed to elucidate complete cellular pathway of mycotoxins toxicity. More important mycotoxins including fumonisins, ochratoxins, trichothecenes, zearalenone, and patulin are discussed in this chapter with focus on toxicity and mechanisms of action in animals and humans.

Introduction

Mycotoxins are secondary metabolites produced by different pathogenic fungi mostly *Fusarium, Aspergillus*, and *Penicillium* (Bennett and Klich 2003). Hot and humid climate is mentioned as an optimum condition to produce mycotoxins. These are low-molecular-weight compounds with different chemical structures which are relatively stable to heat and other treatment process; therefore, they may remain in foods for a long period (Bennett and Klich 2003; Styne et al. 2009). Different foods including wheat, maize, barley, oat, rice, coffee, and fruits can be contaminated with mycotoxins (Voss et al. 2007; Yazar and Omurtag 2008). Mycotoxins can be formed on crops in the field, during harvest, storage, processing, and feeding. Although exposure to mycotoxin is generally mediated by ingestion, dermal and inhalation routes are also important (Peraica et al. 1999; Styne et al. 2009).

Mycotoxins exert toxic effects on many organs of humans and animals most notably the liver, kidneys, lungs, endocrine, nervous, and immune systems (Peraica et al. 1999; Voss et al. 2007; Wild and Gong 2010).

Mycotoxicosis has been considered as an old problem in the world, and some well-documented examples of mycotoxicosis include Balkan endemic nephropathy (BEN). BEN is a progressive chronic nephritis affecting the populations who lived in areas bordering the Danube River in parts of Romania. Mycotoxicosis was due to ochratoxin A (El Khoury and Atoui 2010) and alimentary toxic aleukia (ATA) that

caused deaths of large population in the Orenburg district of the former USSR during World War II due to trichothecenes (Yazar and Omurtag 2008).

Disruption in cell differentiation and cell growth, inhibition of protein synthesis and mitochondrial respiration, inhibition of DNA and RNA synthesis, disruption in the function of different cellular enzymes, depletion of GSH content, and induction of apoptosis through different pathways have been reported as some cellular mechanisms of mycotoxins (Grosse et al. 1997; Liu et al. 2006; Ringot et al. 2006; Liu et al. 2007; Yazar and Omurtag 2008; Wild and Gong 2010).

Because of the importance of mycotoxins on human health, mechanisms of action of the most notable mycotoxins including fumonisins, ochratoxins, trichothecenes, zearalenone, and patulin will be described.

Fumonisins

General Considerations

Fumonisins are a group of toxic metabolites first described and characterized in 1988.

These mycotoxins are produced by a number of *Fusarium* species, mostly *Fusarium verticillioides* (formerly *Fusarium moniliforme*), *Fusarium proliferatum*, and *Fusarium nygamai* (Bennett and Klich 2003).

Fumonisins are commonly produced in hot and dry weather following high humidity. Different environmental factors such as temperature, insect damage, humidity, and rainfall during preharvest and harvest periods could increase the formation of fumonisins (Yazar and Omurtag 2008; Styne et al. 2009).

More than 28 analogous of fumonisins have been discovered, which include the fumonisin A, B, C, and P series (Styne et al. 2009). FB1 (Fig. 1) is the most

Fig. 1 Chemical structure of fumonisin B1 (Bennett and Klich 2003)

common fumonisin produced in nature, comprising approximately 75 % of the total fumonisin content. FB2, FB3, and FB4 are in order less prevalent and have different structures from FB1 in the number and placement of hydroxyl groups on the molecule's hydrocarbon "backbone" (Voss et al. 2007).

Fumonisins are heat-stable toxins, which most generally are found in corn, corn-based foods, and other grains such as rice (Chu 2006). The toxin content is significantly reduced during processes in which the temperature exceeds 150 °C. Alkaline cooking and heating (nixtamalization) of maize induce hydrolysis of fumonisins, but do not completely detoxify contaminated maize. In addition, they may be converted to products of unknown toxicity. Many of mycotoxins are soluble in organic solvents, but fumonisins are hydrophilic agents and usually they are extracted in aqueous methanol or aqueous acetonitrile (Blackwell et al. 1996; Styne et al. 2009).

Mechanism of Action

Fumonisins or their hydrolyzed forms are not metabolized by phase I or II enzymes, and thus their mechanisms of action do not depend on metabolic activation (Voss et al. 2007).

There is structural similarity among fumonisins and the sphingoid bases; sphinganine (Sa) and sphingosine (So) as shown in Fig. 2. Therefore, these mycotoxins can interfere with the de novo synthesis of complex glycosphingolipids. Following disruption in sphingolipid metabolism via inhibition of ceramide

Fig. 2 Chemical structure of sphinganine (*Sa*) and sphingosine (*So*) (Voss et al. 2007)

synthase (Voss et al. 2007; Yazar and Omurtag 2008), different cellular processes, such as cell differentiation, cell proliferation, and cell morphology can be affected (Yazar and Omurtag 2008; Wild and Gong 2010). Also, induction of apoptosis in different cell culture systems including kidney, cardiomyocyte, intestinal, epithelial, and esophageal cells after exposure to FB-1 has been demonstrated (Styne et al. 2009).

Mitochondrial damage and induction of lipid peroxidation were described as other mechanisms in fumonisin-induced toxicity (Abel and Gelderblom 1998; Styne et al. 2009).

Free FB-1 excretion in the feces and Sa/So and SaP/SoP (sphinganine phosphate to sphingosine phosphate) alteration in the plasma and urine have been considered as fumonisins biomarkers (Cai et al. 2007; Kim et al. 2007).

Toxicity in Animals

Fumonisin-induced toxicological and pathological effects have been extensively studied in different animal models. The liver and kidney are the main target organs in fumonisins toxicity, although species, strain, and sex-dependent differences exist (Voss et al. 2007). Toxic effects in both liver and kidney include apoptosis alone or together with increased mitosis as an early microscopic indication of tissue injury (Voss et al. 2001). Long-term chronic exposure induced severe liver lesions that finally developed to cholangioma, fibrosis, and cirrhosis (Lemmer et al. 2004). Horses are the most sensitive species to fumonisin toxicity and equine leukoencephalomalacia (ELEM) which has been reported in them was associated with necrosis of white matter, edema and hemorrhage (Voss et al. 2007; Yazar and Omurtag 2008). In pigs, pulmonary edema syndrome (PED) has been recorded and it seems that cardiotoxicity induces pulmonary edema which is generally lethal (Voss et al. 2007; Yazar and Omurtag 2008). Studies in ruminants exhibited fumonisins could induce nephrotoxicity and hepatotoxicity in calves, lambs, and goats (Styne et al. 2009).

Fetotoxicity in the presence of maternal toxicity was reported, although several animal studies indicated that there is little or no transfer of fumonisins through the placenta or to milk (Voss et al. 2007). Another report showed that FB1 could cross the placenta and induced neural-tube defects in animals which is due to alteration of sphingolipid metabolism and reduction in folate concentration (Gelineau-van et al. 2005). Since FB induced membrane disruption, it could lead to decrease of folate absorption by damage to the gastrointestinal system (Wild and Gong 2010).

Toxicity in Humans

The humans' health effects of fumonisins are not proved, but ecological studies reported consumption of FB1-contaminated maize can be associated with high rates of esophageal cancer (Wild and Gong 2010).

It was also reported that consumption of FB1-contaminated maize or maize-based food products during early pregnancy could result in increased risk of neural-tube defects in the developing fetus. The hypothesis includes a critical role of fumonisins in disruption of folate membrane transport (Missmer et al. 2006; Wild and Gong 2010). A provisional maximum tolerable daily intake (PMTDI) for FB1, B2, and B3, single or in combination, of 2 μg/kg body weight/day on the basis of the NOEL of 0.2 mg/kg body weight/day was established by the Joint FAO (Food and Agriculture Organization)/WHO Expert Committee for Food Additives (JECFA) (Yazar and Omurtag 2008; Wild and Gong 2010).

Carcinogenicity

Studies in rodents have shown that FB1 can induce liver and kidney cancers with both cancer-initiating and cancer-promoting effects. Two important mechanisms of FB1-induced carcinogenesis include (1) induction of apoptosis through disruption of ceramide synthase in the development of hepato- and nephrocarcinogenesis in rats and mice (Styne et al. 2009) and (2) the disruption of fatty-acid metabolic pathways as a major event in the development of hepatocarcinogenesis in rats (Styne et al. 2009). International Agency for Cancer Research (IARC) has evaluated the cancer risk of fumonisins to humans and classified them in group 2B (probably carcinogenic) (Yazar and Omurtag 2008; Wild and Gong 2010). As mentioned above, fumonisins can elevate risk of esophageal cancer in humans.

Ochratoxins

General Consideration

Ochratoxins (OT) belong to a family of mycotoxins that are produced by secondary metabolism of *Aspergillus sp.* and *Penicillium sp.* Different types of ochratoxins occur naturally, which include OTA (ochratoxin A), OTB (dechlorinated OTA), and OTC (ethylated OTA), that are often coproduced. *Aspergillus ochraceus* and *Penicillium viridicatum* are two species which were first reported as producers of OTA and occur frequently in nature. OTA (Fig. 3) which is discovered in 1965 is the major toxin in this group (El Khoury and Atoui 2010; Reddy and Bhoola 2010). Some foods that contain OT include cereal, maize, wheat, barley, oats, and coffee beans (Yazar and Omurtag 2008; Reddy and Bhoola 2010). Also, OT has been found in meat, dairy, and baked products, especially swine sausages. Cereals are mentioned as being the major source of OTA contamination, where 50 % of human daily intake of this mycotoxin is due to the consumption of different cereals and derived products. Recently wine was considered as being the second source of OTA human consumption (El Khoury and Atoui 2010; Reddy and Bhoola 2010). OTA has a resistance to acidity and high temperature; therefore, once foodstuffs are contaminated, totally removing this molecule is very difficult (El Khoury and Atoui 2010).

Fig. 3 Chemical structure of ochratoxin A (El Khoury and Atoui 2010)

Mechanism of Action

Several major mechanisms have been hypothesized to be responsible in the OTA-induced toxicity, such as inhibition of protein synthesis, induction of lipid peroxidation, disruption of calcium homeostasis, inhibition of mitochondrial respiration, apoptosis, and DNA damage (Ringot et al. 2006). Disruption of cellular signaling pathway affecting cell viability and cell growth parameters has been shown (Gekle et al. 2005). In some studies, oxidative stress has been considered as a mechanism of OTA toxicity, while pretreatment of rats with retinol (vitamin A), ascorbic acid (vitamin C), or alpha tocopherol (vitamin E) before OTA administration significantly decreased the number of DNA adducts formed in the kidney (Grosse et al. 1997).

Toxicity in Animals and Humans

After oral administration, OTA has 40–60 % bioavailability. Because of high plasma protein binding, its elimination is a slow process, and high concentrations are found in the blood, kidney, and liver (Styne et al. 2009).

OTA has been found as a nephrotoxic agent in all animal species which causes degeneration of the proximal tubules, interstitial fibrosis in the renal cortex, and atrophy in the tubular epithelium (Chu 2006; El Khoury and Atoui 2010). Liver necrosis was also observed in experimental animals after administration of OTA (Gagliano et al. 2006; Reddy and Bhoola 2010). OTA can cross the placenta and accumulate in fetal tissue, inducing different anomalies in rats (El Khoury and Atoui 2010). OTA can also act as a neurotoxic (Brown et al. 1976) and immunotoxic agent on several species of animals (Bennett and Klich 2003). Inhibition of the peripheral T- and B-lymphocytes proliferation, which stops the production of interleukin 2 (IL2) and its receptors, have been reported as some immunosuppressive effects of OTA (Lea et al. 1989). It should be considered that OTA toxicity is depending on the sex, species, and cellular type of animals (El Khoury and Atoui 2010).

OTA is believed to be responsible for a human fatal disease known as Balkan endemic nephropathy (BEN), a progressive chronic nephritis affecting the populations who lived in areas bordering the Danube River in parts of Romania, Bulgaria, and the former Yugoslavia. OTA also is proposed to be main cause of the Tunisian nephropathy (TCIN) affecting the population in Tunisia (Bennett and Klich 2003; El Khoury and Atoui 2010). It has been demonstrated that OTA levels in the food and human serum of endemic areas are higher than in the non-endemic regions (Reddy and Bhoola 2010). Carcinogenicity of OTA in animals has been proved, and IARC classified it in class 2B (possible carcinogen to humans) (Reddy and Bhoola 2010). The JECFA set a provisional tolerable weekly intake (PTWI) of 0.1 μg/kg body weight/day for OTA (El Khoury and Atoui 2010).

Trichothecenes

General Consideration

Trichothecenes (TCs) are one of the major groups of mycotoxins produced by many species of fungi in the genera *Fusarium* (most frequently), *Myrothecium*, *Spicellum*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium*. Based on the structure, they have been classified into four groups (types A, B, C, and D). Type A includes T-2 toxin, HT-2 toxin, neosolaniol (NESO), diacetoxyscirpenol (DAS), and T-2 tetraol. Deoxynivalenol (DON) and nivalenol (NIV) belong to type B group, while type C includes roridins and verrucarins (Chu 2006; Yazar and Omurtag 2008; McCormick 2011). More than 180 different TCs and their derivative have been characterized (Yazar and Omurtag 2008), but only the most important toxins including T-2 toxin (Fig. 4) and deoxynivalenol (Fig. 5) will be described. The TCs' mycotoxins are nonvolatile, low-molecular-weight, and amphipathic molecules that can cross passively through cell membranes (Sokolović et al. 2008; McCormick 2011). These are heat stable and are not degraded during normal food processing or autoclaving (Yazar and Omurtag 2008).

Mechanism of Action

TCs have high-affinity binding to 60s ribosomal subunit and can inhibit protein synthesis through disruption in polypeptide chain initiation, elongation, and termination stages (Bennett and Klich 2003; McCormick et al. 2011). These mycotoxins can interact with protein sulfhydryl group (Ueno and Matsumoto 1975), alter membrane structure, and consequently induce lipid peroxidation (Yazar and Omurtag 2008). Inhibition of DNA and RNA synthesis, inhibition of electron transport activity, inhibition of succinic dehydrogenase activity, disruption of mitochondrial protein synthesis, generation of free radicals, and stimulation of lipid peroxidation have been considered as different mechanisms of TCs

Fig. 4 Chemical structure of T-2 toxin (Bennett and Klich 2003)

Fig. 5 Chemical structure of deoxynivalenol (Bennett and Klich 2003)

(Wannemacher and Wiener 1997; McCormick et al. 2011). Trichothecene-induced cytotoxicity and apoptosis associated with activation of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated protein kinase (ERK), and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in RAW264.7 macrophage cells and U937 monocyte models suggest possible involvement of these kinases in trichothecene-induced apoptosis (Yang et al. 2000).

Toxicity in Animals

Diacetoxyscirpenol, deoxynivalenol, and T-2 toxin are the most studied of the TCs that are produced by the *Fusarium* species. Usually there is high level of DON contamination in corn and wheat. DON as a major toxin of the type B group causes anorexia, nausea, vomiting, and diarrhea after ingestion in animals, and because of this effect, the name "vomitoxin" is also used. After short-term or long-term administration of DON to experimental animals, one of the major effects observed in most species was reduced growth that often was considered as the most sensitive parameter in routine studies of toxicity. At higher doses, the thymus, spleen, heart, and liver were affected (Chu 2006; Bonnet et al. 2012). Both immunostimulatory and immunosuppressive effects of DON depending on dose and exposure frequency have been reported (Pestka 2007). Effects of DON on brain structure and neurochemistry have been established (Bonnet et al. 2012). It was reported that DON administration to pigs can increase norepinephrine and reduce dopamine concentrations in the hypothalamus, frontal cortex, and cerebellum, while 5-HT (5-hydroxytryptamine) content increased in the hypothalamus 1 h after treatment but reduced in the hypothalamus and the cortex at 8 h (Prelusky et al. 1992). The modulation in the expression of neurotransmitters and anorexigenic or orexigenic neuropeptides in the hypothalamus was hypothesized to contribute to the anorexic behavior induced by inflammatory signals following exposure to DON (Bonnet et al. 2012). DON was not mutagenic in bacteria but induced chromosomal aberrations both in vitro and in vivo, suggesting that it is genotoxic. T-2 toxin contamination can occur in cereal grain including barley, corn, oat, and wheat. T-2 toxin is readily metabolized by the gut microflora of mammals to several metabolites. HT-2 toxin is a primary metabolite that is absorbed into the blood after ingestion of T-2 toxin. Metabolism can continue in the liver with biliary excretion. Cytotoxicity, necrosis of skin tissues, and inflammation reaction near the nose and mouth of animals are some of the toxic effects of T-2 toxin in animals (Bennett and Klich 2003; Chu 2006). T-2 toxin has major effects on hematopoietic system while inducing cellular damage in bone marrow and affecting plasma coagulation factors (Chu 2006). Neurological effects, disruption in blood–brain barrier, neuronal protein depletion, gastrointestinal lesions, hepatotoxicity, and immunomodulatory activity of T-2 toxin have been observed (Smith 1992; Sokolović et al. 2008).

Toxicity in Humans

Alimentary toxic aleukia (ATA) has been demonstrated in humans, which caused the deaths of a large population in the Orenburg district of the former USSR during World War II (Yazar and Omurtag 2008).

T-2 toxin and diacetoxyscirpenol seem to be responsible for ATA. Symptoms and signs of this disease include vomiting, gastroenteritis, skin inflammation, bleeding from nose and mouth, atrophy of bone marrow, leukocytosis, leukopenia, and central nervous system disorder (Chu 2006; Yazar and Omurtag 2008).

TCs can be found in the air during the drying and milling process on farms and on the walls of houses with high humidity (Croft et al. 1986; Yazar and Omurtag 2008). It has been reported that "sick building syndrome" can be related to TCs while the symptoms of airborne toxicosis disappeared after completely cleaning of buildings and ventilation systems (Croft et al. 1986).

TCs have been mentioned as agents for weaponization, because they have antipersonnel properties, large-scale, and easy production and can also be delivered as dust, aerosol, and smoke from aircraft (Wannemacher and Wiener 1997).

There are reports that USSR used TCs as a biological warfare agent in Southeast Asia during the 1970s (Watson et al. 1984). The air attack in Laos has been explained as "yellow rain" that consisted of a shower of sticky yellow liquid that seemed like rain as it fell from the sky. More than this, in Iran-Iraq War (1983–1984), TCs were used in combination with mustard by the Iraq government (Ember 1984). IARC placed TCs in group 3, not classifiable as to its carcinogenicity to humans (Yazar and Omurtag 2008). PMTDI levels for DON and T2-toxin according to JECFA are 1 μg/kg body weight/day and 0.06 μg/kg body weight/day, respectively (Yazar et al. 2008).

Zearalenone

General Consideration

Zearalenone (ZEA) also known as F-2 toxin (Fig. 6) is a secondary metabolite biosynthesized by a variety of *Fusarium* fungi, including *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense*, and *F. semitectum* (Zinedine et al. 2007). Wheat, maize, sorghum, and barley are the main sources of this mycotoxin (Yazar and Omurtag 2008). Presence of oxygen and moisture content are important factors for ZEA production and it should be noticed that ZEA and its metabolites are stable during storage, milling, processing, and cooking (Yazar and Omurtag 2008). Following oral administration, ZEA is rapidly and extensively absorbed from the gastrointestinal tract in animals and humans. ZEA bioavailability can be estimated over 80 % in animals (Zinedine et al. 2007). Major biotransformation pathways for ZEA in animals include the following (Zinedine et al. 2007; Minervini and Aquila 2008):

1. Hydroxylation resulting in the formation of α- and β-ZEA, catalyzed by 3α- and 3β-hydroxyl steroid dehydrogenase (HSDs)
2. Conjugation of ZEA and its reduced metabolites with glucuronic acid, catalyzed by uridine diphosphate glucuronyl transferase (UDPGT)

ZEA may co-occur with DON in grains such as barley, oat, wheat, and corn. Generally, DON is found in higher doses than ZEA when it occurs (Yazar et al. 2008).

Fig. 6 Chemical structure of zearalenone (Bennett and Klich 2003)

Mechanism of Action

Zearalenone (ZEA) is classified as a nonsteroidal estrogen or mycoestrogen; sometimes it is named phytoestrogen (Speijers and Speijers 2004). It has been demonstrated that ZEA and some of its derivatives competitively bind to estrogen receptors (ERα and ERβ) present in different organs such as the uterus, breast, and adrenal and pituitary glands (Bennett and Klich 2003; Minervini and Aquila 2008; Yazar and Omurtag 2008). The relative binding affinities of ZEA and its derivatives to the rat uterine cytoplasmic receptor are:

α-zearalanol > β-zearalanol > ZEA > β-Zearalenol (Yazar and Omurtag 2008).

Binding to estrogen receptor initiates estrogen-dependent transcription in cell nucleus (Minervini and Aquila 2008). In the uterus, estrogen response including increase of RNA synthesis, RNA polymerase activity, and synthesis of uterine estrogen-induced protein can occur (Zinedine et al. 2007).

Toxicity in Animals and Humans

Following binding of ZEA and its metabolites to estrogen receptor, different estrogenic effects including decreased fertility; changed weight of adrenal, thyroid, and pituitary glands; change in serum levels of progesterone and estradiol; vulval edema; vaginal prolapsed; and mammary hypertrophy have been demonstrated in animals (Zinedine et al. 2007).

ZEA is related with reproductive problems in specific animals and possibly in humans (Yazar and Omurtag 2008). Other toxic effects of ZEA like hepatotoxicity, dysfunction of the blood coagulation process, and immunotoxicity in animals have been reported (Zinedine et al. 2007).

Potential activity of ZEA to induce growth of human breast cancer cells which are containing estrogen response receptor has been shown (Ahamed et al. 2001). In vitro study using cultures of bovine lymphocytes confirmed DNA-adduct formation following exposure to ZEA (Lioi et al. 2004). IARC in 1993 evaluated ZEA and based on inadequate data in humans and limited evidence in experimental animals was located in group 3 (not classifiable as to their carcinogenicity to humans)

(Yazar and Omurtag 2008). PMTDI level for ZEA is 0.5 μg/kg body weight/day which has been established by JECFA (Zinedine et al. 2007).

Patulin

General Consideration

Patulin (Fig. 7) is produced by many different molds including *Penicillium, Aspergillus, Paecilomyces*, and *Byssochlamys* (Puel et al. 2010). For the first time, in 1940s, patulin as an antibiotic agent was isolated from *Penicillium patulum* and *Penicillium expansum*. During the 1950s and 1960s, it was shown that besides its antimicrobial, antiviral, and antiprotozoal activity, patulin has toxic effects in both plants and animals, and finally, during the 1960s, patulin was classified as a mycotoxin (Şahin et al. 2011). *Penicillium expansum* which is known as the apple pathogen is the main pathogen of patulin formation. It causes decays in apple which is called "blue mold root" (Şahin et al. 2011). Patulin has been shown to produce generally in apple and apple products; in fruits including pear, apricot, peach, and grape; and less commonly in different foods such as cheese and meat (Şahin et al. 2011). Most exposure to patulin occurs through consuming of apple wine and apple juice (Puel et al. 2010). Optimum temperature for growth of *P. expansum* and formation of patulin in fruits is 25 °C. Formation of patulin decreases while the temperature reduces. However, patulin can be produced at low temperatures like 0–4 °C (Jackson and Al-Taher 2008).

Mechanism of Action

Patulin has electrophilic structure and can interact with nucleophilic groups and damage DNA structure (Schumacher et al. 2006). Patulin has high affinity for sulfhydryl groups and reacts with reactive sulfhydryl groups in cellular proteins and amino acids. It inhibits function of many enzymes such as ATPase, alkaline

O
O
O
OH

Fig. 7 Chemical structure of patulin (Bennett and Klich 2003)

phosphatase, and aldolase (Şahin et al. 2011). Patulin induced cytotoxicity in mammalian cells through depletion of cellular GSH and generation of reactive oxygen species (ROS) (Liu et al. 2007).

Apoptosis has been considered as one of the mechanisms of patulin-induced cytotoxicity. Exposure of HL-60 cells (human leukemia cells) to patulin induced mitochondrial-dependent apoptosis through a P53-independent pathway (Wu et al. 2008). In HEK 293 cells (human embryonic kidney), patulin increased phosphorylation of P38 kinase and (c-Jun-N-terminal kinase) JNK, but only P38 kinase signaling was responsible for patulin-induced cell death (Liu et al. 2006). In skin cells of mice, patulin exhibited potential effect to induce DNA damage leading to P53-mediated cell cycle arrest, increased release of cytochrome C protein in cytosol, and enhanced caspase-3 activity (Saxena et al. 2009).

Toxicity in Animals and Humans

Patulin mostly accumulates in erythrocytes; therefore, it can be accumulated in organs such as kidney, spleen, liver, and heart which are supplied with large amounts of blood. Patulin acute toxic effects in animals are vomiting, edema, and convulsion (Şahin et al. 2011).

After subacute administration of patulin to rats, weight loss, gastric damages, and alteration in renal function have been observed (Puel et al. 2010). Cerebral bleeding, tremor, and involuntary movement in the muscles are neurotoxic effects of patulin (Şahin 2011).

Treatment with patulin increased defective embryos in rats and induced growth retardation and hypoplasia of the mesencephalon and telencephalon (Smith et al. 1993).

Immunotoxicity of patulin, including reduction of secretion of IFN-γ and IL-4 by human macrophages and reduction of secretion of IL-4, IL-3, and IL-10 by human blood mononuclear cells and T cells, has been shown (Luft et al. 2008; Puel et al. 2010). Patulin was placed in group 3 by the IARC (Puel et al. 2010). Since 2003, European regulation sets a maximum level of 50 μg/L for fruit juices and derived products, 25 μg/L for solid apple products, and 10 μg/L for juices and foods destined for babies and young infants. Today, the US Food and Drug Administration (FDA) limits patulin to 50 μg/L. PMTDI for patulin of 0.4 μg/kg body weight/day is established by JECFA (Puel et al. 2010; Şahin et al. 2011).

Conclusion and Future Directions

Mycotoxins are secondary metabolites of fungi and cause toxicity in both animals and humans. Cereals are the most important source of human food that can be contaminated by mycotoxin during harvest, storage, processing and feeding. Mycotoxins exert toxic effects in different organs; however, hepatotoxicity, nephrotoxicity, and carcinogenicity are mostly due to fumonisins and ochratoxins, while

hematoxicity is related to trichothecenes. Zearalenone exhibits estrogenic effects by way of binding to estrogen receptor. Several studies showed cellular mechanisms of mycotoxin toxicity including disruption in protein synthesis through inhibition of polypeptide chain initiation, elongation and termination stages, inhibition of DNA and RNA synthesis, formation of reactive oxygen species, and induction of apoptosis using different in vitro and in vivo models. More studies are necessary to elucidate complete cellular signaling pathway of mycotoxin toxicity.

Cross-References

► Aflatoxins and Their Management
► Biotoxins and Food Safety

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Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment

5

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Abstract

Ricin and abrin belong to the group of type 2 ribosome-inactivating protein, which has a heterodimeric structure consisting of an A-chain linked by a disulfide bond to a B-chain. The B-chain facilitates internalization of the A-chain, which then exerts its toxic effects by inhibiting protein synthesis leading to cell death. These two plant toxins are highly toxic to human, and initial manifestations of ricin or abrin intoxication are generally nonspecific in

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nature. It is hence difficult to diagnose ricin and abrin poisoning through clinical symptoms alone. In view of their high toxicity, stability, relative ease of production and the worldwide availability of their source plants, ricin and abrin are considered to be potential chem-bio weapons of interests to terrorists. While the detection of ricin or abrin in adulterated food and beverages is relatively straightforward, the diagnosis of trace levels of either toxin in biofluid samples is challenged by their short half-life in circulation due to efficient sequestering of these toxins into the target organs. This chapter provides an overview of the latest developments, from the authors' laboratory as well as from open literature, in diagnostic protocols for trace detection of ricin and abrin in biofluids as well as an extensive coverage of various experimental medical countermeasures being developed to tackle this rising threat in chem-bio terrorism.

Introduction

Abrin and ricin are plant toxins derived respectively from castor beans (*Ricinus communis* L.) and rosary peas (*Abrus precatorius* L.). They belong to the group of type 2 ribosome-inactivating proteins (RIPs) (Garber 2008). Both toxins are heterodimeric glycoproteins consisting of two polypeptides designated as the A-chain (approximately 30 kDa) and B-chain (approximately 32 kDa) polypeptide linked by a disulfide bond. At the cellular level, the B-chain is responsible for the binding and internalization of these toxins into the cells. The B-chain, which is a lectin, binds specifically to eukaryotic cell surface receptors, such as glycoproteins and glycolipids containing β-1,4-linked galactose residues, to facilitate the toxin internalization (Olsnes 2004). Once inside the cells, the A-chain exerts its toxic effects on the 60S ribosome to inhibit elongation factor EF-1 and EF-2, preventing protein synthesis, which ultimately leads to cell death (Franz and Jaax 1997). The protein synthesis inhibition process involves catalytic inactivation of the ribosome by the toxin's A-chain, which occurs through the removal of an adenine from position 4,324 of the 28S rRNA in the 60S ribosomal subunit. The depurinated rRNA is then unable to bind protein elongation factor-2, which leads to cessation of protein synthesis, and evental cell death (Audi et al. 2005).

Ricin and abrin were first isolated as galactose-binding lectins (Olsnes 2004). For ricin, the nomenclature introduced by Funatsu's group is in use until today (Funatsu et al. 1978). They separated ricin into two variants, ricin D and ricin E, which have similar molecular mass but different isoelectric points. Ricin D is found in large grain seeds, whereas small grain seeds contain both ricin D and ricin E. Ricin D is known to be a Sepharose-binding protein that exhibits higher cytotoxicity and has an isoelectric point of 7.34, while ricin E is the Sepharose nonbinding fraction, with an isoelectric point of 8.8, which exhibits lower toxicity to malignant cells (Funatsu et al. 1978).

Abrin exists as multiple protein variants in the rosary pea seed. Two abrin proteins have been purified to their crystalline form, which are subsequently termed as abrin A (Sepharose nonbinding protein) and abrin C (Sepharose-binding protein).

Abrin C is made up of two protein subunits, known as "*a*" and "*d*" subunits, while the two subunits in abrin A is termed as "*b*" and "*c*" subunits. Between them, abrin C exhibits higher toxicity in mice (Wei et al. 1974). The A-chain of abrin shares 102 amino acid homology with the A-chain of ricin, while the B-chain of abrin shares 59 % sequence identity with the B-chain of ricin (Olsnes 2004).

The seeds of both plants also contain other strong hemagglutinins, which are made up of two A-chain-related polypeptides and two B-chains (Olsnes 2004). Unlike the RIP toxins, these hemagglutinins are either nontoxic (Abrus agglutinin) or comparatively less toxic (Ricinus agglutinin) (Olsnes 2004). Besides hemagglutinin, a toxic alkaloid known as ricinine could be extracted from castor beans into crude preparations of ricin (Johnson et al. 2005). However, it is structurally distinct from ricin toxin and is relatively much less toxic. In view of the focus on type 2 RIPs in this chapter, neither hemagglutinin nor ricinine will be covered in this review.

Origin, Distribution, Uses, and Misuses

Ricin toxin is derived from the *Ricinus communis* L. plant, which is widely distributed around the world. As this plant is commonly found in the wild and often used as an ornamental plant in domestic gardens, it is easily accessible. Castor beans from the *Ricinus communis* plant is used to produce castor oil, which has a wide variety of commercial applications. From castor meal, the by-product of castor oil production, ricin is readily extracted (Roxas-Duncan and Smith 2012). Intoxication by castor beans is common in countries where the plant is abundant with five beans sufficient to induce a toxic dose (Olsnes 2004). The accessibility and high toxicity of ricin toxin renders it a high-risk asymmetric threat agent to many national security and public health bodies. As a result, ricin is classified as a Category B agent by the US Centers for Disease Control and Prevention (CDC). Agents in this category are considered moderately easy to disseminate, able to cause morbidity and low mortality. Ricin is also monitored as a Schedule 1 agent under the Chemical Weapons Convention. This concern is justifiable as weapon-grade ricin was manufactured and tested in artillery shells in the 1940s within the United States. However, the thermal instability of ricin constrained its utility for conventional warfare, and ricin-containing bombs or W bombs were never used in battle (Franz and Jaax 1997). This did not however deter Iraq from manufacturing approximately 10L of concentrated ricin solution in 1989 and loading them onto artillery shells (Roxas-Duncan and Smith 2012). While the utility of ricin toxin in conventional warfare is uncertain, its potential as a weapon of terror has been amply demonstrated. In 1978, ricin was used successfully for assassinating the Bulgarian journalist Georgi Markov (Franz and Jaax 1997). Interests in utilizing ricin toxin soon spread to terrorist groups, and ricin was found in Afghanistan after the collapse of the Taliban government in 2001. A detailed chronology of ricin use in bioterrorism and crime is covered in an excellent review article (Roxas-Duncan and Smith 2012).

Abrin is derived from the jequirity bean or seeds of *Abrus precatorius* L. plant (*Fabaceae* or *Leguminosae*) that has >30 common names, one of which is rosary pea plant. This plant is native to Southeast Asia and grows well in both tropical and subtropical areas of the world where it has been introduced. It has been recorded in Ayurvedic medicine that the leaves of *Abrus precatorius* are laxative, expectorant, and aphrodisiac, while the seeds are reportedly purgative, emetic, tonic, antiphlogistic, aphrodisiac, and anti-ophthalmic. The ready availability of abrin toxin and its high toxicity lead to concerns that it could pose a severe threat to public health. Hence, abrin is also classified as a Category B agent by the US Centers for Disease Control and Prevention (CDC) and placed in the category of "Biological Select Agents or Toxins" by the US Department of Health and Human Services (HHS). This classification is made despite the fact that abrin has not been considered for any weapon program nor reported to be used in any terrorist attack.

Toxicity, Clinical Signs, and Symptoms

Possible routes of exposure for both toxins include respiratory (inhaled aerosol), gastrointestinal (ingested), and parenteral (injected). Animal studies have suggested that the toxicity and morbidity symptoms are dependent on both the amount of toxin internalized and route of exposure. It is generally agreed that lethality increases when the route of entry moves from ingestion, injection, to inhalation. Most reported cases of human poisoning involved ingestion of plant seeds, either accidentally or being administered as folk medicine (Roxas-Duncan and Smith 2012; Worbs et al. 2011). The estimated LD_{50} (median dose that is lethal to 50 % of a population) for human is 1–20 mg/kg, which is based on extrapolation from actual cases involving castor bean poisoning (Audi et al. 2005). Oral abrin LD_{50} in human is estimated to be 0.1–1 mg/kg (Dickers et al. 2003).

In comparison, the oral LD_{50} determined in the mouse and rat models is about 20–30 mg/kg for ricin toxin (Audi et al. 2005; Garber 2008; He et al. 2010a) and 2.3 mg/kg for abrin toxin (Garber 2008). In such toxicity studies, oral poisonings are usually carried out by gavage to ensure accurate dosing. However, Roy's research group cautioned that this mode of forced feeding may invoke a different toxicity outcome as compared to an actual scenario of human poisoning involving consumption of toxin-contaminated food samples. In particular, the group demonstrated that oral feeding induces lower toxicity than gavage as ricin binds to the tongue and esophageal mucosa tissues, which are the first site of exposure during normal ingestion, thereby resulting in smaller quantity of ricin entering the stomach (Roy et al. 2012). At the other end of the spectrum, in the interest of producing a consistent model, Smallshaw's group fasts their mice 20 h prior to intragastric gavage and food is withheld for an additional 4 h following the challenge, resulting in an LD_{50} that is 10 μg/kg, a value that is approaching the LD_{50} of intraperitoneal injection (Smallshaw et al. 2007).

When ricin is introduced through the intraperitoneal route, its LD_{50} in mice is reported to range from 2.4 to 36 μg/kg (Worbs et al. 2011). On the other hand, intraperitoneal LD_{50} for abrin is reported to be 0.6 μg/kg, while abrin C is more toxic, with an LD_{50} of 0.2 μg/kg (Wei et al. 1974). When inhaled as aerosols, there is no significant reported difference in lethality between ricin and abrin in rats. The LCt_{50} values reported, for ricin and abrin, respectively, were 4.54–5.96 and 4.54 mg min m^{-3} (Griffith et al. 2007). This is despite the fact that abrin is several times more toxic in systemic and oral poisoning cases.

The initial manifestations of ricin or abrin intoxication following ingestion are generally nonspecific in nature and with symptoms resembling food-borne (toxic mushrooms or solanine-containing potatoes), chemical (e.g., strong detergents, caustics, arsenic, colchicines, and heavy metal), or infectious gastroenteritis (Staphylococcus aureus, salmonella, and shigella). Following ingestion, symptoms may appear between 4 and 6 h later but, depending on the dose, may be delayed to 10 h post-ingestion (Roxas-Duncan and Smith 2012). Poisoning symptoms, which vary among individuals, include initial appearance of nausea, abdominal pain, vomiting followed by diarrhea, cramps, dilation of pupils, fever, dehydration, anuria (absence of or defective urine production), sore throat, headache, hypotension, heartburn, internal bleeding of the stomach and intestines, and failure of the liver, spleen, and kidneys. The cause of death is related to toxin-induced damage to the endothelial cells, resulting in a vascular leak syndrome characterized by hypoalbuminemia and edema. This leads to vascular collapse and shock, with death occurring after 3 or more days.

Besides the famous use of injected ricin for the assassination of Georgi Markov, there are many other cases of attempted suicide where ricin seed extracts were self-injected (Worbs et al. 2011). From these cases of parenteral exposure, the symptoms of systemic ricin poisoning is reported to be similar to sepsis, which includes fever, headache, dizziness, nausea, anorexia, hypotension, and abdominal pain, observed 10–12 h postexposure (Audi et al. 2005). Yet in a more recent review, it was reported that both oral and systemic poisoning by ricin shared similar symptoms (Worbs et al. 2011). Profound hypoglycemia, in a reproducible dose- and time-related manner to the amount of ricin administered intraperitoneally, has been observed among intoxicated mice (Pincus et al. 2002). This observation suggested that hypoglycemia is a possible additional symptom of ricin intoxication.

For both toxins, there are no recorded clinical cases of aerosol exposure in human. Using nonhuman primates as human surrogates to study toxic effects of aerosolized ricin, the LD_{50} for ricin was determined to be 5.8 ug/kg. These animals showed symptoms of depression, shortness of breath, and anorexia as early as 12–16 h postexposure. Fever develops within 24 h, with death due to respiratory failure, generally occurring 35–40 h postexposure (Roy et al. 2012). Unfortunately, the respiratory illness arising from inhaled ricin aerosols resembles respiratory problems arising from inhaled irritant gases, metal or polymer fumes, paraquat, and infectious agents (e.g., anthrax, influenza, and Q fever). It is thus difficult to diagnose ricin and abrin poisoning through clinical symptoms alone.

Detection and Diagnosis

Due to similarities with more commonly encountered illnesses, recognition of poisoning by either plant toxin will be challenging and dependent on the existence of a credible threat. In its absence, during an outbreak of severe gastrointestinal or respiratory illness, differential diagnosis and rapid progression of illness may heighten the suspicion for exposure to either toxin. However, definitive confirmation of involvement of either toxin would require epidemiological information around cases of accidental poisoning to suggest a common-source etiology. In the case of demonstrating their use in bioterrorism, confirmatory evidence of trace quantities of either toxin in adulterated samples and biological samples would be required.

Detection of Toxins in Food and Beverages

For direct detection of toxin in sample matrices without prior sample enrichment, antibody-based detection methods are most often cited as the preferred methods. However, such immunological-based detection methods have yet to be validated in any retrospective clinical cases, and toxin concentrations in human following toxic exposures remain uncertain. Using the assumption that 50–250 ml or grams of contaminated drink or food has been consumed by a 70 kg human and the estimated LD_{50} of these toxins in human, the proposed limits of detection (LOD) is tabulated in Table 1 in accordance to the route of entry of these toxins. Using these derived limits of detection, detection methods garnered from literature reports, published over the period of 2005–2013, suggested that such tests have sufficient sensitivity to avoid a false negative in adulterated food and beverage samples (Table 1). The tabulated data indicated that the test sensitivity improved with longer assay durations going from immunochromatographic tests (ICT) to conventional enzyme-

Table 1 Estimated LOD required to detect a dose of toxin lethal to a 70 kg human

Toxin	LD_{50}	Lethal dose of 70 kg man (mg)	Concentration in 50–250 ml or g (mg/ml or mg/g)		Derived LOD (ug/ml)
Ricin (oral)	1–20 mg/kg[a]	70–1,400	1.4–28	0.28–5.6	280
Ricin (ip)	2.4–36 ug/kg[b]	0.168–2.52	0.0034–0.0504	0.00067–0.010	0.67
Ricin (inhaled)	5.8 ug/kg[c]	0.259	0.00518	0.001036	1.04
Abrin (oral)	0.1–1 mg/kg[a]	7–70	0.14–1.4	0.028–0.28	28
Abrin (ip)	0.04 ug/kg[b]	0.0028	0.000056	0.0000112	0.01
Abrin (inhaled)	3.3 ug/kg[d]	0.231	0.00462	0.000924	0.924

[a]Human
[b]Mouse
[c]Macaque
[d]Rat

Table 2 Tests that detect ricin and their performance characteristics

Principle of test	LOD or dynamic range (ng/ml)	Remarks	Reference
ELISA (Tetracore)	5–500	Detection depends on type of beverage (PBS, water, apple juice)	Garber 2008
ICT (Tetracore)	5 (LOD)	Hook effect observed at concentration above 250 ng/ml. Food contributes to background signal	
	5–250		
ICT	1	Signal plateau from 10 to 250,000 ng/ml	Griffiths et al. 2013
	1–10,000	No hook effect	
ELISA	0.15	PBS	
Immuno-PCR	0.01–0.1	Egg, milk, ground beef	He et al. 2010b
ECL	0.04	Juice, dairy products, soda, vegetables, bakery products, chocolate, and condiments	Garber and O'Brien 2008
Bioassay + ECL	0.1	Electrochemiluminescence (ECL)-based detection for functional ricin A-chain RNA N-glycosidase activity	Keener et al. 2006

Table 3 Tests that detect abrin and their performance characteristics

Principle of test	LOD or dynamic range (ng/ml)	Remarks	Reference
ELISA (Tetracore)	5–500	Detection depends on type of food	Garber et al. 2008
Biotin–streptavidin ELISA	0.125–31.25	Suitable for detection from water, soil, food, and blood	Mu et al. 2007
ECL	0.1–0.5	Juices, dairy products, soda, chocolate drink, and condiments	Garber et al. 2008
ICT	3	Milk, orange juice, water	Gao et al. 2012

linked immunosorbent assay (ELISA). The sensitivity can be further improved by signal amplification of the ELISA, usually at the expanse of time, ease of use, and instrumentation. The signal amplification approaches cited include the use of biotin–streptavidin, chemiluminescent substrate (ECL), and immuno-PCR techniques (Tables 2 and 3). On the other hand, while mass spectrometry techniques offer definitive identification of either toxin in complex sample matrices, the sensitivity of such techniques could be up to a threefold magnitude lower than those afforded by immunological protocols, and extensive sample enrichment processes are required (Kanamori-Kataoka et al. 2011).

This approach of approximating a working limit of detection for toxins in food and beverage samples would not be appropriate if a different quantity of contaminated sample is consumed over time. As shown by Garber, ricin and abrin have prolonged stability and persists in adulterated samples (Garber 2008). Purified ricin,

which exists as a white powder, is stable over a wide pH range when dissolved in water, and inactivation requires heating at 80 °C for an hour. Abrin solution, on the other hand, loses its toxic actions when heated to temperatures of 80 °C and beyond. Solid forms of ricin would require either higher temperatures or extended duration of heating to ensure inactivation (Parker et al. 1996). Hence, diagnostic tests would be required when an individual is presented at the hospital with suspected symptoms of intoxication. In such cases, diagnostic tests are usually carried out using the biofluids obtained from the individual.

Diagnosis of Oral Poisoning

Literature on detection of either toxin by the oral route of intoxication is sparse. He's research group reported a transit time of 6 h for ricin to pass from the stomach into the blood stream in a mouse gavage model (He et al. 2010a). With high ricin doses, ricin was reported in fecal samples as early as 2 h, but with lower ricin doses, positive detection was delayed to between 6 and 24 h. However, He's research group did not provide the diagnostic window for which ricin remained detectable by his assay method.

As the diagnostic window for ricin oral intoxication is a useful parameter for managing suspected clinical cases, a rat gavage model was developed to study ricin toxicosis and to establish the diagnosis window for immuno-detection of the toxin from analysis of whole blood, feces, and urine collected from the intoxicated animal model (Foo et al. 2012). This ELISA kit was validated to have an analytical LOD of <0.25 ng/ml in PBS and 0.3–0.4 ng/ml in whole blood and feces. The rats were force-fed at three ricin doses to represent three levels of intoxication: 14 mg/kg (mild), 28 mg/kg (morbid), and 56 mg/kg (lethal). As expected, ricin detection in various specimen matrices (blood or feces) was time and dose dependent (Figs. 1 and 2). At the lethal dose of 56 mg/kg, all intoxicated rats ($n = 3$) died within 2 days of post-gavage challenge. In this lethal poisoning model, the ELISA kit was able to detect ricin 4 h after gavage in all blood specimens collected at an estimated ricin concentration of 4 ng/ml, which rose to 8.5–9 ng/ml at 24 h before dropping back down to 2.8 ng/ml at 48 h (Fig. 1b). At a morbid ricin gavage dose of 28 mg/kg, which was carried out on a larger test cohort ($n = 12$), no lethality was observed and rats exhibited diarrhea for 2–3 days before recovering slowly. Positive detection with the ELISA kit was again achieved at 4 h after oral gavage, but at a lower concentration range of 1.2–2.7 ng/ml (Fig. 1a). The number of ricin-positive blood specimens dropped sharply after 4 h, which was similar to that reported by He et al. Twenty hours after oral gavage, no ricin was detected from any of the collected blood specimens. Hence, the diagnostic window for detecting ricin in whole blood samples is determined to be between 4 and 20 h following gavage challenge involving morbid to lethal challenge doses of ricin.

For fecal specimens, a wide diagnostic window was demonstrated up to 7 days post-ricin ingestion, depending on the ricin gavage dose (Fig. 2). For rats that

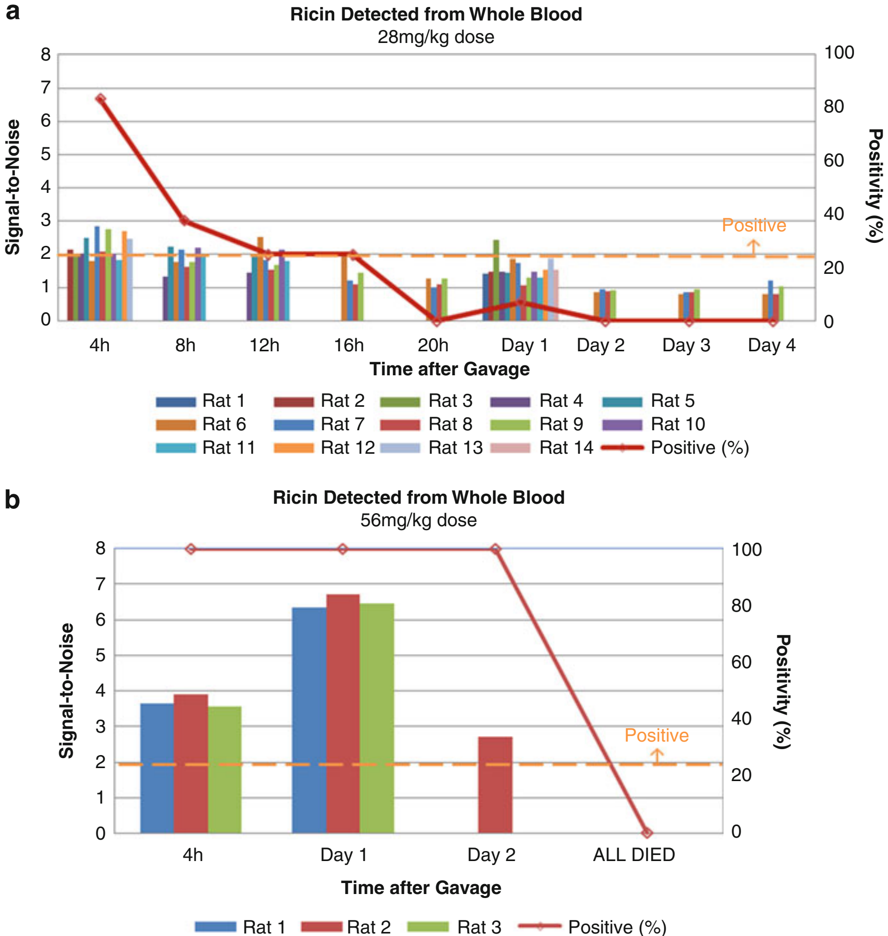

Fig. 1 Figure illustrates the number of whole blood samples gathered from intoxicated rat at various durations post-gavage that provided positive detection response (*right* y-axis) to ricin toxin at a signal-to-noise ratio (*left* y-axis) greater than 2 (S:N > 2). (**a**) Ricin detected in whole blood from intoxicated rats at a morbid ricin dose of 28 mg/kg; (**b**) ricin detected in whole blood from intoxicated rats at a lethal ricin dose of 56 mg/kg. Day 2: two rats died before testing

received a mild dose of 14 mg/kg (n = 4), ricin was detectable in fecal specimens up until the 6th day post-gavage. With a morbid ricin dose of 28 mg/kg, the ELISA kit was able to detect ricin in rat feces from 4 h post-gavage to the end of the 7-day trial. Beyond these 7 days, ricin remained detectable in 50 % of the fecal specimens collected. This prolonged excretion of ricin in fecal samples following gavage feeding is similar to what Roy et al. have previously reported (Roy et al. 2012). It should also be noted that overall, fecal specimens recorded much

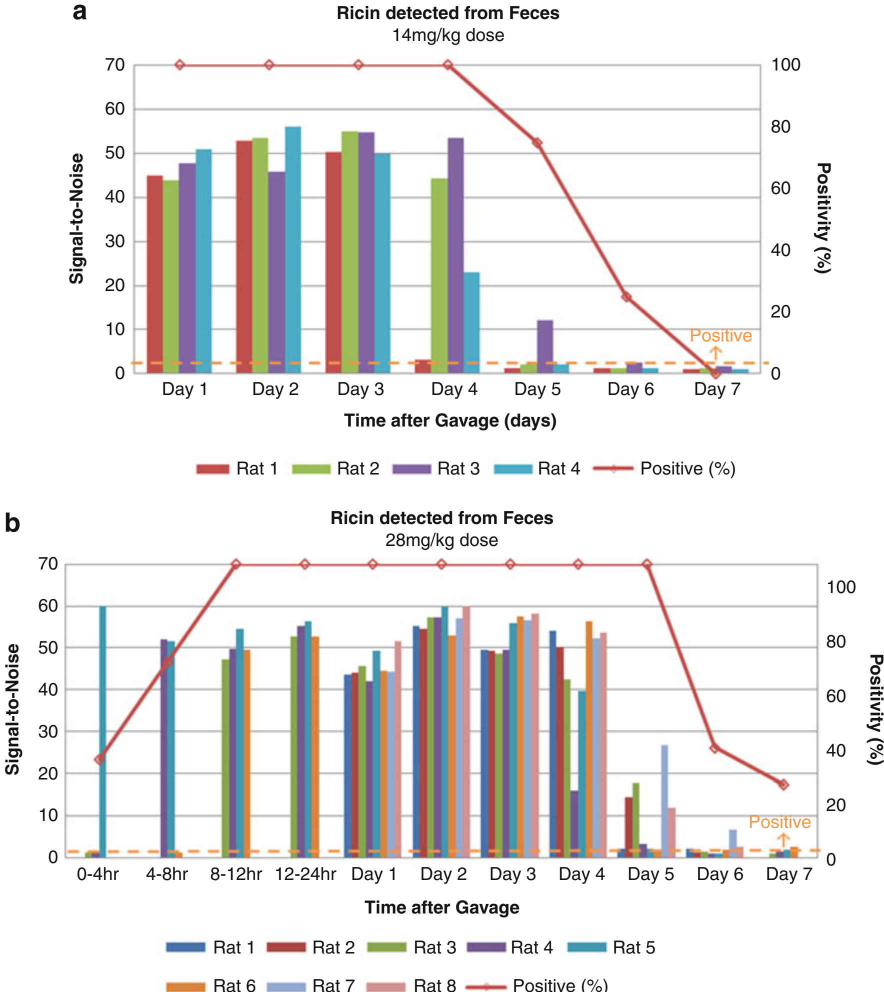

Fig. 2 Figure illustrates the number of fecal samples gathered from intoxicated rat at various durations post-gavage that provided positive detection response (*right* y-axis) to ricin toxin at a signal-to-noise ratio (*left* y-axis) greater than 2 (S:N > 2). (**a**) Ricin detected in feces from intoxicated rats at an asymptomatic ricin dose of 14 mg/kg; (**b**) ricin detected in feces from intoxicated rats at a morbid ricin dose of 28 mg/kg. 0–4 h and 4–8 h: three out of four rats produce feces

greater signal-to-noise ratio, almost 20 times higher, when compared to blood specimens. These results suggested that fecal samples are the most suitable clinical specimen to be tested in suspected cases of oral ricin poisoning, while blood would be the alternative when fecal specimens cannot be obtained.

Urine sample, which is a potential alternative to fecal samples, has been reported to yield only low molecular weight degradation forms of ricin rather than the intact

whole toxin structure (Fodstad et al. 1976). As ricin absorption through the gut and intestinal tract occurs over 2–7 days (He et al. 2010a) while the reported half-life for urinary elimination of ricin is 8 days, the long half-life for urinary excretion of ricin degradation products may potentially be useful in extending the diagnostic window beyond 7 days. Although one of the antibodies used in Foo's study could detect a 15 kDa ricin metabolite, the appearance of this 15 kDa peptide varies significantly between animals (Foo et al. 2012). The authors did not further pursue the detection of ricin metabolites in urine samples in view of this reproducibility challenge and the outstanding diagnostic window demonstrated for detecting ricin in fecal samples.

The detection of ricinine and L-abrine, two small molecules coexisting with ricin and abrin in the seeds of castor plant and rosary peas, in urine samples of intoxicated models has been suggested to be suitable surrogate markers for the diagnosis of intoxication by crude ricin and abrin samples. Indeed, ricinine is detected in urine at 24 and 48 h, while L-abrine is detected at 24 h but not 48 h after the small molecules were introduced into rats through intramuscular route (Johnson et al. 2005, 2009). In a recent case of a patient suffering from intentional ricin intoxication through injection and oral intake of a castor bean extract, peak ricinine levels were detected in the serum and urine samples, between 10 and 29 h postexposure, using a combination of solid-phase extraction and isotopic dilution liquid chromatography–mass spectrometry analysis (Reed et al. 2013). The ricin intake was likely to be above lethal levels as the patient died shortly (38 h postexposure) after admission (4 h postexposure) despite being accorded intensive medical care. Additional studies on animal models will be required to determine the true utility of such surrogate markers to diagnose suspected ricin and abrin poisoning at nonlethal exposure levels.

Diagnosis of Inhalation Poisoning

Inhalation of aerosolized ricin or abrin is considered the most dangerous mode of exposure as exceedingly low lethal doses are observed in experimentally challenged animals. However, there are no recorded clinical cases of human poisoning with aerosolized ricin to advance our understanding of this mode of intoxication. Initial studies carried out with inhalation animal models were used to elucidate the pathological changes of the immediate tissues and localization of ricin with immunohistological techniques. The latter became the key means to confirm the involvement of such toxins in the cause of death during forensic investigations (Griffith et al. 2007; Roy et al. 2012). Further improvements in diagnostic capability to detect aerosolized ricin exposure next appeared in 2013 when Griffiths's research group reported the development of an ELISA technique capable of detecting ricin metabolites in 24-h pooled urine samples gathered from mice, which were subjected to head-only exposure of a $3 \times LCt_{50}$ dose of ricin aerosol. Interestingly, this highly sensitive ELISA protocol, with an LOD of 0.15 ng/ml, was not able to detect ricin in either blood or blood cell pellets during this initial

24 h. Ricin concentrations detected in urine samples peaked at the 8–12-h window with an estimated level of 0.9 ng/ml. In contrast, nasal lavage samples demonstrated very fast clearance, with 5 ng/ml detected in the first hour and almost none after that. Lung lavage recovered washings with ricin concentrations of 10 ng/ml or higher and a detection window between 4 and 16 h postexposure (Griffiths et al. 2013). This appeared to be the most suitable clinical sample for early diagnostic testing to confirm suspected poisoning by aerosolized ricin or abrin.

Medical Management Options and Prognosis

Clinical Management

Currently, treatment for ricin and abrin poisoning remains largely symptomatic and supportive as there are no specific treatment protocols or antidotes available. Henceforth, the major treatment goals for a patient with oral or dermal ricin poisoning have been on the provision of exemplary resuscitative care (Audi et al. 2005). Such resuscitative care could take the form of aggressive fluid resuscitation to improve perfusion, vasopressor therapy, and replenishing of electrolytes to maintain fluid and electrolyte balance. Failure to do so may lead to development of shock, myoglobinuria, and renal failure. Thus, symptoms associated with vascular leak syndrome (VLS) should be closely monitored and treated within an intensive care unit.

When oral poisoning by either of these toxins is suspected, activated charcoal is often recommended for non-vomiting patients to prevent further systemic absorption of these toxins. However, charcoal is likely to be of little value for large molecules such as ricin and abrin toxin. On the other hand, in view of the necrotizing action of these toxins, once gastrointestinal signs appeared, gastric lavage is not advisable for fear of inducing even greater damage to the stomach mucosa tissues. For inhalational exposure, specific resuscitative care may include oxygen, bronchodilators, the use of anti-inflammatory agents and analgesics, endotracheal intubation, and the use of positive-pressure ventilator therapy in addition to fluid and electrolyte replacement (Audi et al. 2005).

Experimental Vaccines

While none of the experimental medical countermeasures against ricin and abrin toxins has received regulatory approval, published animal data have suggested that prior vaccination and/or prompt administration of antitoxin antibody following exposure could disrupt the intoxication process and improve prognostic outcome. The development of various experimental medical countermeasures for both toxins is further elaborated in the following sections.

Abrin was studied in the early 1990s as a potential selective antitumor agent in the form of an immunotoxin. This occurred soon after the DNA sequence of intact

abrin and cDNA sequences of abrin A-chain were reported. To understand abrin's toxicity profile, the researchers identified amino acids at the highly conserved residues after aligning the amino acid sequences of 2 ricin and 8 type 1 RIPs and X-ray crystallographic structure of ricin. Through amino acid substitution experiment, it was determined that Glu164 and Arg167 are essential for abrin A-chain catalysis, while Trp198 plays an important role in maintaining the conformation of abrin A-chain to reassociate with B-chain (Chen et al. 1997). It was not until 2011 that double mutations of Glu164 to Ala164 (E164A) and Arg167 to Leu167 (R167L) were explored to develop a vaccine candidate for abrin toxin. Han et al. report that the abrin mutant A-chain protects 100 % of mice when the mice are challenged with a $10 \times LD_{50}$ dose of the native abrin through the intraperitoneal route (Han et al. 2011).

Similar considerations were undertaken by researchers designing the ricin vaccine. Smallshaw's team attempted to mutate the amino acids at ricin A-chain's enzymatic site (Smallshaw et al. 2007). In addition, they also observed that a deglycosylated ricin A-chain could still lead to local or systemic VLS and decided to also mutate the amino acids at the VLS-inducing sites to obtain a technically safe vaccine. After a series of trials and errors, the group has selected V76M/Y80A (RiVax), as their ricin vaccine candidate (Smallshaw et al. 2007). RiVax was shown to protect mice against ricin challenge in both gavage and aerosol-inhalation challenge models (Smallshaw et al. 2007). RiVax has met many milestones, which include the commercial large-scale fermentation of the recombinant protein and the successful completion of phase I human safety trials (Vitetta et al. 2006) and phase 1B clinical trial in collaboration of Soligenix, Inc.

Another approach to ricin vaccine development was made by overcoming the stability and solubility of the recombinant ricin A-chain construct. It was observed that native ricin A-chain is unstable at pH 7.0 and at temperature >37 °C, where it exists in a partially unfolded "molten globule" state. This species cannot be refolded to the native state by manipulation of buffer conditions, or through the introduction of ribosomal RNA components, which are substrates for ricin A-chain. It was only through the introduction of intact ribosomes that ricin A-chain could be induced to refold and regain its N-glycosidase activity (Argent et al. 2000). While the recombinant ricin A-chain is able to elicit protective immunity, its low thermostability and tendency to undergo partial unfolding are undesirable characteristics for a vaccine candidate. This lead to the proposed study of a recombinant ricin A-chain lacking the C-terminal domain (residues 199–267) and deletion of an exposed loop (residues 34–43). Indeed, this new construct, rRTA 1-33/44-198 (RV*Ec*), was determined to have effectively no toxic N-glycosidase activity, being significantly more thermostable and remained completely folded at temperatures up to 53 °C (McHugh et al. 2004). This construct has been manufactured as a cGMP product and is currently in phase 1 clinical trials, being evaluated for possible human adverse reactions when used as a ricin vaccine candidate (Porter et al. 2011).

In 2013, O'Hara's research team compared the efficacy of these two ricin vaccine candidates by testing both vaccine candidates at various immunization doses after mixing with Alhydrogel as the adjuvant (O'Hara et al. 2013). Qualitatively, both

RiVax and RV*Ec* are equally effective at eliciting protective immunity at the doses tested with all immunized animals surviving a 10 × LD_{50} ricin challenge. However, quantitatively, RV*Ec* tends to elicit higher titer antibody response in every experiment group at a comparable time point. The antibodies reacting to RV*Ec* were determined to be acting on epitopes at the immuno-dominant α-helix structure (residues Y91 to F108 of ricin A-chain), while RiVax generated antibodies that reacted to epitopes spanning the entire ricin A-chain. However, further studies revealed that the majority of toxin-neutralizing antibodies elicited by RiVax were confined to residues 1–198 (O'Hara et al. 2013). This may explain the observed equivalency in protective immunity offered by RV*Ec* and RiVax.

Experimental Use of Neutralizing Antibodies

While the development of vaccine candidates looks promising and active immunization could protect animal or human from the lethal effects of ricin or abrin poisoning, it may not be realistic to perform a mass vaccination program to protect the entire civilian population against such esoteric toxin threats. Alternative therapeutic measures such as immunotherapy to target and remove circulating toxins were also investigated. Early studies with immunotherapy studies demonstrated considerable promise against animals poisoned by ricin toxin (Roxas-Duncan and Smith 2012). As the antibody technology advances, more approaches were reported to develop improved neutralizing antibodies against ricin toxin. Among them, two stood out: a single-domain antibody devoid of light chains, rVH_{PT}, and the "24B11" antibody that was directed against ricin B-chain to block ricin attachment to galactoside receptors (Wang et al. 2006).

To make an antibody that is safer for human use, other research groups applied a different approach of converting neutralizing antibodies into chimeric versions by fusing the mouse variable region genes with those from the human constant region gene. A monoclonal anti-ricin antibody, 4C13, was converted into a chimeric antibody, c4C13, using such a technology (Wang et al. 2007). Similarly, Maddaloni's research team converted one of their anti-ricin A-chain antibodies, with the strongest protective efficacy, mRAC 18, into a chimeric antibody. The resultant antibody retains the same passive immuno-protection effects in vivo as its parental antibody (Maddaloni et al. 2004). In contrast to the findings made by Maddaloni's team, Prigent's group observed that anti-ricin B-chain antibodies protect mice more efficiently than the anti-ricin A-chain antibodies (Prigent et al. 2011). They used a combination of 3 neutralizing antibodies that was able to protect mice up to 7.5 h after a 5 × LD_{50} ricin intranasal challenge.

Instead of working with monoclonal antibodies, Holley et al. work with sheep polyclonal anti-ricin antibodies. The key consideration in working with polyclonal antibodies was a concern that a panel of monoclonal antibodies would be required to fully neutralize a toxin, which would have made monoclonal antibodies an exorbitant therapeutic option. Moreover, sheep is traditionally a good source for producing large amounts of antivenom products with a good safety record. In their

studies, they found that despeciated sheep antibody in the conformation of $F(ab')_2$, but not Fab', extended protection after inhalation ricin challenge. The antitoxin protected 100 % of the mice challenged with a 3 × LCt_{50} aerosolized ricin dose even when the antitoxin was administered as late as 16 h post-inhalation challenge (Griffith et al. 2007).

In comparison, research into development of anti-abrin neutralizing antibodies is much less active, and only two reports are found in our review of the literature. Zhou's research team used phage display technology to select anti-abrin human monoclonal antibodies from a human naïve scFv library, which have detection limits of 35–75 ng/ml when analyzed using surface plasmon resonance (SPR) binding kinetics assays (Zhou et al. 2007). Using a more conventional method, a monoclonal antibody that was mapped to abrin A-chain, D6F10, was found to protect mice from abrin challenge when given 1 h prior to exposure (Surendranath and Karande 2008). While this field of research remains highly promising, concerns about their cost and limited therapeutic window may limit their practical utility in clinical practice (Barbier et al. 2012).

Targeted Therapy and Intervention Points in Ricin-Mediated Cytotoxicity Pathway

Cell entry by ricin required initial binding, via the ricin B-chain, to a range of cell surface glycolipids or glycoproteins having β-1,4-linked galactose residues (Audi et al. 2005). This saccharide–toxin recognition event was investigated as a target for early therapeutic intervention. In 1973, Lin et al. demonstrated that addition of D-galactose or its derivatives, lactose or raffinose, completely prevented ricin-mediated inhibition of protein biosynthesis in Ehrlich ascites tumor cells. Fodstad et al. (1976) later demonstrated efficacy of lactose in partial protection of mice from lethal ricin challenge (Table 4) when lactose was co-injected with ricin. Dawson et al. (2006) developed carbohydrate-substituted dendrimers as potential ricin B-chain inhibitors. However, as the study found that these multivalent dendrimers were no more potent than monovalent galactose, there was no further work in this area. There were also no report citing the efficacy of lactose or galactose when administered post-ricin challenge.

Other therapeutic measures targeted the intracellular pathways of ricin, where antibodies supposedly would not have access to neutralize or bind ricin for clearance by macrophages. The intracellular pathway commences with internalization of ricin into cells through clathrin-dependent and clathrin-independent mechanisms. Within the cell, ricin is transported in a retrograde manner from early endosomes to the endoplasmic reticulum (ER), through the Golgi apparatus (Audi et al. 2005) before final translocation into the cytosol where ricin A-chain refolds and enzymatically inactivates the 28S RNA of the 60S ribosomal subunit. A breakthrough was made in 2010 by Stechmann's research team (Stechmann et al. 2010), who demonstrated successful application of Retro-1 and Retro-2 chemical entities to inhibit the intracellular retrograde transport of ricin and shiga-like toxin from early

Table 4 Prophylactic and therapeutic measures and points of intervention in ricin-mediated toxicity

Point of intervention	Treatment principle	Treatment drug or compound	Effect	Source
Pre-intoxication	Vaccine	Mutant abrin A-chain	Confer 100 % protection to the animals when challenge with $10LD_{50}$ dose of native abrin through the intraperitoneal route	Han et al. 2011
		Mutant ricin A-chain (RiVax)	Completed phase 1B clinical trial, in collaboration of Soligenix, Inc.	Smallshaw et al. 2007
		Truncated ricin A-chain (RV*Ec*)	Currently in phase 1 clinical trials	McHugh et al. 2004
Pre- or post- intoxication (depends on route of intoxication)	Antibodies	Sheep polyclonal antitoxin raised using ricin toxoid	Effective treatment up to 16 h post-ricin $3LCt_{50}$ inhalation challenge with 100 % survival in mice	Griffith et al. 2007
		Combination of three monoclonal antibodies	Neutralization of ricin toxicity up to 7.5 h after $5LD_{50}$ ricin intranasal challenge, allowing a 90 % mice survival	Prigent et al. 2011
Post-intoxication				
Before entry of ricin into cells	Competitive binding for cell surface receptor	Lactose	Co-injection with ricin changed organ distribution of ricin (80 % in liver after 30 min, compared to 48 % without lactose); partial **protection** of mice from lethal dose	Fodstad et al. 1976

Ricin in cells				
Early endosome to Golgi apparatus	Inhibition of retrograde transport	Retro-1 and Retro-2	Protection of human cells against ricin; no additive or synergistic effect when coadministered; Retro-2 **protected** mice from lethal ricin challenge (nasal instillation at LD_{90})	Stechmann et al. 2010
Before depurination of rRNA	Inhibition of enzymatic activity of ricin A-chain	Anti-ricin A-chain RNA aptamer (31RA)	Partial **protection** against ricin-induced cytotoxicity in CHO AA8 cells	Fan et al. 2008
Post-depurination of rRNA (downstream pathways)	Preservation of membrane fluidity and prevention of lipid peroxidation[a]	Dexamethasone	Significantly **extended survival time** of mice treated with an IP LD_{100} dose (25 ug/kg) of ricin, **but could not prevent death**	Muldoon and Stohs 1994
	Inhibition of conversion of ornithine to putrescine (polyamine that binds readily to ribosomes, DNA, RNA, and cell membranes)[a]	Difluoromethylornithine	Low single doses of DFMO 2–6 h after ricin **extend the survival time**	
	Activation of cholinergic anti-inflammatory pathways	Nicotine	**Delayed mortality** and reduced systemic organic failure in mice previously given IP lethal doses of ricin (50 or 100 ug/kg)	Mabley et al. 2009
	Suppression of ricin-induced stress signaling pathways	Small molecules PW66, PW69, and PW72	Delayed ricin-induced cell death	Wahome et al. 2012
	Moderate signal transduction of inflammatory cytokines	Liposome-encapsulated N-acetylcysteine (Lipo-NAC)	Reversed ricin A-chain-induced hepatotoxicity	Buonocore et al. 2011

(*continued*)

Table 4 (continued)

Point of intervention	Treatment principle	Treatment drug or compound	Effect	Source
After ingestion (prior vomiting)	Gastrointestinal decontamination	Gastric lavage	Limited efficacy; can be considered if patient presents **within 1 h post-ingestion**	Audi et al. 2005
		Activated charcoal		
Manifestation of symptoms	Symptomatic and supportive treatment for hypotension	Vasopressor (dopamine) therapy, fluid and electrolyte management, and avoidance of shock		Audi et al. 2005

Protection: Drug or compound to be administered **prior** to ricin challenge to be effective
IP intraperitoneal, LD_{50} median lethal dose, LCt_{50} the vapor or aerosol exposure (Ct) necessary to cause death in 50 % of the population exposed
[a]Hypothesized role in interference of ricin-mediated toxicity

endosomes to the Golgi apparatus. These small molecules protected human pulmonary carcinoma alveolar basal epithelial A549 cells and Hela cells from the cytotoxic actions of ricin and shiga-like toxins, respectively. Interestingly, these novel chemical entities appear to exhibit specific inhibitory effects as they did not affect the integrity of the Golgi apparatus or the retrograde transport of other compounds. The team further demonstrated the prophylactic protective effects of Retro-2 against lethal intranasal ricin challenge, but similar prophylactic effects in mice was not observed with Retro-1 (Stechmann et al. 2010). If Retro-2 has to be given prior to ricin exposure, the drug delivery pathways and pharmaceutical formulations need to be improved to maximize the benefits of this inhibitor (Barbier et al. 2012). At present, the detailed mechanism through which Retro-1- and Retro-2-mediated inhibition of retrograde trafficking of ricin and shiga-like toxin remains to be established; early evidence suggested targeting and relocation of syntaxin 5 activity by these novel small molecules (Stechmann et al. 2010).

Ricin toxic action and ability to inhibit protein synthesis is mediated through binding of its A-chain to 28S rRNA followed by catalytic depurination of a specific adenine residue from the α-sarcin/ricin loop (SRL). Hence, inhibiting the catalytic activity of the enzymatic moiety of ricin A-chain by small-molecule compounds has been another reported therapeutic approach. In particular, one such specific catalytic blocker reported was an anti-ricin A-chain RNA aptamer (31RA), which partially protected cells against the cytotoxic effects of ricin (Fan et al. 2008). However, most of these in vitro inhibitors often fail to protect animals against actual in vivo ricin challenges. The recent work of Pang et al. (2011), who used the doorstop approach to identify small molecules that prevent the active-site residue of RIPS (e.g., Tyr80 of ricin), from adopting an active conformation, resulted in up to 20 % cell protection against ricin cytotoxic effects. This doorstop approach circumvents the problem of strong electrostatic interactions at the RIP–SRL interface that renders drug-like molecules ineffective in competing against rRNA for binding to RIPs (Pang et al. 2011). Inhibitors based on the oligonucleotide stem-loop RNA inhibitors of ricin A-chain were also developed, but this drug development approach faces additional obstacles with respect to bioavailability and hydrolysis by circulating nucleases (Amukele et al. 2005).

While the detailed mechanisms behind ricin cytotoxic effects are being investigated, some research groups have turned to cell-based, high-throughput screening of novel chemical entities from commercially available chemical libraries to uncover alternate novel therapeutic entities and to reveal all possible pathways associated within ricin-induced toxicity in vivo. Using a cell-based high-throughput screening (HTS), Wahome et al. (2012) have identified three compounds, PW66, PW69, and PW72, which significantly delayed ricin-induced cell death without any demonstrable effect on ricin's ability to arrest protein synthesis in cells or on its A-chain enzymatic activity. Instead, all three compounds appeared to function by blocking downstream proinflammatory cytokine production that is associated with toxin-mediated apoptosis. PW66 eliminated ricin-induced TNF-α secretion by J774A.1 macrophages and concomitantly blocked activation of p38 MAPK and JNK signaling pathways. PW69 suppressed activity of the executioner caspases 3/7

in ricin-treated cells, while PW72 suppressed ricin-induced TNF- α secretion, but not p38 MAPK and JNK signaling. The actual molecular targets of the three compounds have yet to be identified, but the roles that these targets play in ricin-induced stress activation pathways can be central to understanding the connection between ricin lethality and the accompanying inflammation and apoptotic events. Other compounds that have been identified using a more selective screen are dexamethasone and difluoromethylornithine (DFMO). Both compounds were reported to significantly extend the survival duration among ricin-challenged mice (Muldoon and Stohs 1994). It is postulated that the action of dexamethasone in inhibiting phospholipase activation, which is associated with lipid peroxidation, serves to delay ricin lethality. The mechanism of DFMO is less clear. DFMO is an inhibitor of ornithine decarboxylase, an enzyme responsible for conversion of ornithine to putrescine. Putrescine is a polyamine that binds readily to ribosomes, DNA, RNA, and cell membranes. The physiological role of polyamines and whether they modulate ricin toxicity remains to be ascertained.

Ricin has also been shown to activate phagocytes in vivo that generate excessive amounts of reactive oxygen species (ROS), which also exacerbated the inflammatory process and cause further tissue damage. These oxidative stress-mediated mechanisms result in hepatic lipid peroxidation, glutathione (GSH) depletion, DNA single-strand breaks, and increased urinary excretion of carbonyl compounds as well as increases in kidney lipid peroxidation with concomitant decreases in GSH (Buonocore et al. 2011). To counter the downstream inflammatory effects induced by ricin poisoning, various anti-inflammatory substances, such as nicotine, have also been investigated (Mabley et al. 2009). Nicotine administered 2 h after ricin injection in mice was determined to significantly delay and reduce ricin-induced mortality, an effect correlated to the reduced serum levels of TNF-α and markers of kidney and liver dysfunction in nicotine-treated animals. Nicotine administration also attenuated the increase in ricin-induced oxidative stress levels in the kidney and liver (Mabley et al. 2009). However, nicotine is a highly toxic chemical, its use as a therapeutic compound in humans would need to be reviewed thoroughly. A summary of all the abovementioned experimental medical countermeasures and their respective targeted points of intervention is provided in Table 4.

At first glance, therapeutic approaches that target post-ricin cellular internalization pathways do not seem to fare better in terms of extending the current therapeutic window afforded by neutralizing antibodies. It should be pointed out that these noval chemical entities therapeutic approaches are not limited to extracellular sites, unlike the antibodies. Their efficacy could possibly be further enhanced by modifying the drug delivery method and by combining compounds targeting different pathways of ricin toxicosis into a treatment regime. A sterling example of how drug delivery can improve the treatment outcome was observed in Buonocore et al. (2011)'s work involving the use of liposome-encapsulated N-acetylcysteine (Lipo-NAC) to increase intracellular delivery of NAC to target cells poisoned by ricin A-chain. The targeted drug delivery approach enhances NAC-mediated protection against intracellular-reactive oxygen species, ultimately

reversing ricin A-chain-induced hepatotoxicity. For the treatment to be effective, it is also essential to perform toxicokinetic–toxicodynamic studies to identify the main target organ(s) for different routes of intoxication. The liposome-encapsulated therapeutic compounds can then be directed to these specific sites via intravenous injection or aerosol delivery depending on the main site of injury. In this way, a combination of inhibitors of retrograde transport, ricin A-chain enzyme activity, and downstream stress-mediated pathways and inflammation, with immunotherapeutics, may provide an effective medical countermeasure to ricin and abrin toxicity, beyond what could be offered by conventional antibodies and vaccine approaches.

Conclusion and Future Directions

In this chapter, a background description of the enzymatic function and structure of ricin and abrin toxin has been provided. The toxic actions displayed by toxins at the molecular, cellular, and physiological levels of poisoning are presented with respect to its functional structure. Unlike the detection of heat-stable ricin or abrin in adulterated food and beverages, detection of trace levels of either toxin in biofluids (diagnostics) is much more difficult. The challenge of diagnosis and treatment of ricin or abrin arises from efficient sequestering of these toxins from the circulatory system through binding to a ubiquitous galactose-recognition cell surface receptor and rapid internalization. As such, defining the window for diagnosis following the appearance of intoxication symptoms remains a fairly tricky business. For the oral route of exposure to ricin, fecal samples represent the most suitable diagnostic sample as a prolonged diagnostic window is possible. Where such samples are not available for immediate point-of-care testing, a customized assay for whole blood for maximizing availability of ricin and high sensitivity has also been described.

In the area of medical countermeasures, given the speed of both cell targeting and rapid depurination of ribosomal RNA, one school of thought advocates prophylaxis. To this end, based on alteration of ricin active sites, two types of vaccines have been developed. While mass vaccination for the general population may be cost-prohibitive, vaccination of personnel prior to engaging in war may have its utility. More instant protection in this sense can probably be provided by antitoxins produced in sheep and horses, though they do not allow for administration in humans more than once in a lifetime. Despeciated antibodies and humanized monoclonal antibodies have been developed to lower the immunogenic risk of administration. However, the window of therapeutic opportunity for antibodies remains limited as it is effective only against circulating toxins. To extend the therapeutic window, other innovative therapeutic approaches that target various post-internalization pathways of these toxins have been reported in this chapter. While promising solutions in the form of small molecule-based inhibitors of ricin retrograde transport, specific inhibitors of ricin A-chain enzymatic site, and modulators of ricin-induced stress signaling pathways have been found, further drug

development is required to ensure safe and efficient delivery of this small molecule therapeutics to their target sites. In addition, detailed elucidation of the mechanism of action of these novel chemical entities and their toxicity would be required before they could be used in man.

It has been noted that there is a relative paucity of research efforts to develop diagnostics and therapeutic options for abrin poisoning. In view of the higher toxicity of abrin toxin, more emphasis in this field would be essential.

Cross-References

▶ Biotoxins and Food Safety
▶ Immunoneutralization of Abrin
▶ The Biowarfare Agent Ricin

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Aflatoxins and Their Management

6

Hassan Yazdanpanah and Samira Eslamizad

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Abstract

Contamination of foods with aflatoxins (AF) has received a great awareness during the last few decades. AF are highly substituted coumarins containing a fused dihydrofurofuran moiety which are produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi. AF contamination can occur in various

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commodities including cereals, nuts, dried fruits, cocoas, oil seeds, spices, and copras in the field and/or during storage. The toxicological consequences of AF in populations are quite varied due to a wide range of exposures leading to acute and chronic effects. They are known mutagenic, teratogenic, carcinogenic, and immunosuppressive toxins. In addition, contamination of foods with AF may create significant direct and indirect economic consequences both for producer and consumer countries. Therefore, many countries have set legislation with regard to AF in foods. Concerning management strategies for AF, a number of methods have been investigated to prevent AF contamination to remove AF from the contaminated foods and feeds, to detoxify AF in contaminated foods and feeds, or to prevent AF effects. One possible approach to the management of the risks associated with AF contamination is the use of the integrated system of Hazard Analysis and Critical Control Points (HACCP). This proposed control program for processed foods/feeds should be based on the HACCP approach and should involve strategies for prevention, control, good manufacturing practices, and quality control used at all stages of production from the field to the final consumer. In this chapter, various aspects of AF including producing fungi, occurrence, legislations, toxicokinetics, toxicology, and management strategies are reviewed.

Introduction

Mycotoxins are organic and complex secondary metabolites produced by various fungi species. They are mainly produced by fungal genera including *Aspergillus, Fusarium, Penicillium, Claviceps*, and *Alternaria* under proper conditions. Some of the factors that influence the growth of fungi on crops and their subsequent mycotoxin production include plant genetics; exposure to fungal spores; weather conditions and climate during planting, growing, and harvesting; insect damage; crop management; and use of fungicides. Mycotoxin-producing fungi are commonly subdivided into field fungi and storage fungi (Rodrigues and Naehrer 2012).

Just a few hundred mycotoxins out of the thousands of existing ones are associated with foodstuffs and only a handful present food safety challenges (Murphy et al. 2006). Aflatoxins, aflatoxin M1(AFM1), ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (FM), zearalenone (ZEN), and patulin (PTN) are among the most important mycotoxins. Mycotoxin contamination can occur in all agricultural commodities in the field and/or during storage, if conditions are favorable to fungal growth. Mycotoxins may contaminate a wide range of agricultural products including cereals, nuts, dried fruits, coffees, cocoas, spices, oil seeds, fruits, etc. When mycotoxins present in foods in sufficiently high levels, they can produce toxic effects that range from acute to chronic (like cancer), mutagenic, and teratogenic effects (Murphy et al. 2006).

AF are considered to be the group of mycotoxins of greatest concern from a global perspective. They have become recognized as ubiquitous contaminants of the human foodstuff supply throughout the economically developing world (Kensler et al. 2011). Various food commodities including cereals, nuts, dried fruits, cocoas,

oil seeds, spices, and copras may be contaminated with AF (Murphy et al. 2006). The toxicological consequences of AF in populations are quite varied due to a wide range of exposures leading to acute and chronic effects. They are known mutagenic, teratogenic, carcinogenic, and immunosuppressive toxins (Kensler et al. 2011). In addition to health risks to populations, contamination of foods with AF may create significant direct and indirect economic consequences both for producer and consumer countries. In this chapter, different aspects of AF are discussed.

Historical Perspective

The AF were discovered in the late 1950s and early 1960s following the severe outbreak of turkey "X" disease which resulted in the deaths of numerous turkeys and other farm animals fed diets containing certain lots of peanut meal originating in South America. Experiments revealed that toxicity was associated with the presence of *Aspergillus flavus*, and when the fungus was inoculated into uncontaminated peanut meal, it produced toxins similar to those found in the contaminated meal. Therefore, the isolated toxins were named "aflatoxin" (*Aspergillus flavus* toxin) (Ayub and Sachan 1997; Kensler et al. 2011).

Producing Fungi and Production Conditions

AF are secondary fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi. AF production in innate substrates depends upon the various factors, that is, type and moisture content of substrate, physical damage of the kernels, fungal species, temperature, humidity, and minerals (Abrar et al. 2013). The producing fungi are ubiquitous and can affect many dietary staples of developing countries. Fungal invasion and contamination often start before harvest, and AF accumulate postharvest when food commodities are stored under conditions that promote fungal growth. AF occur mostly in tropical regions with high humidity and temperature (between 24 °C and 35 °C) (Wild and Gong 2010; Williams et al. 2004). Grains must be kept dry, free of insects, and free of damage. Grains stored at warm temperatures (>20 °C) under high moisture/humidity (>14 %) and/or inadequately dried can potentially become contaminated with AF. These conditions allow mold "hot spots" to occur in the stored grain (Richard 2007).

Chemistry, Occurrence in Foods, and Legislations

Chemistry

Chemically, the AF are highly substituted coumarins containing a fused dihydrofurofuran moiety. The naturally occurring AF are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), of which

AFB1 is the most abundant, carcinogenic, and toxic one (Wild and Gong 2010). The four major AF (AFB1, AFB2, AFG1, and AFG2) are called based on their relative chromatographic mobility during thin-layer chromatography and their fluorescence under ultraviolet irradiation (blue or green) (Bennett and Klich 2003). The blue fluorescent toxins (B) are characterized by fusion of a cyclopentenone ring to the lactone ring of the coumarin moiety, while the green fluorescent toxins (G) contain a fused lactone ring (Kensler et al. 2011). AFM1 and AFM2 are the hydroxylation products of AFB1 and AFB2, respectively, and found in milk and milk products (Wild and Gong 2010).

Occurrence in Foods

AF contamination can occur in various commodities in the field and/or during storage, if conditions are favorable to fungal growth. Many crops including cereals, nuts, dried fruits, cocoas, oil seeds, spices, and copras are contaminated with AF. Typical occurrence average ratio for AFB1 and AFB2 (mainly produced by *Aspergillus flavus*) is approximately 4:1. Typical average occurrence ratio for AFB1 and the sum of AFB2, AFG1, and AFG2 (the G toxins are mainly produced by *Aspergillus parasiticus*) is approximately 1:0.8, although variations do occur for both ratios (FAO 2004). Although contamination by the fungi may be universal, the levels or final concentrations of AF in the grain product can vary from less than 1 μg/kg to greater than 12,000 μg/kg. Indeed, in a recent outbreak of AF-induced death of people in Kenya, individual daily exposure of AFB1 was estimated to be 50 mg/day (Kensler et al. 2011).

A number of studies on the occurrence of AF in foodstuffs and feedstuffs have been published. Examples are given in Table 1 as well as in the following text.

The data show that AF are present in foodstuffs and feedstuffs and that maximum contamination levels exceeding the maximum levels or guidance values are likely to occur. AF production occurs mainly in regions with tropical or subtropical climates (Streit et al. 2012). Therefore, from a European perspective, the most common source of AF exposure is imported feed such as copra, palm kernel, peanut cake, and corn gluten meal (depending of origin). It has been stressed that as a consequence of rising average temperatures, patterns of mycotoxins occurrence in Europe are expected to change. For supporting this statement, Southern Europe was used as an example. It has been reported that while the importance of DON is about to decrease, *A. flavus* infection and AF contamination, which were uncommon in Europe, have become increasingly important. In 2003, in northern Italy, a hot and dry growing season resulted to severe corn infection with *A. flavus*. Analysis of AFB1 showed an incidence of 75 % with a mean contamination of 4.4 μg/kg. Using this corn as feedstuff for dairy cattle resulted in a widespread milk contamination with AFM1, and several thousand tons of milk exceeding the EU legal limit were discarded (Streit et al. 2012).

Rodrigues et al. (2011) analyzed various mycotoxins including AF in 324 grain, feed, and feed commodity samples, which were sourced directly at animal farms or

Table 1 Occurrence of AF in foodstuffs and feedstuffs

Food type	Country	Contaminated/ total	AF	Concentration (μg/kg)	Method	Reference
Rice	Canada	99/199	AFB1	<0.002–7.1	High-performance liquid chromatography (HPLC)	(Bansal et al. 2011)
Rice	IR Iran	59/71	Total AF (AFT)	2.097–10.94	HPLC	(Mazaheri 2009)
Wheat	Tunisia	10/46	AFT	0.15–18.6	HPLC	(Ghali et al. 2010)
Pistachio	IR Iran	3,699/10,068	AFB1	5.9 (Mean)	HPLC	(Cheraghali et al. 2007)
Hazelnut	Turkey	43/51	AFT	<0.625–10	Enzyme-linked immunosorbent assay (ELISA)	(Aycicek et al. 2005)
Infant milk food, milk-based cereal, weaning food, infant formula, and liquid milk	India	76/87	AFM1	0.063–1.012	ELISA	(Rastogi et al. 2004)
Rice	Vietnam	51/100	AFB1	3.31 (mean)	HPLC	(Nguyen et al. 2007)
Commodities, feeds, and feed ingredients	Egypt	3/16	AFB1	0.4 (mean)	HPLC	(Rodrigues et al. 2011)
Commodities, feeds, and feed ingredients	Jordan	9/20	AFB1	2 (mean)	HPLC	(Rodrigues, et al. 2011)
Commodities, feeds, and feed ingredients	South Africa	3/77	AFB1	0.3 (mean)	HPLC	(Rodrigues et al. 2011)
Peanuts and peanut products	Brazil	14/100	AFB1	Peanuts: 24.0–87.5	Thin-layer chromatography (TLC)	(Hoeltz et al. 2012)
				Peanut products: 22.0–84.6		

feed production sites in Middle East and Africa between February and October 2009. The incidence of AF in samples varied from 0 to 94 %. The mean level of AF contamination in total samples ranged from 0.2 μg/kg to 116 μg/kg. Warmer countries, such as Nigeria, Kenya, and Ghana, had a higher incidence of AF, while more temperate countries exhibited a totally different contamination pattern (Rodrigues et al. 2011). In a survey between January 2009 and December 2011, a total number of 7,049 corn, soybean/soybean meal, wheat, dried distillers grains with solubles, and finished feed samples from Americas, Europe, and Asia were analyzed for various mycotoxins including AF (Rodrigues and Naehrer 2012). The results showed that from 4,627 samples analyzed for AF, these toxins were present in 33 % of the samples with the average of 21 μg/kg. The maximum level of AF was found in corn samples at the level of 6,105 μg/kg (Rodrigues and Naehrer 2012).

To assess the incidence of mycotoxins in feed and feed raw materials, a 2-year survey program (from October 2003 to December 2005) was done by a feed additive producer (Binder et al. 2007). From North Asia, a total number of 3,420 samples were analyzed. The low proportion (0.03) of positive AFB1 samples was notable. From Southeast Asia, a total number of 2,040 analyses were undertaken. The incidence of AFB1 was 0.34 and the highest level found was 347 μg/kg. In South Asia, there was a clear indication of high AF occurrence (0.63). The average and median contamination levels were 52 μg/kg and 24 μg/kg, respectively (Binder et al. 2007).

Mycotoxin contamination of foodstuffs and feedstuffs in IR Iran has been reviewed (Yazdanpanah 2006). Pistachio nuts produced in IR Iran during March 2002 to February 2003 were analyzed for presence of AF (Cheraghali et al. 2007). In this regard, 3,356 pistachio nut samples were collected. After dividing samples to subsamples, 10,068 AF analyses were carried out. Among 10,068 samples analyzed, AFB1was detected in 3,699 samples (36.7 %), with the mean and median of 5.9 μg/kg and 0.1 μg/kg, respectively. The AFT was detected in 2,852 samples (28.3 %) with the mean and median of 7.3 μg/kg and 0.4 μg/kg, respectively (Cheraghali et al. 2007). In IR Iran, a survey of AFB1 was performed on 90 samples collected from Tehran retail market in June 2005 (Yazdanpanah et al. 2013). The results showed that none of the bread and wheat flour samples were contaminated with AFB1. The mean AFB1 levels in rice, puffed corn snack, and peanut samples were 4.17 μg/kg, 0.11 μg/kg, and 1.97 μg/kg, respectively. The level of AFB1 in 3 samples (one rice and two peanut samples) was found to be higher than 5 μg/kg (Yazdanpanah et al. 2013).

In IR Iran, 51 maize samples, intended for animal feed and human consumption, were collected from the four main maize production provinces and analyzed by HPLC for contamination by AF (Ghiasian et al. 2011). AFB1 was detected in 58.3 % and 80 % of the maize samples obtained from Kermanshah and Mazandaran provinces, respectively. The level of AFB1 in 15.68 % of the total samples was above the maximum tolerated limit (5 μg/kg) for AFB1 in maize in IR Iran. The mean contamination level of AFT (23.86 μg/kg) in the positive samples was higher than maximum tolerated limit for maize in IR Iran (20 μg/kg) intended for animal feed (Ghiasian et al. 2011).

Legislations

Widespread concern about the potential toxic effects of AF in humans and animals as well as possible transfer of residues into milk and edible animal tissues has demanded the need for establishment of control measures and limits by international authorities (Abrar et al. 2013; Kensler et al. 2011). Over the years, the number of countries which have set regulations for AF has markedly increased. The regulations for AF are often detailed and specific for various foodstuffs, feedstuffs, and dairy products (FAO 2004). The data published by FAO showed that the maximum tolerated levels for AFB1 in food have not changed dramatically in 2003 compared to the situation in 1995, although the range of limits has narrowed a little (1–20 μg/kg). In 2003, many countries regulated AFT sometimes in combination with a specific AFB1 limit.

Compared to 1995, the range of limits (0–35 μg/kg) has narrowed a little. AFM1 has been regulated in 60 countries in 2003, a more than threefold increase as compared to 1995. Many AF regulations exist for feedstuffs. Concerning AFB1 in feedstuffs for dairy cattle, many more countries (39) have set regulations in 2003, compared with those in 1995 (25 ones). In IR Iran, in 1997, Iranian National Standards Organization set maximum tolerated limits for mycotoxins in foods and feeds (FAO 2004).

Toxicokinetics

After absorption of AFB1 from the small intestine of broilers, it readily binds to plasma albumin, which serves as the major transporter of AFB1 in the blood. AFB1 is "procarcinogen," and enzymatic bioactivation is a prerequisite for its carcinogenic action. In the liver, AFB1 is oxidized by microsomal mixed function oxidase to several water-soluble metabolites (Abrar et al. 2013). Cytochrome P450 (CYP) enzymes are responsible for metabolism of AFB1 to the electrophilic, reactive, and major carcinogenic metabolite AFB1-8,9-epoxide (AFBE) or to the less mutagenic forms such as AFM1, aflatoxin Q1 (AFQ1), or aflatoxin P1 (AFP1) (Abrar et al. 2013; Bennett and Klich 2003; Murphy et al. 2006). In humans, epoxidation is catalyzed by CYP1A2 and CYP3A4 (Kensler et al. 2011). It is believed that AFBE formation and its subsequent covalent binding to DNA, RNA, and proteins play a critical role in both acute and chronic toxicity (Abrar et al. 2013). AFBE can take several pathways, one resulting in toxicity, another in cancer, and others in AFBE excretion (Murphy et al. 2006). The AFBE can react by interacting with DNA to produce a promutagenic AFB1-N^7-guanine adduct. In DNA, this adduct is unstable, rapidly undergoes depurination, and is excreted in the urine (Kensler et al. 2011). Formation of AFB1–DNA adducts (such as with N^7-guanine) leads to gene mutations and cancer (Murphy et al. 2006). A specific mutation of codon 249 is suspected to occur in the human p53 tumor suppression gene by AFB1–DNA adducts (Gomaa et al. 2008; Murphy et al. 2006). It has been shown that AFB1–DNA adducts can result in GC to TA transversions (Bennett and Klich 2003).

Among patients with hepatocellular carcinomas (HCC) in areas of high-risk AF exposure, this mutation was found with greater frequency (Murphy et al. 2006). AFB1 is more carcinogenic and mutagenic than AFG1 (Wild and Gong 2010). AFG2 and AFB2 are relatively nontoxic unless they are first oxidized metabolically to AFB1 and AFG1 in vivo (Kensler et al. 2011). Production of 8,9-dihydro-8,9-dihydroxy-AFB1 is the result of metabolic processing of the AFBE which causes injury of cell and eventual cell death (Caloni and Cortinovis 2011). AF toxicity may also arise through the intracellular reactive oxygen species generation during the metabolic processing of AFB1 by P450 system in the liver. These species may attack membranes as well as soluble cell compounds, eventually leading to the impairment of cell functioning and cytolysis (Abrar et al. 2013). In addition, AFB1 inhibits protein synthesis interfering with the formation of enzymes which are necessary for metabolism and energy and fat mobilization (Caloni and Cortinovis 2011).

Detoxification of the AF exo- and endo-epoxides is mainly through glutathione *S*-transferase-mediated conjugation with reduced glutathione (Wild and Gong 2010).

In humans, there are a number of urinary and serum biomarkers which were validated to accurately predict AFB1 cancer risk. AF-N^7-guanine in the urine serves as an elegant biomarker of biologically effective dose. Urinary measures of AFM1, the AF–albumin adduct, and AF-mercapturic acid are used as biomarkers of internal dose (Kensler et al. 2011). Serum AFB–albumin adducts which are positively associated with hepatocellular carcinoma in humans were found to be widely used in epidemiologic studies. Analysis of serum adducts indicates a positive correlation between dietary AFB1 exposure and serum AFB–albumin adducts (Rawal et al. 2010).

Toxic Effects of Aflatoxins on Animal and Human Health

AF are associated with both toxicity and carcinogenicity in human and animal populations (Bennett and Klich 2003). AF are immunosuppressive, carcinogenic, teratogenic, and mutagenic (Richard 2007). Among all known naturally occurring AF, AFB1 is the most toxic one. The degree of toxicity and mutagenic potency of AFB1 and its metabolites decrease as follows: AFB1 > aflatoxicol (AFL) > AFG1 > AFM1 > AFL-H1 > AFQ1 > AFB2 > AFP1 > AFG2 > AFB_{2k} > AFG_{2k} (Santacroce et al. 2008). Aflatoxicosis is the poisoning that results from ingesting AF (Williams et al. 2004). There are 2 forms of aflatoxicosis: (1) acute intoxication, which results in direct liver damage and subsequent illness or death, and (2) chronic subsymptomatic exposure. In all species, the dose and duration of exposure to AF clearly have a major effect on the toxicology and may cause the following consequences: (1) large doses of AF results into acute illness and death, usually through liver cirrhosis; (2) chronic sublethal doses of AF have immunologic and nutritional effects; and (3) all doses of AF have a cumulative effect on the risk of cancer (Williams et al. 2004).

AF were found to be moderately to highly toxic and carcinogenic in almost every animal species tested, including monkeys (Trucksess 2012). Concerning biological effects of AF, in most animal species, a wide variety effects such as toxicity, genotoxicity, carcinogenicity, teratogenicity, and impairment of immune and reproductive system were reported (Santacroce et al. 2008). The main factor in tolerance relates to the nature of the digestive system. Chickens, ducks, and ducklings are more sensitive, and ruminants are more tolerant. Breed variety, nutrition, sex, age, environmental stress, and presence of other disease agents are other factors contributing to differences in animal susceptibility to AF (Trucksess 2012).

Acute Toxicity

Exposure to large doses of mixed AF may cause acute toxicity with lethal effect. Animal species respond differently in their susceptibility to the acute and chronic toxicity of AF, and no animal species is resistant to the AF acute toxic effects. The AFB1 acute toxicity varies very much between animal species. For AF, LD50 value ranges from 0.5 to 10 mg/kg body weight for most species. AF effect is influenced by several factors including exposure level, duration of exposure, environmental factors, nutritional status, age, and health (Gnonlonfin et al. 2013). Exposure to small doses (a total of 2–6 mg) distributed over a prolonged period could lead to cancer, whereas exposure to estimated mixed total doses ingested with food of around 6,000 mg was reported to acute cause fatal toxicity in adult humans (Gnonlonfin et al. 2013). In humans, acute aflatoxicosis has been reported in several developing countries. Clinical manifestations of aflatoxicosis include vomiting, abdominal pain, pulmonary edema, disruption of blood clotting mechanism, reduced liver function, icterus, a decrease in essential serum proteins that are synthesized by the liver, necrosis of the liver, coma, convulsions, and death with cerebral edema and fatty involvement of the liver, kidney, and heart (Gnonlonfin et al. 2013; Kensler et al. 2011; Rawal et al. 2010). Other symptoms of acute to subacute aflatoxicosis include abdominal pain, vomiting, and edema of the lower extremities. Severe acute liver injury with high morbidity and mortality has been associated with high dose of AF exposure. Consumption of AF-contaminated food can result in outbreaks of sudden death within a population (Gnonlonfin et al. 2013). In the 1974, AF poisoning in India resulted from consumption of heavily contaminated maize. There were at least 97 fatalities (Kensler et al. 2011), and some adults may have eaten 2–6 mg of AF in a single day (Bennett and Klich 2003). In Kenya, in 2004 and 2005, acute aflatoxicosis caused more than 150 deaths (Kensler et al. 2011). In April 2004 in rural Kenya, one of the largest aflatoxicosis outbreaks occurred, resulting in 317 cases and 125 deaths. The source of the outbreak was AF-contaminated homegrown corn with an average concentration of 354 μg/kg (Trucksess 2012). It has been reported that despite eating similar quantities of maize as females, males were more likely to die from aflatoxicosis due to weaker male immune systems (Gnonlonfin et al. 2013).

Chronic Toxicity

Decreased milk or egg production, decrease in growth rate, and immune suppression are the symptoms of chronic exposure to AF in animal kingdom. In addition, liver damage is apparent due to the yellow color. AF affects all poultry species. Although relatively high AF levels are necessary to cause mortality, intake of low concentration of toxins over a long period of time leads to poor feed efficiency, poor growth, suboptimal production, and immunosuppression (Gnonlonfin et al. 2013). Regarding the carcinogenicity of AF, it has been shown that consumption of low levels for a prolonged period can result in primarily liver cancer in several animal species, including aquatic vertebrates (Santacroce et al. 2008). A wide variation exists in species susceptibility to AFB1 hepatocarcinogenesis. Fish and poultry responded to doses as low as 15–30 μg/kg. Rats responded at levels of 15–1,000 μg/kg, whereas mice showed no effects to levels as high as 150,000 μg/kg (Rawal et al. 2010).

A big part of the world population is chronically exposed to AF as evident from the presence of AFM1 in human breast milk as well as umbilical cord blood samples in several countries (Gnonlonfin et al. 2013). For humans, AFB1 is mainly considered as an agent promoting liver cancers, although lung cancer is also a risk among workers handling contaminated grain (Williams et al. 2004). AFB1 is a hepatocarcinogen and has been classified as group 1 human carcinogen (IARC 1993), but may be only part of the total answer to human liver cancer (Richard 2007). Hepatitis B can act synergistically with AF to increase the risk of HCC. According to the World Health Organization, in developing world including Asia and the Pacific Basin (excluding Japan, Australia, and New Zealand), sub-Saharan Africa, the Amazon Basin, parts of the Middle East, the Central Asian Republics, and some countries in Eastern Europe, chronic hepatitis B virus infection occurs more frequently (high infection >8 %). While in the rest of Europe, infection rates are below 1 % (EFSA 2007). Epidemiological studies of human populations exposed to diets naturally contaminated with AF revealed an association between the high incidence of liver cancer in Africa and elsewhere and dietary intake of AF (Turner et al. 2002). Often up to 1 in 10 of the population in sub-Saharan Africa is infected with hepatitis B and C, and AF intake raises the risk of liver cancer by more than tenfold compared to the exposure of both hepatitis alone (Gnonlonfin et al. 2013; Turner et al. 2003). Thus, AFB1 is an independent and possibly strongly potentiating factor for human HCC (Murphy et al. 2006). It has been reported that uncontrolled exposure to AF may cause 4.6–28.2 % of all liver cancer cases globally, with Southeast Asia, China, and sub-Saharan Africa bearing the brunt of the burden (Tillett 2010). In some countries, like Gambia and China, where hepatitis B virus and AF contamination occur together, hepatomas are the predominant cancer (64 % of cancers) and may be a predominant cause of death. It has been reported that 10 % of males' deaths or 10 % of all adults' deaths in Gambia or China (Qidong) were due to liver cancer, respectively. Greater potency of AF in hepatitis B virus-positive people is partly due to this finding that hepatitis B virus positivity reduces the person's ability to detoxify AF (Williams et al. 2004).

AF are immunotoxic to both livestock and humans. It has been reported that in animals, AFB1 induce thymic aplasia, suppress phagocytic activity, reduce T-lymphocyte function and number, and reduce complement activity. In poultry and rats, it has been shown that exposure to AF in contaminated food leads to suppression of the cell-mediated immune responses (Williams et al. 2004). Some of these effects may be mediated through altered cytokine expression (Wild and Gong 2010). In animals exposed to AF, suppression of lymphoblastogenesis, thymic and bursal involution, impairment of delayed cutaneous hypersensitivity, and graft-versus-host reaction also occurred (Williams et al. 2004). Reduced humoral immunity was shown in AF-exposed animals as was increased susceptibility to infections or reduced response to vaccines (Wild and Gong 2010). There are few studies regarding the immunologic suppression effect of AF in human populations. It has been estimated that 30 % of Gambian children are exposed to food with AF levels greater than 100 μg/kg. Previous studies in poultry have shown that when feeds contain similar levels of contamination, immune competence is compromised (Turner et al. 2003). Turner et al. (2003) reported that children are naturally exposed to AF through the diet at levels that compromise the immune system in other species and observed a highly significant association between AF exposure and reduced salivary secretory IgA. In another study in Gambia, children with malaria parasitemia had significantly higher mean AF–albumin adducts, but that there were no marked associations with experience of malaria infection and antibody titer to asexual stages of *Plasmodium falciparum* or lymphoproliferative responses (Wild and Gong 2010). In Ghana, in one study, alterations in different lymphocyte subgroups in relation to AF–albumin adduct level were reported. In another study, high AF–albumin adducts were associated with alterations in some lymphocyte subsets (Wild and Gong 2010). Totally, the studies of immunomodulation in AF-exposed populations are inconclusive. However, the data suggest that in populations exposed chronically to AF, effects on immune parameters could occur (Wild and Gong 2010).

Chronic AF exposure has major effects on nutritional status in animals. In animals exposed to AF, the efficiency of food use is consistently lower. In poultry, a 7–10 % drop in food conversion efficiency is observed, and decreased growth rates are a consistent sign of chronic AF exposure. In animals, it is well established that dietary AF reduces the rate of growth and other measures of productivity (Williams et al. 2004). Limited evidence suggests that growth suppression may also occur in humans. It has been reported that children in Togo and Benin who ate foods contaminated with high levels of AF were stunted and underweight, symptoms normally associated with malnutrition (Gnonlonfin et al. 2013). In Benin, the effects of AF exposure on growth were assessed in a longitudinal study over an 8-month period. There was a strong negative correlation between AF–albumin adducts and height increase over the 8-month follow-up. The highest quartile of biomarker was associated with a mean 1.7 cm reduction in growth over 8 months compared with the lowest quartile (Kensler et al. 2011). In Benin and Togo, a striking inverse association was found between AF–albumin adducts and growth in a cross-sectional study of children aged 1–5 years. Children who were stunted or

underweight had 30–40 % higher mean AF–albumin levels. In a subsequent 8-month longitudinal study, there was a strong negative correlation between AF–albumin adducts and height increase over the 8-month follow-up. These studies were extended to consider in utero exposure in a group of Gambian children and again an association was found between exposure and impaired growth, on this occasion in the first year of life. The mechanisms by which AF may exert an effect on growth are currently unknown, although the possibility of a compromised intestinal integrity, through altered barrier function as a consequence of endothelial cell toxicity or immune suppression, is a valid hypothesis to explore further (Wild and Gong 2010). In the blood, urine, and livers of children with symptoms of nutritional deficiencies (e.g., kwashiorkor), higher AF levels have been found in comparison with similar age-matched children. In comparison with AF-negative kwashiorkor children, AF-positive kwashiorkor children showed significantly greater severity of edema, increased number of infections, lower hemoglobin levels, and longer duration of hospital stay. It seems that protein deficiency reduces the capacity of the liver to detoxify AF; thus AF may be a contributory factor in increasing the morbidity of children suffering from other disease (Gnonlonfin et al. 2013).

There are few studies concerning the reproductive health effect of AF, and they have been reviewed by Shuaib et al. (2010). The available studies have largely focused on birth outcomes such as low birth weight and contamination of breast milk by AF. Six studies found marked associations or correlations between low birth weight and AF, while one study did not find any correlation. One study found maternal serum AF to be a risk factor for jaundice in infants. One study found a higher concentration of AF in the semen of infertile men. The findings showed a higher rate of AF contamination of maternal breast milk in developing countries, at levels beyond the acceptable limits. Totally, the reviewed studies were unable to draw definitive conclusions about the reproductive health effects of AF (Shuaib et al. 2010). However, considering the high contamination rate of breast milk by AF and the known toxic effects of AF on other organ systems, stakeholders in affected countries should take urgent steps to reduce exposure of vulnerable populations to the toxic effects of AF (Shuaib et al. 2010).

Aflatoxin Management Strategies

Many developing countries have found that reducing concentration of mycotoxins in foods will not only reduce financial burden on health care but also confer international trade advantages (Gnonlonfin et al. 2013). A great deal of research has been done for several years to find methods to reduce AF in contaminated agricultural produce. Recently, these AF management strategies have been reviewed by (Abrar et al. 2013; Gnonlonfin et al. 2013; Stoev 2013). A number of methods have been investigated to prevent AF contamination, to remove AF from the contaminated foods and feeds, to detoxify AF in contaminated foods and feeds, or to prevent AF effects. One possible approach to the management of the

risks associated with AF contamination is the use of the integrated system of HACCP. This proposed control program for processed foods/feeds should be based on the HACCP approach and should involve strategies for prevention, control, good manufacturing practices, and quality control used at all stages of production from the field to the final consumer (Stoev 2013). As an example, IR Iran, in the past decade, has implemented effective interventions to control AF in pistachio nuts. Implemented interventions such as establishing an efficient decision-making system; focusing on preventive methods; applying HACCP, good agricultural practice, and good storage practice guidelines; and using accurate and sensitive sampling and analytical methods proved to be effective. The statistics published by the European Commission (EC) regarding Rapid Alert System for Food and Feed (RASFF) for AF contamination in IR Iran pistachio nuts confirms a significant reduction in AF contamination in pistachio nuts exported from IR Iran. As a consequence, in regard to AF contamination, IR Iran experience to prevent and control AF contamination in pistachio nuts was fruitful (Cheraghali and Yazdanpanah 2010).

Preventive Measures of Aflatoxin Contamination of Foods/Feeds

The occurrence of fungi and mycotoxins can be decreased by application of a variety of preventative measures both preharvest and postharvest including appropriate control measures, timely harvesting, cleanup, drying and storage practices, management of insect infestation, crop rotation, creating of plant cultures resistant to fungi infestation, and others (Stoev 2013).

Biological strategies, such as toxigenic fungi, have been developed for prevention of AF contamination. In Nigeria, less toxigenic strain of *A. flavus* was isolated from soils. In the United States, such atoxigenic strains of *A. flavus* and *A. parasiticus* upon introduction to soil of developing crops have led to AF contamination in peanuts ranging from 74.3 % to 99.9 % of the original seen contamination. Postharvest (storage) AF contamination was reduced by 95.9 % through field application of non-toxigenic strains of *A. flavus* and *A. parasiticus* (Gnonlonfin et al. 2013).

Appropriate use of pesticides during the production process could help in reducing the fungal infection or insect infestation and subsequent mycotoxin contamination. Fungicides such as itraconazole and amphotericin B have been shown to effectively control the AF-producing *Aspergillus* species (Gnonlonfin et al. 2013).

Another tool is growing resistant varieties, which leads toward safety measure against AF contamination in field crops (Abrar et al. 2013). Rapid drying of agricultural products for lowering the moisture level is very critical. It has been shown that drying harvested maize to a moisture content of 15.5 % or lower within 24–48 h reduces the risk of fungal growth and subsequent AF biosynthesis (Gnonlonfin et al. 2013). In corn, several studies have shown that there is a positive correlation between AF contamination and insect damage. Therefore, for reduction

of mycotoxin contamination, proper management of insect pests through appropriate control strategy is needed (Gnonlonfin et al. 2013). The best method for controlling mycotoxin contamination is prevention through preharvest management. However, when mycotoxin contamination occurs, the hazards associated with various mycotoxins must be managed through postharvest procedures (Stoev 2013).

Physical Methods of Aflatoxin Decontamination of Foods/Feeds

Various physical methods including thermal inactivation, irradiation, cleaning, washing, segregation, mechanical sorting and separation, solvent extraction, etc., can be used to reduce or eliminate the risk of AF contamination in various types of foods. Regarding the effectiveness of cleaning for AF decontamination, an average reduction of about 40 % in concentration was usually reported (Stoev 2013). Significant amounts of AF can be removed from grains by immersing them in water and removing the upper floating fraction. It has been reported that sorting, winnowing, washing, and crushing combined with dehulling of maize grains were relatively effective in achieving a significant AF removal (Gnonlonfin et al. 2013).

Damaged or inadequately developed nuts highly contaminated with AF can be removed using automated sorting and segregation of peanuts. Fluorescence sorting is used mainly for screening and decontamination of corn, cottonseed, and dried figs, whereas electronic sorting is another method for peanuts decontamination, which is based on the color of roasted, blanched peanuts (Stoev 2013). In peanuts, it has been shown that a significant proportion (80 %) of the toxin is often associated with the small and shriveled seeds and moldy and stained peanuts, which can be removed by sorting (Gnonlonfin et al. 2013). AF can be eliminated from food commodities by utilization of various solvents, but the barriers for commercial exploitation of such methods are high prices and possible solvents residues (Abrar et al. 2013; Stoev 2013).

It has been shown that solar radiation, as a physical method of decontamination, is an inexpensive way of partial detoxification of AF in contaminated coconuts, peanuts, sesame, and corn (Stoev 2013). It has been shown that thermal inactivation could be a suitable method for AF decontamination in pistachio nuts (Yazdanpanah et al. 2005). In this regard, the effect of roasting on AF reduction in pistachio nuts was investigated. Although all treatment protocols showed some degree of AF degradation (ranging from 17 % to 63 %), roasting spiked samples at 120 °C for 120 min and 150 °C for 30–120 min caused substantial reduction of AF. Treatment of naturally contaminated whole pistachio kernels at 150 °C for 30 min significantly reduced level of AF contamination in samples (up to 81 % reduction in AFB1 level in pistachio nut with original contamination of 235 μg/kg). Degradation of AF was both time and temperature dependent (Yazdanpanah et al. 2005).

Chemical Methods of Aflatoxin Decontamination of Foods/Feeds

Numerous chemopreventives have been assessed for their effectiveness in AF decontamination. Various chemicals such as sodium bisulfite, ammoniation, hydrogen peroxide, ozone, propionic acid phosphine, fungicide, sodium bentonite, clay-based inorganic adsorbents, and limewater have been used to destroy or degrade AF effectively, but most of them are impractical or potentially unsafe to use due to the formation of toxic residues or the effect on nutrient content, flavor, odor, color, texture, and/or the functional properties of the product. Ammonization and reaction with sodium bisulfite are two techniques for detoxification of AF that have received considerable attention (Abrar et al. 2013).

Use of Different Compounds and Other Methods Preventing the Aflatoxin Effects

There is the possibility of addition of various chemicals or feed additives in order to fix and neutralize mycotoxins (Stoev 2013). Dietary strategies can prevent ingestion or absorption of mycotoxins in prepared foods and feeds. In this regard, food components (phenolic compounds, coumarin, chlorophyll and its derivatives, fructose, aspartame), antioxidant compounds (selenium, vitamins, provitamins), mineral and biological binding agents (hydrated sodium calcium aluminosilicate, bentonites, zeolites, activated carbons, bacteria, and yeast), and medicinal herbs and plant extracts can be used. Chlorophyllin and oltipraz and/or dietary intervention like broccoli sprouts and green tea was found to be effective in preventing the production of epoxide (that leads to chromosomal damage) or increasing detoxification processes. Enterosorption based on the use of certain clay minerals (such as Novasil) was found to be especially useful in binding mycotoxin from contaminated feedstuffs (Gnonlonfin et al. 2013). For example, hydrated sodium calcium aluminosilicate clay is very useful for preventing aflatoxicosis in farm animals and for reducing AF concentrations in milk (Stoev 2013). In a trial in Ghana, ingestion of capsules containing a clay compound resulted in a marked reduction of the biomarker of AF exposure (Gnonlonfin et al. 2013).

Food/Feed Processing as a Method of Aflatoxin Decontamination

Processing can be defined as any physical, chemical, or biological treatment that is applied to a raw material to produce the final consumer product and includes any procedure from dry and wet milling of grains, baking, extrusion, and steaming to feeding cereal-based complete feeds to animals to produce meat or milk. During processing, the stability of mycotoxins may be affected by biological or chemical reactions and factors such as temperature, moisture content, pressure, pH, buffering conditions, and the presence of other constituents and enzymes (Stoev 2013).

Stability of AF to heat in processes such as baking and extrusion depends on pH and temperature. For example, higher temperatures or alkaline processes (such as the use of leavening agents or tortilla production) can reduce AF levels. In the manufacture of tortillas, AF can be significantly decreased during the treatment of corn with limewater. AF are successfully eliminated during refining of oil. AF levels can be considerably decreased with addition of sodium chloride during the cooking of unshelled peanuts under pressure (Stoev 2013). In IR Iran, in Damghan city (Semnan Province), the pistachio nuts are roasted with lemon juice. In an investigation, the efficacy of lemon juice and/or citric acid in AFB1 degradation in pistachio nuts was evaluated (Amirahmadi et al. 2005). The results showed that roasting pistachio nuts with lemon juice at 90 °C or 120 °C for 30 min was not effective on AFB1 degradation. However, a synergistic effect on AF degradation was observed between heating pistachio at 120 °C for 1 h and adding lemon juice and citric acid. When pistachio nut samples (with AFB1 level: 268 μg/kg) are roasted with a mixture of lemon juice (15 ml), citric acid (2.25 g), water (30 ml), and sodium chloride (5 g) at 120 °C for 1 h or 150 °C for 30 min, AFB1 was degraded 58 % or 47 %, respectively. These roasting procedures improved taste, flavor, physical appearance, and acceptability of pistachio nuts (Amirahmadi et al. 2005).

In wet milling, a large percentage of AF are removed in the steep water. In dry milling, AF concentrate in the bran and offal fractions of wheat and germs (Stoev 2013).

Conclusions and Future Directions

From a global perspective, AF are considered to be the group of mycotoxins of greatest concern. They have become recognized as ubiquitous contaminants of the human foodstuff supply throughout the economically developing world. A big part of the world population is chronically exposed to AF which is associated with both toxicity and carcinogenicity in human populations. Due to unavoidable and unpredictable nature of AF, the contamination of foods with these fungal toxins presents a unique challenge to food safety. Many developing countries have found that reducing concentration of AF in foods will not only reduce financial burden on health care but also confer international trade advantages. Therefore, AF contamination must be managed through using proper management strategies. One possible approach to the management of the risks associated with AF contamination is the use of the integrated system of HACCP. This proposed control program for processed foods/feeds should be based on the HACCP approach and should involve strategies for prevention, control, good manufacturing practices, and quality control used at all stages of production from the field to the final consumer. AF reduction and control are dependent on the concerted efforts of all sectors involved in the food production chain. The key actions include AF awareness as a public health issue, strengthening laboratory and surveillance capacities, as well as establishing early warning system and training of farmers.

Cross-References

▶ Biotoxins and Food Safety
▶ Current Insights into Mycotoxins

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Toxins Relevant to Gastrointestinal Disorders

7

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Abstract

Gastrointestinal diseases (GIDs) remain a major disease burden in both developing and developed countries worldwide. The major source of GI tract infection is through contaminated food and water. Food contamination, whether accidental or deliberate, can occur anywhere along the food processing line, from the source to the consumer. A number of viral, bacterial, and parasitic pathogens are involved in the causation of the GID. Infections of the GI tract can range from mild self-limiting diarrhea to severe life-threatening situations. Bacterial toxins

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secreted during the growth of bacteria have significant implications in eliciting diarrhea. Toxins may have different modus operandi and include those acting on the cell surface and those acting on the intracellular targets. Surface-acting ones may comprise toxins that act on the immune system (superantigens) and cell membranes and pore-forming toxins. Intracellular-acting toxins may constitute those acting on protein synthesis and signal transduction and cytoskeleton structure. Enzymatic toxins include those possessing ADP-ribosyltransferase activities.

Detection of these pathogens or toxins often requires multiple steps such as extraction, sample cleanup, or pre-concentration for toxins or multiple cultures for pathogens, prior to measurement, resulting in longer analysis times. Recent efforts in the development of a variety of immunobased diagnostic assays have reduced the analysis time without compromising the sensitivity, with the enzyme-linked immunosorbent assay (ELISA) format being the most common.

In this chapter few selected toxins including superantigens, cholera toxins, *Bacillus cereus* toxins, *C. perfringens* enterotoxins, and Shiga-like toxins; clinical features; mode of action; and diagnostic methods are discussed.

Introduction

Gastrointestinal diseases (GIDs) have substantial morbidity and mortality with an estimated 70 million affected annually in the USA. These do come with a significant burden on the cost and have been estimated at US$142 billion per year (Peery et al. 2013). Infections of the GI tract range in their symptoms from mild self-limiting episodes to severe and life-threatening diarrhea. Diarrhea kills 2,195 children every day, and 1 in 9 children deaths is due to diarrhea worldwide, making it the second leading cause of death among children under 5 years of age. It is estimated that diarrhea accounted for 9.9 % of the 6.9 million deaths among children in 2011 (Lanata et al. 2013; Liu et al. 2012; Fischer-Walker et al. 2013). The major source of GID outbreaks is through contaminated food and water. According to a report from the Ministry of Health (MOH), Singapore, a total of 627 reported food poison cases were recorded in 2011. A recent report in December 2012 has registered 453 affected cases connected to food consumption (MOH Report 2012, 2014).

Several organisms have been implicated as important causes of these deaths, yet there has not been a review using standardized methods to determine the importance of all of the common pathogens. Important bacterial species include *Bacillus cereus*, *Clostridium perfringens*, *Campylobacter*, *Escherichia coli* (*ETEC*, *EPEC*, *STEC*), *Helicobacter*, *Listeria monocytogenes*, *Salmonella* spp. (excluding *S. typhi*), *Shigella*, *Yersinia enterocolitica*, and *Vibrio cholerae* O1 and O139. Viruses of the GI tract include norovirus, rotavirus, calicivirus, astrovirus, and small round viruses, and common parasites include *Cryptosporidium*, *Entamoeba histolytica*, and *Giardia lamblia*.

In this chapter bacterial toxins of GI significance will be discussed. The most common toxins include cholera toxin of *Vibrio cholerae*, Shiga-like toxin (SLT) produced by *Escherichia coli* (STEC), *Clostridium perfringens* enterotoxin (CPE), and hemolytic (HBL) and nonhemolytic (Nhe) enterotoxins of *Bacillus cereus*.

Bacterial Causes of Food Contamination, Mode of Action, Detection and Treatment Options

There are two forms of food contamination events, viz., food poisoning and foodborne infection. Food poisoning occurs after consumption of food containing toxins, which may be chemical like heavy metals or of bacterial origin like *Clostridium spp*., *Staphylococcus aureus*, and *Bacillus cereus*. The bacteria multiply and produce toxin within contaminated food. Heating may destroy the bacteria in food preparation events, but the toxin is unaffected and, when consumed, may cause an effect within hours. In foodborne infections, the food may simply act as a vehicle for the pathogen like *Salmonella* or *Campylobacter*.

The digestive tract is one of the ecosystems that harbor a large variety of bacteria. Among them, certain bacteria have developed various strategies, including the synthesis of virulence factors such as toxins, to interact with the intestinal mucosa, and are responsible for various pathologies. These toxins are of different sizes, structures, and modes of action and are able to interact with the gastrointestinal mucosa. Some toxins, termed enterotoxins, directly stimulate fluid secretion in enterocytes or cause their death, whereas other toxins pass through the intestinal barrier and disseminate by the general circulation to remote organs or tissues, where they are active. After recognition of a membrane receptor on target cells, toxins can act at the cell membrane by transducing a signal across the membrane in a hormonelike manner, by pore formation, or by damaging membrane compounds. Other toxins can enter the cells and modify an intracellular target leading to a dysregulation of certain physiological processes or disorganization of some structural architectures and cell death. Moreover, they are being used in therapeutics as protective antigens in vaccines, in immune modulation, or in the specific delivery of a protein of interest into target cells (Popoff 2011).

Gastrointestinal Symptoms

There are basic symptoms indicating a GI tract problem that include the following:

Nausea and vomiting can vary from an unsettled feeling in the stomach to the violent action of immediate vomiting. Patients with nausea and vomiting symptoms should assume the ingestion of a reactive food (i.e., food containing toxins) or poisoning with a pathogen.

Bloating can result from excessive gas in the digestive system, failure of the digestive tract to sustain youthful peristaltic contractions, or a lack of sufficient

quantities of digestive enzymes and bile acids to rapidly break down food. Intestinal gas results from food fermentation and swallowing air while eating.

The ability to identify contaminated food samples is of great importance to the food processing industry as well as to regulatory agencies. There is a need for accurate techniques to rapidly detect the presence of foodborne pathogens.

Diarrhea is the increased frequency of bowel movements, which are also loose or watery. Protracted bouts of diarrhea can result in nutritional deficiencies due to poor absorption of essential nutrients.

Abdominal pain appears in different patterns and with varying intensities. Cramping occurs because of muscle spasms in abdominal organs. Severe cramping pain, often called colic, usually occurs from problems with strong allergic response to food. Abdominal cramping near the navel is typically from the small intestine. Other symptoms such as fever and bloody stools may also be present.

It is generally impossible to distinguish in clinical symptoms the etiologic agent of diarrhea; nevertheless, patient recent history and laboratory investigation of samples can aid in precise diagnosis. This is important during outbreak investigations to instigate appropriate epidemiologic studies and control measures.

Bacterial Causes of Diarrhea

Escherichia coli (***E. coli***): One of the most versatile pathogens that can be part of the normal gut flora. However, some strains like enterotoxigenic *E. coli* (ETEC) that produces toxins, both heat-labile toxins (LTs) and heat-stable toxins (STs), and *E. coli* O157:H7 (Shiga toxin-producing *E. coli*, STEC) that produces Shiga-like toxins (STx 1 and STx2) have the ability to cause disease in humans through the presence of specific virulence factors. STx is alternatively known as verotoxin or VT, and the STEC as verotoxigenic *E. coli* or VTEC. Undercooked or raw ground beef has been implicated in many of the cases of human illness. The infective dose of *E. coli* O157:H7 may be as low as 10 cells. Diagnosis of an infection is typically done by isolating toxins from *E. coli* O157:H7 or by isolating the bacterium itself from human stool samples.

Symptoms can range from mild gastroenteritis to severe bloody diarrhea, mostly without fever (Table 1), through to two serious conditions known as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) that affect the blood, the kidneys, and in severe cases the central nervous system. The time from consumption of the STEC to diarrheal illness is about 3 days. Diarrhea may turn bloody after 1 or 2 days and, if the HUS develops, in 5–7 days. The rate at which the HUS develops during an outbreak varies widely from 0 % to 15 %, and death from STEC-mediated HUS occurs in approximately 5 % of patients, particularly in infants, young children, and the elderly.

Transmission occurs through consumption of contaminated food or water, from direct or indirect contact with animals that carry STEC. Infection is readily spread

Table 1 Clinical features of few bacterial diarrhea infections

Bacterial pathogen	Incubation period	Duration of illness	Symptoms			
			Diarrhea	Nausea, vomiting	Abdominal cramps	Fever
ETEC, *STEC*	1–2 days	2–3 days	Severe	No	Mild–moderate	Mild
Vibrio	8 h–2 days	3 days–1 week	Severe	Mild	Mild–moderate	Mild
Bacillus cereus	8–12 h	1–2 days	Mild–Moderate	Mild–Moderate	Moderate–Severe	No
Clostridium perfringens	8 h–1 day	1 day	Moderate	No	Moderate–Severe	No

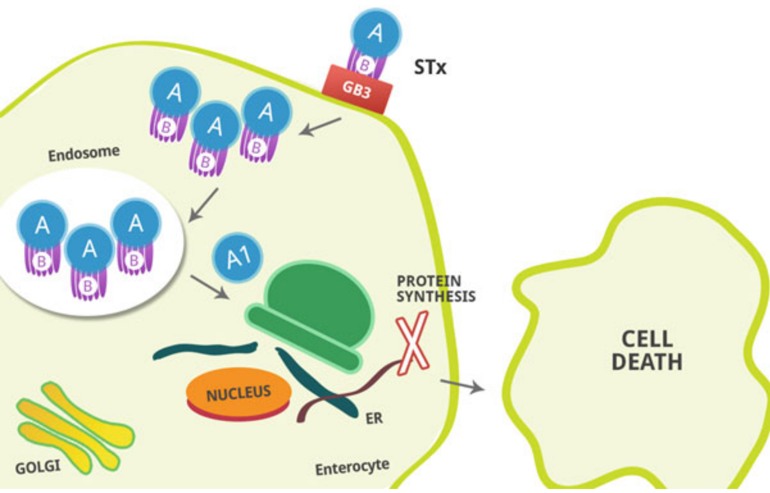

Fig. 1 Mechanism of action of *E. coli* Shiga-like toxin (*STx*). STx internalization by endocytosis and subsequent transport through the endoplasmic reticulum (*ER*) and inactivating with ribosome causing cell death. (*A & B* are Shiga toxin subunits, *GM1* GM1 ganglioside receptor, *A1* enzymatic fragment of A subunit, *Gb3* globotriaosylceramide receptor)

between family contacts, particularly those who may be caring for infected children, and in settings such as children's day-care centers.

Mechanism

All members of the STx protein family are structurally and functionally closely related. STx1 and STx2 are about 70 % similar at the amino acid level, have the same mode of action, and belong to the class of AB5 toxins. They consist of the A subunit (~32 kDa), which is cleaved by the mammalian, membrane-anchored protease furin to yield an enzymatically active fragment of ~27.5 kDa and non-covalently linked, five identical receptor binding B subunits (~7.5 kDa). The pentamer of identical B subunits from STx allows the toxin to bind to its receptor, the glycolipid globotriaosylceramide or Gb3 on the host cell (Fig. 1). Each STxB chain subunit can bind three molecules of Gb3.

Upon binding of toxin to its receptor on the surface, the toxin is internalized by receptor-mediated endocytosis and is transported to the Golgi and to the endoplasmic reticulum, from which the A subunit is translocated to the cytoplasm, where it can gain access to the ribosomal target. Dislocation across the endoplasmic reticular membrane has been mediated by binding to a chaperone recently identified to be clathrin-associated Hcs70 co-chaperone RME-8 that regulates endosomal trafficking of STx (Popoff et al. 2009). The A subunit of Stx gets activated by proteolytic cleavage into A1 and A2 products which remain held by a disulfide bond. The A1 chains dislocate from the ER, leaving the A2 portion associated with the pentameric B ring. The STxA1 acts to remove an adenosine residue (depurinates) from the 28S rRNA. The destruction of the 28S rRNA prevents further protein

Table 2 Toxins expressed among the different types of *Clostridium perfringens*

Type	Toxins			
	Alpha	Beta	Epsilon	Iota
A	Yes	No	No	No
B	Yes	Yes	Yes	No
C	Yes	Yes	No	No
D	Yes	No	Yes	No
E	Yes	No	No	Yes

synthesis in the host cell. Both epithelial and endothelial cells intoxicated with Stx may undergo an apoptotic cell death after intoxication (Tesh 2010).

Detection

The standard method of diagnosis includes enrichment of sample followed by polymerase chain reaction (PCR) or CHROMagar STEC screening test. Confirmation of pathogen may be done by immunomagnetic separation of cells and serogroup-specific antibodies. STx1 have high homology to Shiga toxin, and hence, STx2 can be used for the STEC detection from enriched samples using a variety of immunoassays including ELISA, immunochromatography test, flow cytometry, latex agglutination test, immune-PCR, and optical immunoassay (OIA). These laboratory methods can detect the STEC and SLTs ranging from 10,000 cells to 100,000 cells per gram of test matrices or SLTs in the range of 200 pg/mL–20 ng/mL of matrix (Hirvonen et al. 2012; Zhang et al. 2008). However, with recent molecular typing using real-time PCR and microarray, the sensitivity has greatly enhanced with a range of two colony-forming units 2 CFU–10 CFU per gram of sample (Koppel et al. 2013; Suo et al. 2013; Trevisani et al. 2013).

Clostridium perfringens

Clostridium perfringens are gram-positive organisms found in soil, sediments, and areas subject to human or animal fecal pollution. *C. perfringens* food poisoning is caused by ingestion of a large number of toxin-producing *C. perfringens* bacterial spores. Spores withstand cooking and can germinate upon favorable conditions and produce enterotoxins. *C. perfringens* pathogenesis occurs mainly through contaminated meat products and they commonly produce alpha toxin; rarely beta toxin-producing strains are associated with acute necrotizing disease of the small intestine presenting with abdominal cramps and diarrhea. The infective dose is greater than 10^8 vegetative cells. Diagnosis of *C. perfringens* poisoning is confirmed by detecting the toxin in the feces of patients.

Classification: *C. perfringens* have five subclasses A–E, based on the production of four major toxins, namely, alpha, beta, epsilon, and iota (Table 2). *C. perfringens* strains produce another important toxin, a 35 kDa protein known as *C. perfringens* enterotoxin (CPE), responsible for several human gastrointestinal diseases including type A food poisoning and many cases of antibiotic-associated diarrhea (AAD). CPE specifically interacts with different claudins (claudin-3 to claudin-9 and

claudin-14), which are a subclass of tight junction proteins implicated in epithelial barrier function (Mitchell and Koval 2010). Tight junctions consist of several proteins, including transmembrane proteins linked to the actin cytoskeleton by scaffold proteins. Of these transmembrane proteins, claudins are the primary structural determinants of paracellular permeability. Claudins are 20–27 kDa transmembrane proteins that span the membrane bilayer four times.

Mechanism of Action

After ingestion of contaminated food, *C. perfringens* passes from the stomach to the small intestine where it multiplies and sporulates. During sporulation, CPE is expressed and accumulates within the bacterium until it is discharged when the sporulating cells lyse. Upon release into the intestinal lumen, CPE binds to intestinal epithelial cells, typically to the claudin-3 or claudin-4, and initiates a cascade of events wherein six small complexes oligomerize to form hexameric complex that forms pores in the plasma membrane. These hexameric pores increase plasma membrane ion permeability allowing extracellular calcium influx that induces cell death by apoptosis or oncosis (Chakrabarti and McClane 2005). With very high CPE doses, where many pores form, the increase in cellular Ca^{2+} levels is so rapid and massive that oncosis develops due to strong calmodulin and calpain activation. With lower CPE doses, there is a slower and lesser (but still potent) increase in cellular calcium ion levels that cause a lesser activation of calmodulin and calpain. Activation of those cytosolic proteins leads to mitochondrial membrane depolarization inside the CPE-treated cell, which results in substantial cytochrome C release that triggers a rapid apoptotic response involving caspase 3/7 activation (Fig. 2). The resultant morphologic damage from CPE-induced oncosis or apoptosis exposes the basolateral membranes on the CPE-treated cell and adjacent cells present in the intestinal epithelium. This exposure allows unbound CPE access to additional CPE receptors on the basolateral membranes, from which the receptor-bound CPE can interact with occludin. Those interactions cause formation of additional ~155 kDa complex and initial formation of the ~200 kDa CPE complex, containing occludin. Formation of the ~200 kDa complex triggers internalization of tight junction proteins such as occludin, thereby damaging tight junction structure and function. That tight junction disruption produces paracellular permeability alterations which contribute to CPE-induced diarrhea during GI disease.

ELISA is one of the common methods to detect CPE that are mostly monoclonal antibody based. However, detection of CPE-positive and CPE-negative *C. perfringens* in foods and the environment can be achieved by molecular typing methods like PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP); these methods may generally require 1,000 bacterial cells per gram to detect them as positive.

Bacillus cereus

B. cereus are gram-positive organisms that can be found in the soil and in the water. They are mainly associated with rice dishes, occasionally pasta, meat, or vegetable dishes, dairy products, soups, sauces, and sweet pastry products (Reis et al. 2014).

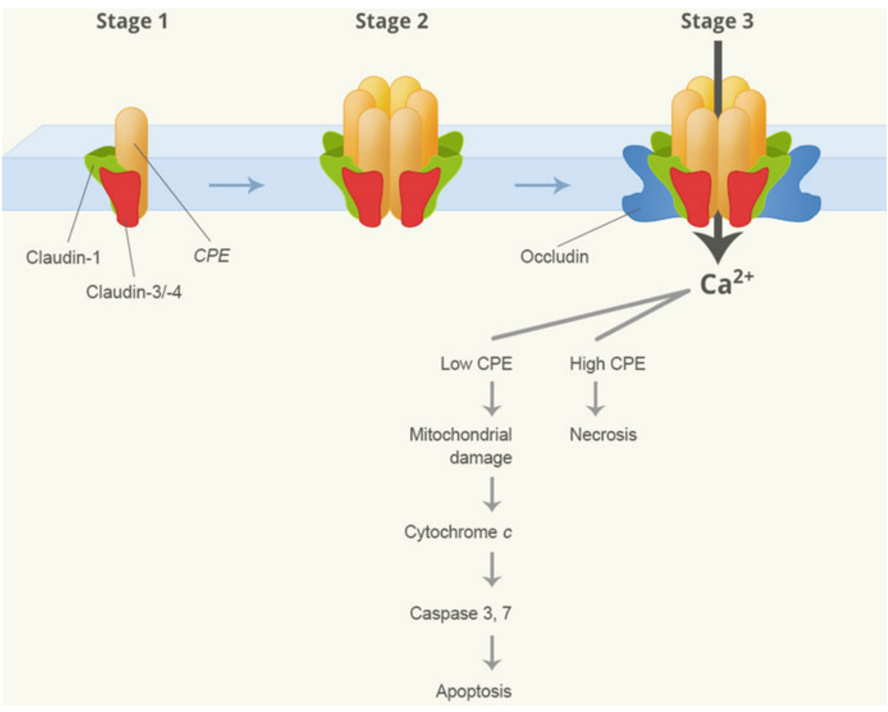

Fig. 2 A schematic of *Clostridium perfringens* enterotoxin (*CPE*) mode of action divided into three stages: (*1*) formation of CPE and claudin complex, (*2*) formation of CPE and claudin large complex, and (*3*) mature stage with binding of claudins and pore formation

Two different foodborne diseases are attributed to *B. cereus*: emetic syndrome and diarrheal syndrome. Both diseases have mild manifestation and, in most cases, are self-limited, although severe cases resulting in the death of the patient have been reported (Reis et al. 2013). They have been associated with food poisoning by ingestion of a large number of their vegetative forms, sporulated forms, or preformed toxins in contaminated food. Once the intestine is reached, they tend to colonize and produce enterotoxin during their growth, and these toxins are heat stable and adopt a similar mode of action to cholera. The mechanism of action of such toxins is not completely known, but it is believed that diarrhea is caused by the formation of pores in the cellular membrane, which induces the loss of Na^+ and Cl^- ions and water, resulting in an electrolyte imbalance (Bhunia 2008).

The toxins that are considered the main virulence factors of the diarrheal syndrome are hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K (CytK). In addition enterotoxin FM (EntFM), enterotoxin T (BceT), and hemolysin II (Hly II) were also described as potential diarrheal toxins (Kotiranta et al. 2000; Hendriksen et al. 2006). HBL is a three-component toxin consisting of two lytic proteins, L1 and L2, that are encoded by *hblD* and *hblC* genes, respectively, and a binding component B encoded by *hblA* gene. Each of the L1, L2, and

B proteins has a molecular weight of 40 kDa, and the corresponding genes are located on the same operon. The presence of all three components is necessary for the toxin activity (Lindback and Granum 2006; Beecher and Wong 2000). HBL has hemolytic as well as dermonecrotic and vascular permeability activities.

Nhe and Hbl are both tripartite pore-forming toxins that require the combined action of the three proteins NheA, NheB, and NheC or Hbl-B, Hbl-L_1, and Hbl-L_2, respectively. Sequential binding of multicomponent toxins has been suggested for Nhe toxin like that described for staphylococcal toxins (Lindback et al. 2010), although for the latter toxin the model proposed suggested that both NheB and NheC are capable of associating with the cell surface.

Detection

Various methods have been developed for detecting *B. cereus*, *B. cereus* spores, or *B. cereus* enterotoxins. A sensitive chemical assay is employed to detect cereulide based on high-performance liquid chromatography with ion trap mass spectrometry (LC-MS). DNA-based methods, such as polymerase chain reaction (PCR) and real-time PCR, have been developed for the detection of cereulide and enterotoxins. Furthermore, immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and colony immunoblot assay, have been developed to detect the diarrheal toxins. The use of immunomagnetic separation in assays is increasing because magnetic handling is quick, is efficient, and only gently affects the target analytes. The assay sensitivity was determined to be 10 CFU/mL (Chu et al. 2009). Recently, a number of molecular methods comprising multiplex PCR and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with rapid detection have been developed (Kim et al. 2012; Abdou et al. 2012; Tsilia et al. 2012).

Staphylococcal Enterotoxins

Staphylococcal enterotoxins (SEs) are the most widely studied of the toxic foodborne proteins. Although these toxins are produced by various strains of *Staphylococcus*, evidence has shown that they are primarily produced by the *Staphylococcus aureus* strain. *S. aureus* is a facultative anaerobic bacteria that expresses a wide array of virulence factors including enzymes, cytotoxins, exotoxins, and exfoliative toxins. Staphylococcal enterotoxins (SE) are the focus of this chapter. Currently there are nine SEs (A, B, C, D, E, G, H, I, J) that have been identified in a wide variety of food products: meat, poultry and egg products, milk and dairy products, and bakery products (Le Loir et al. 2003). These toxins also are highly heat stable, making them a potential health hazard when they are present. The infective dose of toxins is estimated to be 0.1 μg/kg body mass (Evenson et al. 1988). Detection of the presence of SEs is typically done through isolation in the suspected food source.

SEA and SEB are the most common types related to food poisoning. SEB in addition has been considered as a potential bioweapon. SEB is quite stable to heat,

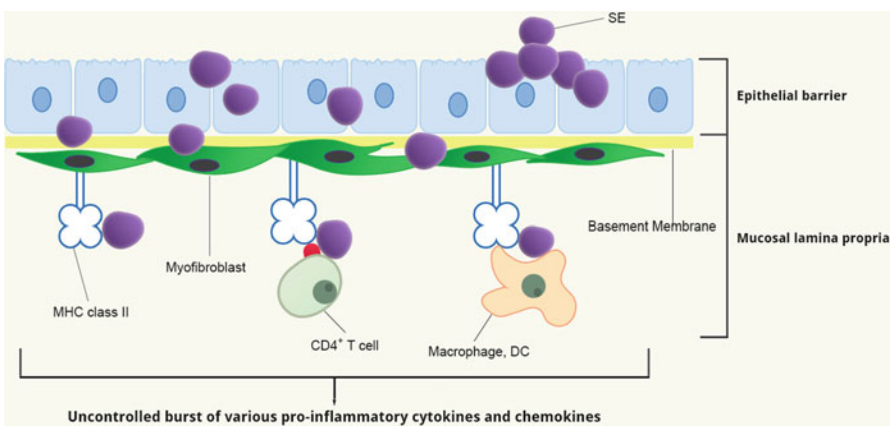

Fig. 3 Schematic representation of the SE-mediated gastrointestinal (GI) inflammatory injury. SE crosses the intact intestinal barrier and binds to class II MHC molecules expressed on the subepithelial myofibroblast cells. MHC class II–SE–TCR interactions may result in hyperactivation of the APCs and the T cells leading to the uncontrolled burst of proinflammatory cytokines and chemokines causing the superantigen-mediated acute inflammation and shock

proteolytic digestion, and a wide pH range, also making it easy to produce and distribute. A very small amount (0.004 μg/kg) is effective at inducing symptoms, and a dose of 0.02 μg/kg could be lethal. The fact that a low dose of SEB is sufficient to incapacitate people is another factor that makes it a potential weapon. Inhalation of SEB leads to shortness of breath and chest pain for several hours after exposure. With heavy exposure, more serious symptoms could occur such as high fever, pulmonary edema, possible acute respiratory distress syndrome, or septic shock.

SED positions itself as the second most common toxin associated with food poisoning. SEs are classified as superantigens and have the ability to stimulate large population of T-helper cells leading to massive cytokine response. Superantigens are bivalent molecules that have been shown to simultaneously bind to major histocompatibility complex (MHC) and the T-cell receptor (TCR) variable domains. Bacterial superantigens also known as pyrogenic toxins and include SEs A–E, and G–I mature SEs have a length of 220–240 amino acids with a molecular size of about 25 kDa. SEA and SEB exhibit binding activity of TCR. Zn binding sites in these SEs were found to facilitate binding to class II MHC molecules. MHCs are expressed by antigen-presenting cells (APC). The mode of action of the SEs is presented in Fig. 3. This MHC class II–SEs–TCR tri-molecular interaction leads to an uncontrolled release of various proinflammatory cytokines including IFN gamma, TNF-α, IL-1β, IL-6, and IL-8, the key cytokines/chemokines causing superantigen-mediated acute inflammation and shock. Whereas T cells are normally only activated in an antigenic-specific way, their interaction with SEs leads to a massive proliferation and differentiation of T cells mostly associated with acute inflammatory responses.

Detection

SEs are a leading cause of gastroenteritis (vomiting and diarrhea) resulting from consumption of contaminated food. The potency of SEs requires that methods of detection be sensitive enough to measure contamination at levels as low as nanograms per gram food. Immunological testing, especially enzyme-linked immunosorbent assay, has been the method of choice for SE detection because antibodies to the toxin are available. Of the ELISA-based methods proposed for the identification of staphylococcal enterotoxins, TECRASET-VIA (TECRA International Pty Ltd., NSW, Australia) polyvalent ELISA has been evaluated exhaustively and approved by AOAC International (Bennett and McClure 1994; Bennett 2005). And an improved enzyme-linked fluorescent assay has been developed with greater sensitivity 1 ng per gram (1 ng/g) within an assay time of 1.5 h.

However, fiber-optic biosensors and surface plasmon resonance sensors have been developed to analyze SEs in food with greater sensitivity (Rasooly 2001). These biosensors detected SEB at concentrations of ~5 ng/g in various foods using fluorescent-labeled antibodies. An evanescent wave biosensor was used to detect a similar toxin (SEA) in food at 10–100 ng/g. The various detection methods developed recently for the sensitive detection of SEs are presented in Table 3.

Cholera Toxin

Cholera is an acute infection of the GI tract caused by the comma-shaped gram-negative bacterium *Vibrio cholerae*. It has a long history of association with several pandemics and has two biotypes, viz., classical and El Tor. The symptoms of cholera are entirely caused by the production of an enterotoxin named cholera toxin (CT).

The clinical features of cholera are summarized in Table 1 and present with characteristic watery diarrhea termed as rice watery stools. This results in severe dehydration, metabolic acidosis (bicarbonate loss), hypokalemia (potassium loss), and hypovolemic shock.

The cholera toxin (CTx) secreted by *Vibrio cholerae* has been characterized and contains five binding (B) subunits of ~11.5 kDa, an active (A1) subunit of ~23.5 kDa, and a bridging piece (A2) of ~5.5 kDa that links A1 to the 5B subunits.

Mode of Action

Once entered the cell cytosol CTx targets heterotrimeric G proteins and induces production of cyclic AMP (cAMP). Buildup of this molecule leads to secretion of chloride ions into the intestinal lumen, which in turn prompts massive fluid loss in the form of secretory diarrhea.

Superficially similar in structure to STx is cholera toxin (CTx), produced by the bacterium *Vibrio cholerae*. It also has an activated A1 chain, disulfide linked to an A2 chain that remains tightly associated with a pentameric ring of B subunits. The CTxB subunits can each bind three molecules of its lipid receptor GM1 ganglioside on the surface of the cell. The toxin is composed of a 27 kDa monomer A subunit linked to the B domain by a trypsin-sensitive loop and a long a-helix, which inserts

Table 3 Recent diagnostic methods developed for the detection of SEs in various samples

Name of assay	Type	Assay time	Sample type	Detection limit (LOD)	References/ comments
Array-based detection of bacterial toxins	Immunofluorescent-based microarray analytics	<90 min	SEB in buffer or raw milk	0.5–1 ng/ml	Weingart et al. (2012)
Biosensor detection of SEB in food	Sandwich immunoassay	20 min	SEB-spiked food homogenate supernatants	0.1 ng/ml	Sapsford et al. (2005)
Electrical percolation-based biosensor for real-time direct detection of SEB	Single-walled carbon nanotubes (SWNTs)–Ab complex as BIO semiconductor (BSC)	10 s	Milk/food supernatants/ purified SEB in buffer	1–5 ng/ml	Bruck et al. (2013)
Handheld assay for rapid field testing of SEB in water	Singleplex (one-line) & Multiplex (2-line-2 dot) HHA models	15 min	SEB-spiked water samples	4.55 ng/ml	Wade et al. (2011)
Gold nanoparticle-based enhanced chemiluminescence (ECL) immunosensor for SEB detection	ECL immunosensor–sandwich ELISA type	10 min	SEB-spiked food samples	0.01 ng/ml	Yang et al. (2009)
Lateral flow immunoassay (LFA)	Double-Ab sandwich immunochromatographic method (strip assay)	<10 min	SEB-spiked food samples/culture supernatants	10 ng/ml	Chiao et al. (2013)
Electrochemical immunosensor for SEB detection	Magnetosome-based electrochemical immunosensor	<30 min	Spiked milk sample supernatants	0.017 ng/ml	Wu et al. (2013)
Magnetic immunoassay for detection of SEs in complex media	Sandwich-type immunoassay using magnetic nanoparticles	25 min	Neat milk samples	0.3 ng/ml SEA	Orlov et al. (2013)
MS-based SEB detection in aerosol	MALDI-TOF–TOF tandem MS	5 min	Peptide mixture after tryptic digestion of aqueous sample	3.9 fmol (833 ng/ml toxin)	Alam et al. (2012)

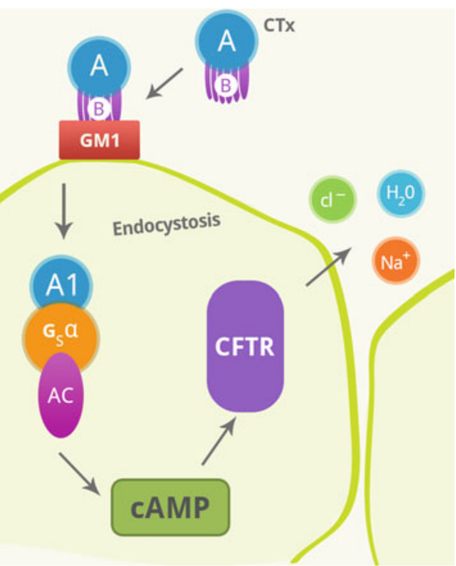

Fig. 4 Mechanism of action of cholera toxin (*CTx*). (*A & B* cholera toxin subunits, *GM1* GM1 ganglioside receptor, *A1* enzymatic fragment of A subunit, $G_s\alpha$ G protein, *AC* adenylate cyclase, *cAMP* cyclic adenosine monophosphate, *CFTR* cystic fibrosis transmembrane conductase regulator)

inside the core of the B pentamer, thus anchoring the two subunits. For full activity, the A subunit needs to be proteolytically cleaved and reduced at the disulfide bridge between cysteines 187 and 199 to give two fragments: the enzymatic subunit A1 and the linker fragment A2. Ubiquitins have been assumed to play a role in the dislocation of CTx across ER membrane.

To retain its activity, CTxA1 undergoes substrate-mediated folding in the cytosol including ADP-ribosylation factor 6 (ARF6) and heat shock protein (Hsp90). The enzymatically active domain A1 (an ADP-ribosyltransferase) binds NAD and transfers the ADP-ribose group to an Arg residue located within the central portion of several GTP-binding proteins such as G_s, G_t, and G_{olf}. Upon ADP-ribosylation of G_s, in particular, the adenylate cyclase is permanently activated, causing an abnormal intracellular cAMP accumulation in the cytosol. cAMP increase in epithelial cells sparks phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) and activation of chloride channels in the plasma membrane, among other events (Fig. 4; De Haan and Hirst 2004). This triggers high levels of chloride secretion and mucus shedding into the intestinal lumen; recent studies have provided evidence that *V. cholera* associates with these secreted clumps of mucin (Ritchie et al. 2010). To maintain osmotic balance of the luminal fluid, water is also released, and the overwhelming abundance of fluid produces the massive secretory diarrhea experienced by cholera patients (Field et al. 1989).

Treatment for Diarrhea

Accurate identification of the causative pathogen is necessary for determining a suitable treatment strategy, predicting the course of disease, prevention, and control

measures. Diarrhea can lead to severe dehydration with patients losing copious fluid (electrolytes and water) to the secretory diarrhea in hours, which is indicated by sunken eyes, loss of skin elasticity, and barely detectable blood pressure. Untreated patients can lose up to 10 % of their body weight within 24 h.

Administration of oral rehydration therapy (ORT) is quite effective to replace the massive loss of fluid by the patients. In severe cases, intravenous fluids and electrolyte replacement can be used to prevent or treat dehydration. In conjunction with hydration, treatment with broad spectrum antibiotics is recommended for severely ill patients for better management of diarrhea.

For cholera treatment, doxycycline is recommended as first line for adults, while azithromycin is recommended as first line for children and pregnant women. During an epidemic or outbreak, antibiotic susceptibility should be monitored through regular testing of sample isolates.

Currently there are two oral vaccines that are available in the market to prevent the cholera episodes. Dukoral consists of recombinant CTx B subunits and killed *V. cholerae* strains. It is relatively expensive and requires 2–3 doses for better protection. Another oral vaccine Shanchol recently approved by World Health Organization (WHO) lacks the recombinant CTxB subunit and is of cheaper cost than Dukoral. It is a killed whole cell formulation containing *V. cholerae* O1 and O139 serotypes (WHO 2010).

At present, there is no available therapeutic for treating staphylococcal exotoxin-induced shock except for the use of intravenous immunoglobulins; however, it has been shown that dexamethasone potently inhibits staphylococcal exotoxin-induced T-cell proliferation, cytokine release, and activation markers in human peripheral blood mononuclear cells (Krakauer and Buckley 2006). In very severe cases, respiratory support may be required.

Conclusion and Future Directions

In conclusion, thorough understanding of the epidemiology of food poisoning and foodborne illnesses is required in different geographical landscape. This could help in planning strategies to prevent and control the incidence. Moreover, the extreme complexity and mechanism of action of the various biotoxins of GID importance necessitate the rapid and accurate diagnosis for appropriate interventions. Not much commercial assays are available to detect the enterotoxins of GID relevance, and most of them are merely R&D tools and not suitable for point-of-care analysis. Focus should be more towards the development of on-site diagnostic kits with good performance for better management of outbreak situations.

For *Bacillus cereus* enterotoxins, the mechanism of action is poorly understood. A remarkable feature of *B. cereus* toxin Hbl is the requirement of three toxin subunits (B, L_1, L_2) for pore formation. Many bicomponent bacterial toxins have been described, such as the classic AB toxins that are composed of a cellular binding and an enzymatic effector subunit. Some bacteria also express bicomponent pore-forming toxins, such as staphylococcal leukocidins, that cause lysis of

cells of the leukocytic lineage. However, Hbl and the structurally similar Nhe toxin of *B. cereus* are the only known pore-forming toxins described to date that require the assembly of three subunits on the cellular membrane to induce lysis. Future research could be directed to have a clear understanding of the toxin pathogenesis and intracellular targets of *B. cereus* toxins for therapeutic interventions. Research is ongoing to explore and utilize *C. perfringens* toxins' properties in the treatment of targeted cancer therapy similar to the botulinum toxins that are currently used in practice for the treatment of a variety of neurological diseases.

Cross-References

► Aptamers as New Agents Against Biotoxins
► Biotoxins and Food Safety
► Immunosensors: Using Antibodies to Develop Biosensors for Detecting Pathogens and Their Toxins
► Structure, Genetics, and Mode of Disease of Cholera Toxin

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Section II

Risk Assessment of Biotoxins

The Biosecurity Threat Posed by Biological Toxins

8

Brenda A. Wilson and Mengfei Ho

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Abstract

Biological toxins are highly diverse and produced in nature by a wide variety of organisms. A number of key features favor the potential threat of these biotoxins as bioterror agents, including their high potency, the relatively long latency period before symptoms are manifested, the difficulty in detecting or diagnosing their presence and identity, and their relative ease in production and stability in the environment. All of these features also create major challenges in developing tools and reagents to combat toxin-mediated diseases. So far, there have been a limited number of toxins that have drawn attention for potential use as bioterror agents, but there are many more naturally occurring toxins that have been isolated, purified, and characterized, as well as cloned and modified to make different recombinant variants. Current treatments for toxin exposure are limited to vaccination or passive immunization with antibodies; however, there are no postexposure therapies available after symptoms have manifested. A vigilant and robust biotoxin research community must be mobilized to not only better characterize the existing biotoxins but also to anticipate new variants or entirely

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new biotoxins that might arise and to develop appropriate antitoxin countermeasures. In addition, a definitive roadmap must also be formulated for safe, documented, and controlled handling of biotoxins during basic research and during development of toxin-based therapeutics for biomedical applications.

Introduction

It can no longer be denied or ignored that harmful or lethal chemical and biological agents can be and have been used as implements of terror by states (larger-scale warfare programs conducted by a sovereign nation) or individuals (smaller-scale acts of terrorism by an individual or small group of individuals). Even a cursory scan of global news events over the past century, including more recent incidents such as the anthrax attacks of 2001, the Syrian sarin chemical weapons attack of 2013, and others reported on various news media, government websites, and medical bulletins, clearly points toward the reality that we are facing a real and serious threat to our biosecurity. Along with the heightened awareness of biosecurity threats through deliberate acts of terrorism since 2001, the threat from naturally occurring or accidental exposures is also increasing at an alarming rate (Wilson 2008; Morens and Fauci 2013). With this backdrop, toxic chemical and biological substances (a.k.a., poisons) emerge as key biothreat agents.

Chemical poisons have historically played important roles as warfare agents (Szinicz 2005; Ganesan et al. 2010), causing debilitating and often lethal injury upon contact, inhalation, injection, or ingestion. Chemical poisons are low-molecular-weight inorganic or synthetic organic compounds and are generally distinguished from biological toxins (or biotoxins), which are poisons that are derived or synthesized from biological sources. The US Code of Law defines a "toxin" as "the toxic material or product of plants, animals, microorganisms (including, but not limited to, bacteria, viruses, fungi, rickettsiae or protozoa), or infectious substances, or a recombinant or synthesized molecule, whatever their origin and method of production" (US Code of Law 2012). Small molecule, peptide, and protein biotoxins constitute key virulence determinants for a large number of pathogenic microorganisms (Alouf and Popoff 2006). Many protein toxins are enzymes that enter eukaryotic host cells and modulate cellular activities through selective action on their cognate intracellular target proteins or substrates.

Biological toxins include some of the most potent poisons known for humans and animals, yet their application as biowarfare agents so far has been limited to some extent by a number of practical and technical issues (Madsen 2001; Henghold 2004; Bigalke and Rummel 2005; Anderson 2012), which make conversion into a bioweapon of mass destruction problematic to implement. Layered onto this are some psychological and political–cultural aspects that appear to have terrorists opting for more immediate and spectacular demonstration of violence as terror agents, such as use of incendiary devices or bombs (Crenshaw 2011). Nevertheless, there are a few notable biotoxins that have been developed in the past as bioterror

agents (or bioweapons), most notably the botulinum neurotoxins (BoNTs), ricin, saxitoxin, and staphylococcal enterotoxin B (SEB) (Salem 2003; Szinicz 2005). Importantly, the formidable arsenal of biological toxins that modulate human/ animal–pathogen or predator–prey interactions has grown tremendously over the last few decades (Table 1). As can be seen clearly from this long list, the diversity of biotoxins that might be used as bioterror agents consequently has also expanded. Moreover, the potential for rapid discovery and development of toxic bioactive molecules has likewise increased exponentially with new advances in synthetic biology-related technologies, including metagenomics, functional genomics, recombinant molecular biotechnologies, protein engineering, as well as high-throughput combinatorial approaches for metabolite isolation, identification, purification and activity screening, and new formulation matrices and protocols. These enabling technologies lend special urgency to intensify our efforts toward understanding toxin-mediated disease processes and developing alternative antitoxin strategies and treatment modalities that are more universally applicable and more rapidly and effectively deployed.

Biotoxins as Agents of Bioterrorism

Toxins as Dual-Use Biological Agents

Many of the biotoxins listed in Table 1 possess potent, yet specific or selective cardiovascular, neurological or other cellular biological activities, which have proven invaluable for furthering our understanding of physiological and neurological processes. Although toxins have obvious harmful effects, their exquisite selectivity and high potency can also be exploited for beneficial purposes, including their use as research tools for cell biology, physiology, and pharmacology and as therapeutic tools for biomedical applications and cosmetics. Consequently, toxins are often considered to be dual-use biological agents (a.k.a., double-edged swords) for their beneficial potential when used in medicine or research and for their harmful potential when encountered in nature or when misused. For instance, some of these toxin activities have use for development of therapeutics against certain cardiovascular or neurodegenerative diseases (Joseph et al. 2004; Katona 2012; Aktories 2013). An array of venom toxins exhibit high selectively toward their cognate targets involved in hemostasis and thrombosis, properties that have made them extremely valuable for diagnostic and biomedical applications (McCleary and Kini 2013). A number of toxins, including diphtheria toxin, ricin, and *Pseudomonas* exotoxin A, are currently serving as the basis for potent anticancer treatments (Pastan et al. 2007). These highly effective recombinant immunotoxins are comprised of the cell-killing activity domain of the toxin fused or linked with an antibody fragment that targets specific surface antigens or receptors on the cancerous cell while leaving other healthy cells intact. The cell-killing domain is thereby selectively delivered into the cancer cell, where it triggers cell death.

Table 1 Selected biological toxins (biotoxins, venoms), their modes of action, and representative structures of toxins within each category

Name	Common source and structure	Mode of action	Bioterror agent?	References
Small molecule toxins				
Marine dinoflagellate-derived and bacterially derived toxins				
Brevetoxins (PbTx)	Cyclic polyketide-derived polyethers produced by marine plankton dinoflagellate protists (*Karenia brevis*, *Chattonella* sp.)	Paralytic neurotoxins that block voltage-gated Na^{2+} channels		(Blunden 2001)
Saxitoxins	Nonterpene alkaloid produced by marine dinoflagellate species (*Alexandrium, Gymnodinium, Pyrodinium*) and freshwater cyanobacteria (*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Planktothrix*)	Paralytic neurotoxins that block voltage-gated Na^{2+} channels	CDC Select Agent, small amount of saxitoxin stockpiled after WWII	(Blunden 2001; Szinicz 2005)
Saxitoxin (PST, TZ, paralytic shellfish toxin)				
Neosaxitoxin (NSTX)				
Gonyautoxin (GTX)				
Anatoxin (very fast death factor, ATX)				
Tetrodotoxins (TTX, pufferfish toxin, tarichatoxin, maculotoxin)	Guanidinium-containing 2,4-dioxaadamantine-like compound produced in a wide range of marine animals by symbiotic bacteria (*Vibrio*, *Pseudomonas*, *Bacillus*, *Alteromonas*, *Aeromonas*, *Micrococcus*, *Pseudoalteromonas*, *Serratia marcescens, Shewanella putrefaciens, Acinetobacter*, *Streptococcus*, *Caulobacter*, *Flavobacterium*, *Plesiomonas*, *Microbacterium arabinogalactanolyticum*, *Nocardiopsis dassonvillei, Actinomycetes*, *Marinomonas*, *Tenacibaculum*)	Paralytic neurotoxins that block voltage-gated Na^{2+} channels	CDC Select Agent	(Hwang and Noguchi 2007)

Mycolactones	Polyketide-derived lipid-like macrolide produced by mycobacteria (*Mycobacterium ulcerans, M. liflandii, M. pseudoshottsii, M. marinum*)	Causes Buruli ulcers by damaging skin, fat, and soft tissues and inhibiting immune responses		(Hong et al. 2008)
Endotoxins	Membrane components (LPS, lipopolysaccharide) of Gram-negative bacteria	Systemic inflammation, endotoxic shock		(De Castro et al. 2012)
Representative structures[a]				
Brevetoxin B ChemSpider ID: **9041149**		Saxitoxin ChemSpider ID: **34106**	Tetrodotoxin ChemSpider ID: **9349691**	
Mycolactone ChemSpider ID: **4445303**		Endotoxin ChemSpider ID: **23112074**		
Marine mollusk-derived toxins				
Onchidal	Sesquiterpene-acetate metabolite produced by marine mollusks (sea slugs, *Onchidella binneyi*, other *Onchidella* species)	Neurotoxin that acts as irreversible acetylcholinesterase inhibitor		(Abramson et al. 1989)

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Representative structures[a]				
Onchidal ChemSpider ID: **4945331**				
Amphibian-derived toxins				
Bufotoxins Bufotalin Bufogenine	Steroidal digoxin-like cardiac glycosides found in eggs and skin granular epidermal glands of psychoactive toad species (common European toad *Bufo bufo, Bufo melanostictus*)	Cardiotoxin, digitalis-like inhibition of myocardial Na^{2+}/K^{+} ATPase		(Shimada et al. 1985)
Bufotoxins Bufotenin (5-MeO-DMT, bufotenin) Bufotenin (5-HO-DMT)	Tryptamine-derived alkaloid compounds produced by wide variety of plant species (*Anadenanthera peregrina* seeds, *Diplopterys cabrerana* leaves) and skin parotid glands of psychoactive toad species (Colorado River toad *Bufo alvarius*)	Psychoactive, hallucinogenic, binds serotonin receptors, used as entheogen by South American shamans		(Shimada et al. 1985)
Batrachotoxins (BTX)	Steroidal alkaloids produced by certain Central and South American poison dart/arrow frogs (Dendrobates, *Phyllobates*)	Neurotoxin, activates Na^{2+} ion channels, digitalis (digitoxin)-like cardiotoxin, causes ventricular fibrillation and cardiac arrest		(Daly et al. 2005)
Samandarins Samandaridine Samandarin	Steroidal alkaloids produced by skin glands of the fire salamander (*Salamandra salamandra*)	Neurotoxic, cardiotoxin, causes muscle convulsions, high blood pressure, hyperventilation		(Daly et al. 2005)

Epibatidine Histrionicotoxins Pumiliotoxin 251D	Alkaloids produced by skin glands of certain Central and South American poison dart/arrow frogs (Ecuadorian phantasmal *Epipedobates tricolor*, harlequin *Oophaga histrionica*, *Dendrobates*, *Phyllobates*, *Minyobates*) and certain toad species (*Melanophryniscus*)	Cardiotoxins, analgesic effects through binding nicotinic acetylcholine receptors to cause release of dopamine and norepinephrine, paralytic effects through binding muscarinic acetylcholine receptors to block adenylyl cyclase		(Daly et al. 2005)
Representative structures[a]				
Batrachotoxin ChemSpider ID: **10310314**		Samandarin ChemSpider ID: **16736008**		
Epibatidine ChemSpider ID: **2332841**		Histrionicotoxin ChemSpider ID: **4941928**	Bufotoxin ChemSpider ID: **16735711**	

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Plant-derived toxins				
Strychnine Brucine	Terpene indole alkaloid produced in ripe seeds (Saint Ignatius' bean) of *Strychnos nux-vomica* or *S. ignatii* trees	Neurotoxin that blocks glycine and acetylcholine receptors to cause muscle convulsions, asphyxia	Rodent and small animal pest poison, cases of accidental or suicidal human poisoning	(Makarovsky et al. 2008)
Strychnine ChemSpider ID: **389877**		Brucine ChemSpider ID: **390579**		
Fungal-derived toxins (mycotoxins)				
Trichothecenes Type A T-2 Diacetoxyscirpenol Satratoxin-H Type B Nivalenol Vomitoxin (DON, deoxynivalenol) Fumonisins (B1, B2)	Epoxy-containing sesquiterpenoid mycotoxins produced by various fungal species (*Fusarium, Myrothecium, Trichoderma, Trichothecium, Cephalosporium, Verticimonosporium, Stachybotrys, Podostroma cornu-damae*)	Skin-penetrating inhibitors of protein synthesis via reaction with rRNA, skin, and mucosal irritants	T-2 and diacetoxyscirpenol are CDC Select Agents	(Bennett and Klich 2003; Paterson 2006)

Aflatoxins	Polyketide-derived mycotoxins produced by many fungal species of *Aspergillus* (notably *A. flavus*, *A. parasiticus*) that contaminate agricultural foodstuffs (mostly gains, seeds, nuts)	Metabolized by the liver to a reactive epoxide, which can intercalate into DNA, acute hepatic necrosis, cirrhosis, and/or liver cancer	Common food contaminant that is highly regulated by FDA	(Bennett and Klich 2003; Paterson 2006)
Aflatoxin B1, B1 metabolites M1, Q1				
Aflatoxin B2, B2 metabolite M2				
Aflatoxin G1, G2				
Aflatoxicol				
Sterigmatocystin (dermatoxin)				
Citrinin	Polyketide-derived mycotoxin produced by various fungal species (*Aspergillus*, *Monascus*, *Penicillium*)	Skin-penetrating nephrotoxin that damages mitochondria and inhibits respiration		(Bennett and Klich 2003; Paterson 2006)
Cytochalasins	Alkaloid mycotoxins produced by various fungal species (*Helminthosporium dematioideum*, *Phoma*, *Homiscium*, *Curvularia lunata*, *Aspergillus clavatus*)	Cell permeable, bind F-actin and blocks actin microfilament polymerization and cytoskeletal function, inhibits mitosis		(Bennett and Klich 2003; Paterson 2006)
Cytochalasin A-F, H, J				
Ergotamine	Ergot alkaloid produced by fungus *Claviceps purpurea*	Neuroactive vasoconstrictor, binds serotonin receptors		(Bennett and Klich 2003; Paterson 2006)
Gliotoxin	Sulfur-containing amino acid-derived mycotoxin produced by various fungal species (*Aspergillus fumigatus*, *Trichoderma*, *Penicillium*, *Candida*, *Gliocladium fimbriatum*)	Immunosuppressive, anti-inflammatory activity		(Bennett and Klich 2003; Paterson 2006)
Ibotenic acid	Unusual amino acid (precursor to muscimol) produced by mushrooms *Amanita muscaria* and *Amanita pantherina*	Neurotoxic agent, causes brain lesions		(Bennett and Klich 2003; Paterson 2006)

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Muscimol (agarin, pantherine)	Alkaloid produced by mushrooms *Amanita muscaria* and *Amanita pantherina*	Psychoactive neurotoxic agent, selective agonist for $GABA_A$ receptors, sedative–hypnotic effects		(Bennett and Klich 2003; Paterson 2006)
Ochratoxins (A–C)	Polyketide-derived mycotoxin produced by various fungal species (*Aspergillus ochraceus, Aspergillus niger, Penicillium verrucosum, Penicillium carbonarius*)	Neurotoxic, immunosuppressive due to immunotoxicity (immune cell death), carcinogenic by induction of oxidative DNA damage	Common food contaminant	(Bennett and Klich 2003; Paterson 2006)
Patulin	Polyketide-derived mycotoxin produced by various fungal species (*Aspergillus, Penicillium*)	Genotoxic, carcinogen	Common contaminant in apples	(Bennett and Klich 2003; Paterson 2006)
Zeranol (α-zearalanol) Zearalenone (ZEA, RAL, F-2 mycotoxin)	Polyketide-derived mycotoxin produced by various fungal species (*Fusarium, Gibberella*)	Skin-permeable estrogenic toxin, causes infertility, abortion, estrogenic growth promoter, mitogenic for breast cancer cells	Common feed and crop contaminant	(Bennett and Klich 2003; Paterson 2006)
Representative structures[a]				
Trichothecene ChemSpider ID: **20004967**		Satratoxin-H ChemSpider ID: **4515470**	Fumonisin B2 ChemSpider ID: **3314**	

Aflatoxin B1 ChemSpider ID: **13758**	Citrinin ChemSpider ID: **10222475**		Cytochalasin D ChemSpider ID: **23550627**	
Ibotenic acid ChemSpider ID: **1196**	α-Zearalanol ChemSpider ID: **4447689**	Patulin ChemSpider ID: **4534**	Muscimol ChemSpider ID: **4116**	
Gliotoxin ChemSpider ID: **5988**	Ergotamine ChemSpider ID: **7930**		Ochratoxin A ChemSpider ID: **390954**	
Natural peptide-based toxins				
Invertebrate-derived and insect-derived peptide toxins (venoms)				
Conotoxins α-Conotoxins δ-conotoxins κ-conotoxins μ-conotoxins ω-conotoxins	Disulfide bond-containing peptides (10–30 residues) in venom produced by marine mollusks cone snail species (*Conus*)	Neuroactive ion channel blockers α –Paralytic inhibitor of nicotinic acetylcholine receptors in nerves and muscles δ – Inhibits voltage-gated Na^{2+} channels κ – Inhibits K^{+} channels μ – Inhibits voltage-gated Na^{2+} channels in muscles	α-Conotoxin is a CDC Select Agent	(Olivera and Teichert 2007)

(*continued*)

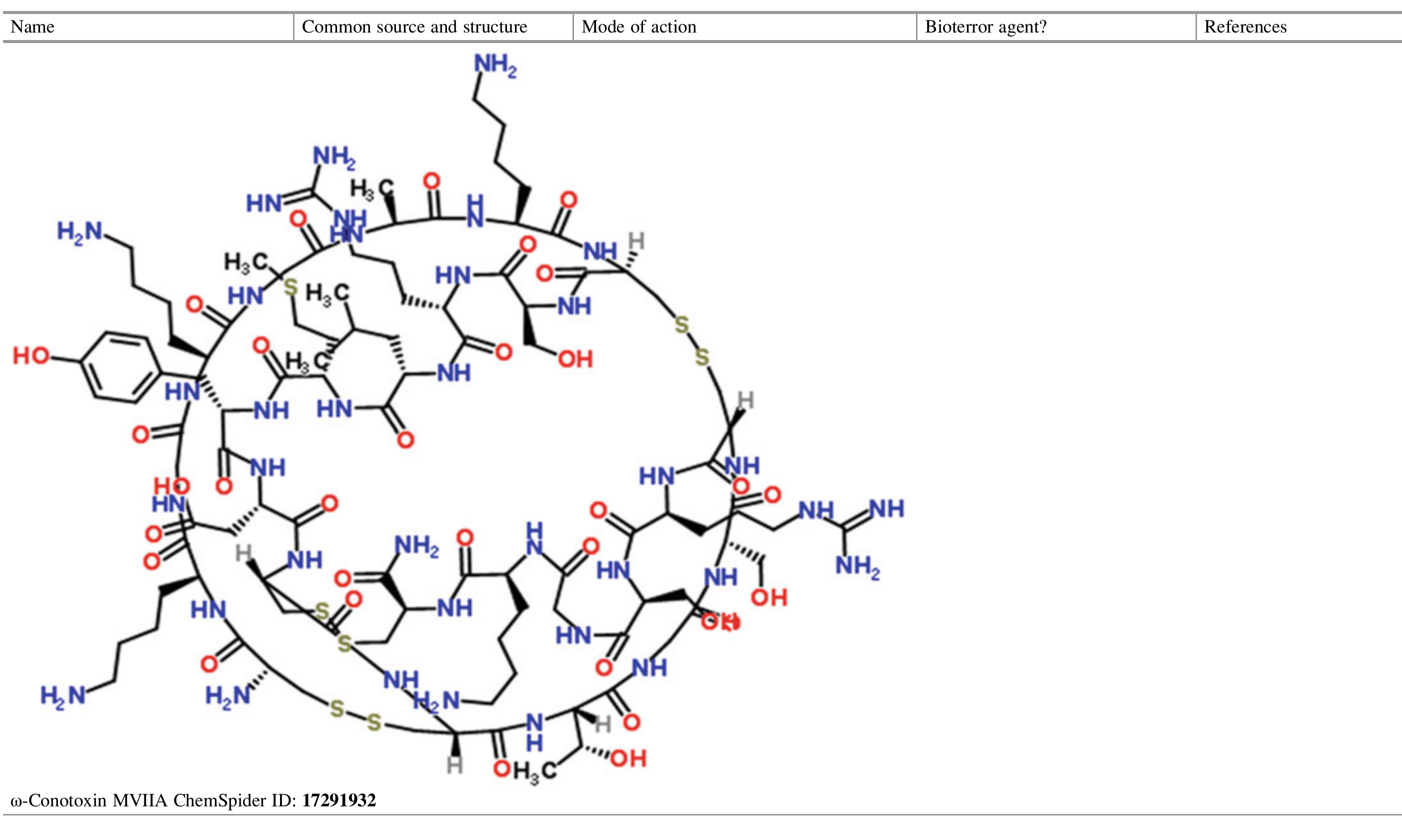
Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Conotoxins		ω – Inhibits N-type voltage-gated Ca^{2+} channels (analgesic pain reliever)	aa	
Con-G, Con-T, Con-R, Con-L, Con-Pr1/2/3, Con-P, Con-E, Con-R1-A, Con-Br		Conantokins – inhibit N-methyl-D-aspartate receptors, sedative		
Contryphans		Contryphans – inhibit Ca^{2+}-dependent K^+ channels or L-type Ca^{2+} channels		
Phoneutria nigriventer toxin-3 (PhTx3)	Peptide found in venom produced by Brazilian wandering spider (*Phoneutria nigriventer*)	Neuropeptide, broad-spectrum voltage-gated Ca^{2+} channel blocker that inhibits glutamate release, Ca^2 and glutamate uptake in synaptosomes		(Escoubas 2006)
Stromatoxin	Inhibitor-cysteine-knot motif-containing 34-mer peptide found in venom produced by African tarantula (feather leg baboon spider, *Stromatopelma calceatum*)	Neurotoxin, blocks delayed-rectifier and A-type voltage-gated K^+ channels		(Escoubas 2006)
Vanillotoxins (VaTx1, VaTx2, VaTx3)	Inhibitor-cysteine-knot motif-containing peptide found in venom produced by Trinidad tarantula (*Psalmopoeus cambridgei*)	Neurotoxin, agonist for V subfamily of transient receptor potential cation channels (pain) and antagonist for voltage-gated K^+ channels (paralysis)		(Escoubas 2006)
Huwentoxins (I, X, II–IV, VII, VIII)	Disulfide bond-containing 25–40-mer peptides found in venom produced by Chinese bird spider (*Ornithoctonus huwena*)	Neurotoxins, paralytic		(Escoubas 2006)
		I, X – inhibit N-type voltage-gated Ca^{2+} channels		
		II–IV, VII–VIII – inhibit voltage-gated Na^{2+} channels		
Charybdotoxins	Disulfide bond-containing (short-chain neurotoxin, SCN) peptides found in venom produced by various scorpions (CTX 37-mer, AgTX 38-mer, ScTX 31-mer, Lq1/Lq2 37-mer, deathstalker Israeli yellow	Neurotoxin, paralytic, block various Ca^2 $^+$-activated, voltage-gated, shaker, or inward-rectifier K^+ channels		(Escoubas 2006)
Charybdotoxin (CTX)				
Agitoxin (AgTX-1, AgTX-2, AgTX-3)				
Scyllatoxin (ScTX, leiurotoxin-I)				

Lq1, Lq2 Margatoxin (MgTX)	scorpion *Leiurus quinquestriatus hebraeus;* MgTX 39-mer, Central American bark scorpion *Centruroides margaritatus;* KTX 38-mer, Middle Eastern and North African black scorpion *Androctonus mauretanicus mauretanicus*; IbTX 37-mer, Eastern Indian red scorpion *Buthus tamulus;* SloTX 37-mer, Hoffmann scorpion *Centruroides noxius;* 34-mer, Tunisian chactoid scorpion *Scorpio maurus palmatus*; dimer of 22-mer and 23-mer, Asian forest black scorpion *Heterometrus fulvipes*)			
Kaliotoxin (KTX)				
Iberiotoxin (IbTX)				
Slotoxin (SloTX)				
Maurotoxin (MTX)				
κ – Hefutoxin (κHTX-1,2)				
Imperatoxin A (IpTXa)	Disulfide bond-containing SCN 33-mer peptide found in venom produced by African scorpion (*Pandinus imperator*)	Cytotoxin, binds to Ryanodine receptors to stimulate Ca^{2+} release from sarcoplasmic reticulum into cytosol, induces muscle contraction		(Escoubas 2006)
Birtoxins	Disulfide bond-containing (long-chain neurotoxins, LCNs) peptides found in venom produced by scorpions (birtoxins ~58-mer South African spitting scorpion *Parabuthus transvaalicus;* BmKAEP 61-mer – Manchurian scorpion *Mesobuthus martensii*)	Neurotoxin, convulsive, tremors, tachypnea (fast breathing), depression, paralytic, blocks voltage-gated Na^{2+} channels		(Escoubas 2006)
Birtoxin				
Bestoxin				
Ikitoxin				
Dortoxin				
Altitoxin				
BmKAEP (*Buthus martensi* Karsch anti-epilepsy peptide)				
Representative structures[a]				

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
 ω-Conotoxin MVIIA ChemSpider ID: **17291932**				

Phoneutria nigriventer toxin-3-4 (PnTx3-4)	ACIPRGEICTDDCECCGCDNQCYCPPGSSLGIFKCSCAHANKYFCNRKKEKCKKA			
Stromatoxin	DCTRMFGACRRDSDCCPHLGCKPTSKYCAWDGTI			
Vanillotoxin	DCAKEGEVCSWGKKCCDLDNFYCPMEFIPHCKKYKPYVPVTTNCAKEGEVCGWGSKCCHGLDCPLAFIPYCEKYRGRND			
Huwentoxin	ACKGVFDACTPGKNECCPNRVCSDKHKWCKWKL			
Charybdotoxin	QFTNVSCTTSKECWSVCQRLHNTSRGKCMNKKCRCYS			
Imperatoxin	TMWGTKWCGSGNEATDISELGYWSNLDSCCRTHDHCDNIPSGQTKYGLTNEGKYTMMNCKCETAFEQCLRNVTGGME7GPAAGFVRK TYFDLYGNGCYNVQCPSQ			
Birtoxin	ADVPGNYPLDKDGNTYKCFLLGGNEECLNVCKLHGVQYGYCYASKCWCEYLEDDKDSV			
Vertebrate-derived peptide toxins (venoms)				
Bungarotoxins α-Bungarotoxin (α-BnTX) γ-Bungarotoxin (γ-BnTX)	Disulfide bond-containing (LCN: α-BnTX 74-mer; γ-BnTX 68-mer) peptide found in venom produced by elapid snakes (Taiwanese banded kraits, *Bungarus multicinctus*)	Neurotoxin, paralytic, irreversible, competitive inhibitor of postsynaptic nicotinic acetylcholine receptors of skeletal muscles and brain		(Mackessy 2010)
Calceptines (CaS, FS2) Calcicludine (CaC)	Disulfide bond-containing SCNs 60-mer peptides found in venom produced by mamba snakes (CaS, black mamba *Dendroaspis polylepis*; CaC, green mamba, *Dendroaspis angusticeps*)	Neurotoxin, block L-, N-, and P-type high-voltage-gated Ca^{2+} channels		(Mackessy 2010)
Dendrotoxins (α-DTX, δ-DTX, DTX-I, DTX-J, DTX-K, BPTI)	Disulfide bond-containing SCNs 57–60-mer peptides found in venom produced by various mamba snakes (*Dendroaspis*)	Neurotoxin, block voltage-gated K^+ channels in neurons to release acetylcholine, results in hyperexcitability, convulsive		(Mackessy 2010)
Cardiotoxins (CTX I–V)	Disulfide bond-containing SCNs 60-mer peptide found in venom produced by Taiwan cobra snakes (*Naja naja atra*)	Neurotoxin, lethal cytotoxin, induces apoptosis via release of cytochrome C		(Mackessy 2010)
Representative structures[a]				
α-Bungarotoxin	RTCHISTSSTPQTCPKG QDICFRKTQCDKFCSIRGAVIEQGCVATCPEFRSNYRSLLCCRTDNCNP			
Calcicludine	WQPPWYCKEPVRIGSCKKQFSSFYFKWTAKKCLPFLFSGCGGNANRFQTIGECRKKCLGK			

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Dendrotoxin	VCRDWFKETACRHAKSLGNCRTSQKYRANCAKTCELC			
Cardiotoxin	LKCNKLVPLFYKTCPAGKNLCYKMFMVATPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN			
Fungal-derived peptide toxins				
α-Amanitin	Cyclic 8-mer peptide produced by several mushroom species (*Amanita phalloides*, *Amanita virosa*, *Amanita bisporigera*, *Galerina marginata*, *Conocybe filaris*)	Lethal cytotoxin, inhibits DNA-dependent RNA polymerase II, kills liver and kidney cells, death due to multiorgan failure		(Bennett and Klich 2003; Paterson 2006)
Phallotoxins	Bicyclic 7-mer peptides produced by death cap mushroom (*Amanita phalloides*) and Blusher mushroom (*Amanita rubescens*)	Hepatotoxin, binds filamentous F-actin and blocks actin depolymerization		(Hallen et al. 2007)
Phalloidin				
Phallacidin				
Phallacin				
Phallisacin				
Phalloin				
Prophalloin				
Representative structures[a]				
α-Amanitin 3	MSDINATRLPIWGIGCNPCVGDDVTSVLTRGEA			
Phalloidin	MSDINATRLPAWLATCPCAGDDVNPLLTRGE			
Bacterial-derived peptide toxins				
Superantigens	22–29-kDa peptides produced by toxinogenic strains of Gram-positive bacterial species (*Staphylococcus aureus*, group A *Streptococcus pyogenes*, *Streptococcus*, *Streptococcus equi*, *Streptococcus dysgalactiae*)	Directly bind to MHC-II molecule on antigen-presenting cells and form complex (with or without peptide antigen) with T-cell receptor on T cells, triggering excessive T-cell activation and cytokine/chemokine release, resulting in toxic shock	Staphylococcal enterotoxins A–E are CDC Select Agents	(Salem 2003; Szinicz 2005; Alouf and Popoff 2006)
Staphylococcal				
enterotoxins (SEA, SEB, SEC1–SEC3, SED, SEE, SEG-SER, SEU)				
Toxic shock syndrome toxin (TSST-1)				
Streptococcal				
enterotoxins				
(SpeA, SpeC, SpeG-M, Ssa, SmeZ1–Z2, SPE-I, SPE-L, SPE-M, SPE-G, SDM)				

Microcystins	Cyclic nonribosomal peptides produced by cyanobacterial species (*Microcystis aeruginosa, Nodularia spumigena, Planktothrix*)	Hepatotoxicity, bind and inhibit protein phosphatases, damage liver		(Dittmann et al. 2013)
Nodularin-R				
Cyanotoxin				
Representative structures[a]				
Superantigens				
Staphylococcal enterotoxin A (SEA)	SEA PDB: 1SXT	TSST-1 PDB ID: 2QIL	SpeC PDB ID: 1AN8	
Toxic shock syndrome toxin (TSST-1)				
Streptococcal enterotoxin (SpeC)				
Microcystins Nodularin	Nodularin ChemSpider ID10471625			
Natural protein toxins				
Invertebrate-derived protein toxins (venoms)				
Latrotoxins	130-kDa dimeric or tetrameric protein found in venom produced by widow spiders (*Latrodectus*)	Neurotoxin, acts presynaptically to release neurotransmitters (acetylcholine)		(Sudhof 2001)
Alpha-latrotoxin				
α, β, γ, δ, ε – vertebrate specific				

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Phospholipase A2 toxins	Dipartite or tripartite peptide complexes – larger ~15-kDa catalytic subunit with Ca^{2+}-activated phospholipase A2 activity, 1–2 other smaller peptides	Neurotoxin, hydrolyses membranes of nerves and muscles		(Alouf and Popoff 2006; Mackessy 2010)
Imperatoxin I (IpTXi)				
β -Bungarotoxin (β-BnTX)				
Taicatoxin (TCX)	Found in venom produced by:			
	IpTXi – African scorpion (*Pandinus imperator*)			
	β–BnTX– Taiwanese banded kraits (*Bungarus multicinctus*)			
	Taicatoxin – Australian taipan snake (*Oxyuranus scutellatus scutellatus*)			
Representative structures[a]				
Phospholipase A2 toxins	Imperatoxin PDB ID: 1IE6	β-Bungarotoxin PDB ID: 1BUN		
Imperatoxin				
β2-Bungarotoxin				

Plant-derived protein toxins				
Type 2 ribosome-inactivating proteins (RIP)	66-kDa AB-type modular proteins produced as single-chain precursor protein	Cytotoxin, binds cell surface galactose-containing glycolipids and enters mammalian cells, where its catalyzes depurination of residue A4324 of 28S rRNA to inactivate ribosomes, blocks protein synthesis and kills cells	CDC Select Agents, ricin developed and field tested, WWI, WWII and after	(Szinicz 2005; Alouf and Popoff 2006; Schep et al. 2009)
Ricin	Modules:			
Abrin (variants a–d)	Carbohydrate-binding lectin domain			
	Adenenine-specific rRNA N-glycosylase catalytic domain			
	Found in seeds of plants:			
	Ricin – the flowering castor oil plant (*Ricinus communis*)			
	Abrin – the Indonesian legume ornamental rosary pea (jequirity bean, *Abrus precatorius*)			
Representative structures[a]				
Type 2 ribosome-inactivating protein toxins	Ricin PDB ID: 2AAI			
Ricin				

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Bacterial-derived protein toxins				
β-Pore-forming toxins				
Cholesterol-dependent cytolysins	Small β-barrel protein produced and secreted as monomer by:	Hemolytic, form pores in cell membranes, cause cell lysis (hemolysis)		(Alouf and Popoff 2006)
Listeriolysin O (LLO)	59-kDa LLO – *Listeria monocytogenes*			
Ivanolysin (ILO)	59-kDa ILO – *Listeria Ivanolysin*			
Pneumolysin (PLY)	53-kDa PLY – *Streptococcus pneumonia*			
Streptolysin O (SLO)	64-kDa SLO – *Streptococcus pyogenes*			
Streptolysin S (SLS)	53-kDa SLS – *Streptococcus pyogenes*			
Intermedilysin (ILY)	53-kDa ILY – *Streptococcus pyogenes*			
Vaginolysin (VLY)	57-kDa VLY – *Gardnerella vaginalis*			
Anthrolysin (ALO)	57-kDa ALO – *Bacillus anthracis*			
	58-kDa BLY – *Clostridium botulinum*			
Botulinolysin (BLY)	59-kDa TLY – *Clostridium tetani*			
Tetanolysin (TLY)	56-kDa PLO – *Clostridium perfringens*			
Perfringolysin O (PLO)	64-kDa ALN – *Arcanobacterium pyogenes*			
Arcanolysin (ALN; pyolysin, PLO)				

Aerolysin-like, cholesterol-independent cytolysins	Small β – barrel protein produced and secreted as monomer by:	Hemolytic, form pores in cell membranes, cause cell lysis (hemolysis)		(Alouf and Popoff 2006)
Aerolysin (ALY)	52-kDa ALY – *Aeromonas hydrophila*			
Epsilon toxin (ETX)	32.5-kDa ETX – *Clostridium perfringens*			
Septicum alpha-toxin (Csa)	47.5-kDa SaT – *Clostridium septicum*			
Staphylococcal	Small β-barrel protein produced and secreted by *Staphylococcus aureus* as 33-kDa monomer that oligomerizes as a heptamer on cell surfaces	Hemolytic, forms pores in erythrocyte membranes, causes red blood cell lysis (hemolysis), skin dermonecrosis		(Alouf and Popoff 2006)
α-Hemolysin (Hla, α-toxin)				
Staphylococcal Panton-Valentine leukocidin (PVL)	Bipartite protein (33-kDa LukS-PV, 34-kDa LukF-PV) cytotoxin produced and secreted by *Staphylococcus aureus*	Hemolytic, forms pores in membranes, lyses leukocytes		(Alouf and Popoff 2006)
Representative structures[a]				
Cholesterol-dependent cytolysins	Streptolysin O PDB ID: 4HSC		Perfringolysin O PDB ID: 1PFO	
Streptolysin O				
Perfringolysin O				
Aerolysin-like, cholesterol-independent cytolysins	Proaerolysin PDB ID: 1PRE		Epsilon toxin PDB ID: 1UYJ	
Proaerolysin				
Epsilon toxin				

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Staphylococcal Panton-Valentine Leukocidin (PVL)	PVL S component PDB ID: 1T5R		PVL F component PDB ID: 1PVL	
Staphylococcal α-hemolysin	α-Hemolysin PDB ID: 3ANZ			
Repeat-in-toxin (RTX) modular pore-forming toxins				
Adenylyl cyclase RTX toxin (CyaA, adenylate cyclase toxin)	220-kDa RTX-type modular protein produced as single-chain polypeptide	Cytotoxin, binds integrin receptors, penetrates cells, converts ATP to cAMP to activate cAMP-dependent signaling pathways that suppress antibacterial responses		(Alouf and Popoff 2006)
	Modules:			
	Ca^{2+}/calmodulin-dependent adenylyl cyclase domain			
	Repeat-in-toxin (RTX) receptor-binding and membrane-penetrating domain			
	Secreted by *Bordetella* species			

MARTX toxins	Multifunctional autoprocessing repeat-in-toxin (MARTX)-type proteins produced as single-chain polypeptide with multiple different modules	Cytotoxin, binds receptors, penetrates cells, cross-links actin, induces apoptosis, modulates cell signaling and cytoskeletal function		(Satchell 2011)
$MARTX_{Vv}$	Modules:			
$MARTX_{Vc}$	Repeat-in-toxin (RTX) domain			
RtxA	Actin-cross-linking domain (ACD)			
	Rho-inactivation domain (RID)			
	Cysteine protease domain (CPD)			
	Membrane-localization domain (MLD)			
	Domains of unknown function (DUFs)			
	Produced/secreted by:			
	556-kDa $MARTX_{Vv}$ – *Vibrio vulnificus*			
	485-kDa $MARTX_{Vc}$ – *Vibrio cholera*			
	421-kDa RtxA – *Aeromonas hydrophila*			
	200–900-kDa MARTX toxins – *Vibrio splendidus, Pseudomonas luminescens, Yersinia enterocolitica, Xenorhabdus bovienii, Xenorhabdus nematophila*			

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Vacuolating toxin (VacA)	139-kDa protein produced as single-chain autotransporter precursor polypeptide that is processed to 88-kDa mature secreted modular protein	Cytotoxin, binds phospholipid and protein receptors, enters cytosol, induces vesicle vacuolation, forms Cl^- channels in mitochondrial membranes, resulting in loss of membrane potential, mitochondrial fragmentation, induction of reactive oxygen species, apoptosis, autophagy, cell death, risk factor for gastric cancer		(Alouf and Popoff 2006; Kim and Blanke 2012)
	Modules:			
	Autotransporter domain			
	Receptor-binding domain			
	Membrane-translocation domain			
	Cl^- channel-forming domain			
	Secreted by *Helicobacter pylori*			
Representative structures[a]				
Repeat-in-toxin (RTX) toxins				
Bordetella pertussis adenylyl cyclase RTX toxin	*Bordetella* AC toxin catalytic domain with C-terminus of calmodulin PDB ID: 1YRT		Pre-cleavage form of cysteine protease domain of *Vibrio cholera* RTXA toxin PDB ID: 3FZY	
Vibrio cholerae MARTX Toxin				
Helicobacter pylori vacuolating toxin (VacA)	VacA p55 domain PDB ID: 2QV3			

AB-type modular single-chain protein toxins				
Clostridial neurotoxins	AB-type modular proteins produced as single-chain polypeptide with multiple modules	Paralytic neurotoxin, binds to ganglioside and protein receptors on presynaptic motor neurons, enters cytosol and cleaves SNARE-motif-containing membrane-fusion proteins (SNAP-25, syntaxin, synaptobrevin), blocks acetylcholine neurotransmitter release to cause paralysis (BoNT – flaccid, TeNT – spastic)	BoNTs are CDC Tier 1 Select Agents, field tested and stockpiled after WWII, increasingly used for clinical applications	(Arnon et al. 2001; Salem 2003; Szinicz 2005; Alouf and Popoff 2006; Katona 2012; Aktories 2013; Barash and Arnon 2013; Dover et al. 2013; Popoff 2013; Relman 2013)
Botulinum neurotoxins serotypes A-H (BoNT/A-H)	Modules:			
	Receptor-binding domain			
	Membrane-translocation domain			
Tetanus neurotoxin (TeNT)	Zn^{2+}-dependent metalloprotease catalytic domain			
	Secreted by:			
	150-kDa BoNTs – *Clostridium botulinum* and certain other clostridial species (*C. butyricum*, *C. baratii*, *C. argentinense*)			
	150-kDa TeNT – *Clostridium tetani*			
ADP-ribosylating toxins	AB-type modular proteins produced as single-chain polypeptide with multiple modules	Cytotoxin, binds receptors on cells, enters cytosol and ADP-ribosylates diphthamide reside of eukaryotic elongation factor-2, blocks protein synthesis, kills cells		(Alouf and Popoff 2006)
Diphtheria toxin (DT)				
Pseudomonas exotoxin A (ETA)	Modules:			
	Receptor-binding domain			
	Membrane-translocation domain			
	NAD^{+}-dependent ADP-ribosyltransferase domain			
	Secreted by:			
	58-kDa DT – *Corynebacterium diphtheriae*			
	67-kDa ETA – *Pseudomonas aeruginosa*			

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
G-protein-deamidating toxins (dermonecrotic toxins)	AB-type modular proteins produced as single-chain polypeptide with multiple modules	Cytotoxin, binds receptors on cells, enters cytosol, and deamidates an active site glutamine residue of small Rho GTPases (CNFs, DNT) or GTPase subunit of heterotrimeric G proteins (PMT) to activate cytoskeletal, calcium and/or mitogenic signaling pathways		(Alouf and Popoff 2006; Wilson and Ho 2010)
Pasteurella multocida toxin (PMT, osteolytic toxin)	Modules:			
	Receptor-binding domain			
	Membrane-translocation domain			
	Glutamine-specific G-protein-deamidase catalytic domain			
Cytotoxic necrotizing factors (CNF1, CNF2, CNF3, CNFy)	Produced by:			
	143-kDa PMT – *Pasteurella multocida*			
	115-kDa CNF1-CNF3 – pathogenic *Escherichia coli*, CNFy – *Yersinia pseudotuberculosis*			
Bordetella dermonecrotic toxin (DNT)	160-kDa DNT – *Bordetella* species			
Burkholderia lethal factor (Blf1)	23-kDa Blf1 effector protein of *Burkholderia pseudomallei*			

G-protein glycosylating toxins	AB-type modular proteins produced as single polypeptide with multiple modules	Cytotoxin, binds receptors on cells, enters cytosol, and glycosylates an active site threonine residue of small Rho GTPases to inactivate cytoskeletal signaling pathways		(Alouf and Popoff 2006)
Clostridial toxins	Modules:			
Toxin A (TcdA)	Receptor-binding domain			
Toxin B (TcdB)	Membrane-translocation domain			
TpeL	UDP-glycoside-dependent glycosyltransferase catalytic domain			
α-Toxin (TcnA)	$InsP_6$-dependent cysteine protease domain			
Lethal Toxin (TcsL)	Secreted by:			
	308-kDa TcdA, 269-kDa TcdB – *Clostridium difficile*			
	191-kDa TpeL – *Clostridium perfringens*			
	250-kDa TcnA – *Clostridium novyi*			
	270-kDa TcsL – *Clostridium sordellii*			
Representative structures[a]				

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Clostridial neurotoxins Botulinum neurotoxin serotype A	BoNT/A PDB ID: 3BTA			
ADP-ribosylating toxins Diphtheria toxin (DT) *Pseudomonas* exotoxin A (ExoA)	DT PDB ID: 1TOX		ExoA PDB ID: 1IKQ	

G-protein-deamidating toxins (dermonecrotic toxins) *Pasteurella multocida* Toxin (PMT) *E. coli* cytotoxic necrotizing factor 1 (CNF1) *Burkholderia* Lethal Factor (Blf1)	Intracellular activity domains PMT-C1-C3 PDB ID: 2EBF	Catalytic domain CNF1 PDB ID: 1HQ0		Blf1 PDB ID: 3TU8
G-protein glycosylating toxins Clostridial toxins Toxin A α-Toxin Lethal toxin	*Clostridium difficile* CdtA PDB ID: 2WN8	*Clostridium sordellii* lethal toxin with UDP-Glc PDB ID: 2VKH	*Clostridium novyi* α-toxin PDB ID: 2VK9	

(*continued*)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
AB-type multi-subunit-complex protein toxins				
ADP-ribosylating toxins Cholera toxin (CT, CTX, CTx) Heat-labile toxin (HLT, LT, HLTx) Pertussis toxin (PT, PTx)	AB-type modular proteins produced as separate subunits that form an AB_5 complex	Cytotoxin, binds ganglioside receptors, enters cells, ADP-ribosylates and activates target GTPase (CT, HLT, Gs α subunit; PT, Gi α-subunit), activates cellular adenylate cyclase, converts ATP to cAMP, stimulates cAMP-dependent signaling, in gut CT and HLT cause massive diarrhea		(Alouf and Popoff 2006)
	Subunits:			
	28-kDa CT, HLT, 26.2-kDa PT S1 catalytic A subunit – NAD^+-dependent ADP-ribosyltransferase			
	11.6-kDa CT B pentamer, PT pentamer (21.9-kDa S2, 21.8-kDa S3, 2 x 12.1-kDa S4, 10.9-kDa S5) – ganglioside-receptor-binding and membrane-translocation subunits			
	Secreted by:			
	CT – *Vibrio cholera*			
	HT – pathogenic *Escherichia coli* (ETEC)			
	PT – *Bordetella pertussis*			

Ribosome-inactivating toxins	AB-type modular proteins produced as separate subunits that form an AB_5 complex	Cytotoxin, binds ganglioside receptors, enters cytosol, depurinates residue A4324 of 28S rRNA to inactivate ribosomes, blocks protein synthesis and kills cells		(Alouf and Popoff 2006)
Shiga toxins (Stx)	Subunits:			
Shiga-like toxins (SLT1 – Stx1, SLT2 – Stx2)	32-kDa catalytic A subunit – adenenine-specific rRNA N-glycosylase			
	7.7-kDa B pentamer – ganglioside-receptor-binding and membrane-translocation subunit			
	Secreted by:			
	Stx – *Shigella dysenteriae*			
	Stx1, Stx2 – pathogenic *Escherichia coli* (STEC, EHEC)			
Anthrax toxins (AnTx)	AB-type modular proteins produced as separate subunits (B component as 83-kDa precursor that is proteolytically processed to remove ~20-kDa peptide) that form an A_3B_7 or A_3B_8 complex	Cytotoxins, PA binds protein receptors, EF and LF translocate into cytosol, LF cleaves MAPK kinases to disrupt mitogenic signaling, EF converts ATP to cAMP and induces cAMP-dependent signaling to cause edema	CDC Tier 1 Select Agents, spores of anthrax toxin-producing bacteria developed and standardized for field use in WWII and later	(Alouf and Popoff 2006)
Anthrax lethal toxin (AnLTx)				
Anthrax edema toxin (AnETx)	Subunits:			
	90-kDa Lethal Factor (LF) – Zn^{2+}-dependent metalloprotease catalytic subunit			
	40-kDa Edema Factor (EF) – Ca^{2+}/calmodulin-dependent adenylyl cyclase subunit			
	63-kDa Protective Antigen (PA) – protein-receptor-binding and membrane-translocation subunit			
	Secreted by *Bacillus anthracis*			

(*continued*)

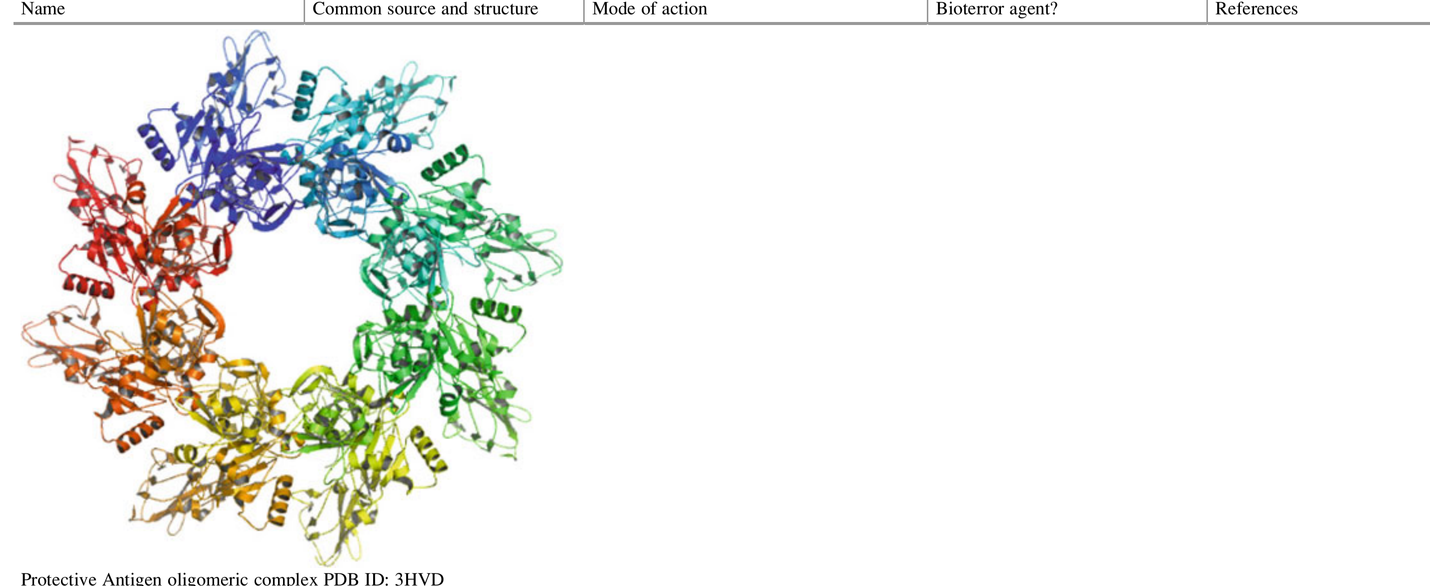
Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
Clostridial binary toxins	AB-type modular proteins produced as separate subunits (B components as 90–100-kDa precursors that are proteolytically processed to remove ~20-kDa peptide) that form an A_3B_7 complex	Cytotoxins, bind cell receptors, enter cytosol, ADP-ribosylate G-actin to prevent actin polymerization, cause cell rounding and changes in cell morphology		(Alouf and Popoff 2006)
Clostridial C2 toxins				
C2 (C2I + C2II)				
Clostridial iota toxins				
Iota toxin (Ia + Ib)				
CST (Sa + Sb)				
CDT (CDTa, CDTb)	Subunits:			
	49-kDa C2I, 45-kDa Ia, 44-kDa Sa, 48-kDa CDTa – NAD^+-dependent ADP-ribosyltransferase			
	80-kDa C2II, 81-kDa Ib, 76-kDa Sb, 75-kDa CDTb – receptor-binding and membrane-translocation subunit			
	Secreted by:			
	C2 toxin – *Clostridium botulinum*			
	Iota toxin – *Clostridium perfringens*			
	CST – *Clostridium spiroforme*			
	CDT – *Clostridium difficile*			

Representative structures[a]				
ADP-ribosylating toxins *Vibrio* cholera toxin, *E. coli* heat-labile toxin *Bordetella pertussis* toxin	Cholera toxin PDB ID: 1S5E	*E. coli* heat-labile toxin PDB ID: 1LTA		Pertussis toxin PDB ID: 1PRT
Ribosome-inactivating toxins Shiga toxin Shiga toxin-2	Shiga toxin PDB ID: 1DM0, 1R4Q		Shiga toxin type 2 PDB ID: 1RAP	
Anthrax toxins Anthrax edema toxin (ET = EF + PA) Anthrax lethal toxin (LT = LF + PA)	Edema factor PDB ID: 1K8T		Lethal Factor PDB ID: 1J7N	

(continued)

Table 1 (continued)

Name	Common source and structure	Mode of action	Bioterror agent?	References
 Protective Antigen oligomeric complex PDB ID: 3HVD				

Clostridial binary C2 toxin	Binary toxin catalytic component C2-I PDB ID: 2J3Z	Binary toxin transport component C2-II PDB ID: 2 J41

[a] Shown are 2D or 3D structures of representative members of each toxin category. The 2D structures were generated using ChemSpider (URL: www.chemspider.com). The ChemSpider identifiers are indicated. Peptide sequences were obtained from Protein Database Bank (PDB) files at NCBI (URL: http://www.ncbi.nlm.nih.gov/protein/). The 3D structures were rendered from PDB files using PyMOL version 0.99. The PDB identifiers are indicated.

Clostridium botulinum neurotoxins (BoNT), serotypes A to H, are considered the most potent protein toxins for humans and are perhaps the most well-known examples of dual-use biotoxins. According to the CDC's National Botulism Surveillance reports from 2001 to 2011 (CDC 2011), there are ~140 cases of naturally occurring botulism per year, which are categorized as food-borne botulism (12 %), infant botulism (68 %), or wound botulism (18 %). Although the protein sequences differ among the toxin serotypes, they all share a common overall structure and mechanism of action (Aktories 2013). The intravenous lethal dose of BoNT serotype A (BoNT/A) is estimated to be 1–10 ng/kg (Arnon et al. 2001). BoNTs are classified as category A select agents and have been the focus of biological weapons development by several countries in the past and more recently by terrorist groups that have attempted the malicious release of BoNTs (Arnon et al. 2001; Szinicz 2005; Katona 2012). In contrast, there have been increasing applications of BoNTs as cosmetic agents for wrinkle removal and as biomedical treatments for neuronal and secretory disorders (Katona 2012; Aktories 2013). One of the greatest concerns regarding the potential use of BoNTs as bioterror agents is their ready availability worldwide as a result of their biomedical or cosmetic applications. Locations for manufacturing, testing, storage, and distribution are all potentially vulnerable points, where relatively large, highly purified quantities of this extremely potent dual-use biotoxin could be intercepted for more nefarious purposes.

What Is the "Real" Potential of Using a Biotoxin as a Bioterror Agent?

There are a number of factors that complicate the process of making a biotoxin into a bioweapon of mass destruction (Madsen 2001; Henghold 2004; Bigalke and Rummel 2005; Anderson 2012; Katona 2012). Factors usually noted as disfavoring development of some biotoxins as biowarfare agents include difficulty in scale-up of production, limited environmental stability, a generally narrow range of host–target specificity, and complicated formulations needed for effective and efficient target delivery. In counterargument, it should be pointed out that many toxins listed in Table 1 are secreted directly into the culture medium by the producing organism, where the toxin would be relatively easy to harvest in crude form simply by centrifuging or filtering to remove the cells. Although purification from crude culture medium preparations is not always easy to achieve for research-grade or biomedical-grade applications, for most nefarious purposes, crude material is more than adequate to serve the intended function. Indeed, some toxins are produced as complexes with other factors that help to stabilize them in the environment. For example, clostridial neurotoxins are produced as high-molecular-weight, multi-protein complexes with hemagglutinins and several nontoxic neurotoxin-associated proteins, which greatly enhance their oral toxicity by stabilizing them during passage through the stomach and gut (Aktories 2013). In addition, exposure of BoNTs to proteases during cultivation or in the host leads to cleavage of exposed loops between the toxic activity module and the delivery

modules, which are still held together by a disulfide bond. This partial proteolytic cleavage also results in enhanced toxicity (Aktories 2013).

Many biotoxins are stable and soluble in aqueous solution or buffer and are colorless, odorless, and tasteless. Some toxins manifest their toxicity overtly by direct and immediate action on host cell membranes. For example, the pore-forming toxins comprise a large family of diverse protein toxins produced by a wide range of organisms, many of which are pathogenic (Alouf and Popoff 2006). Their cytotoxicity is mediated through a shared mechanism of binding to cell surface receptors (often using receptors common to many cell types), followed by oligomerization and penetration of cell membranes to form pores with β-barrel-like structures. The consequence of pore formation is increased ion flux across cellular membranes, which at low doses results in modulation of cell signaling processes and at higher doses results in cell lysis (Alouf and Popoff 2006). These toxins often are associated with the hemolytic, bloody diarrhea or hemorrhagic activity observed in infections caused by pathogens that produce them. In addition to direct injection into the bloodstream, ingestion or application of these toxins to mucosal surfaces could lead to severe damage of the vulnerable tissues, resulting in incapacitation, morbidity, and even death.

On the other hand, a large number of biotoxins, like the organisms that produce them, manifest their toxicities more covertly; that is, they cause disease after a lag period that can range from minutes to hours to days. The insidious nature of these toxins emphasizes the importance of early recognition and response to toxin symptoms. Yet, herein lies the greatest challenge, as they are extremely difficult to detect and identify due to their high potency and the generalized nature of the symptoms they cause (Alouf and Popoff 2006; Aktories 2013).

Many of the biotoxins listed within each of the main categories in Table 1 appear to employ a limited set of similar overall mechanisms for cellular interaction or intoxication and share similar modes of action and cellular or physiological outcomes. However, despite this overall functional similarity, they are highly diverse at the molecular level (sequence and structure) and differ greatly in details of their receptor specificity, entry and translocation pathways, reactions catalyzed, target substrate specificities, and ultimate effects on the host. A large number of protein biotoxins have multiple functional domains (modules) that mediate their toxic activities. This modular nature of protein toxins, in particular, enables a tremendous potential for diversification and combinatorial evolution of biological activities, both in nature and in the modern laboratory.

One of the greatest concerns comes from the rapidity with which current biotechnology capabilities enable the leap from acquisition of a toxin gene to relatively large-scale production of the encoded toxin (Salem 2003; Szinicz 2005; Paterson 2006; Katona 2012; Relman 2013). The cornucopia of genome sequencing data amassed over the past decade has driven home the enormous extent to which genetic exchange has occurred among toxin-producing pathogens. The structural genes encoding most bacterial toxins reside on mobile genetic elements, as parts of extrachromosomal plasmids or phage or as parts of prophage, conjugative plasmids, or transposons that have integrated into the chromosome of the host organism.

Strong evidence now points to the importance of this genetic exchange of not just entire toxin genes but also of domain modules within toxin genes in toxin evolution and diversity (Alouf and Popoff 2006). Consequently, it is essential to learn as much as possible about each of these toxin systems and their components, so as to provide a wide breadth of approaches to combat existing toxin-mediated diseases and to be adequately prepared for any new or unusual toxin-mediated threat, whether natural or deliberate.

Therapies Against Biotoxins: The Critical Need for Postexposure Therapies

The mainstay of defense against biological toxins has been the use of passive or active immunization to prevent and/or neutralize toxin action immediately after exposure. Indeed, three of the previously most devastating toxin-mediated diseases, namely, diphtheria, pertussis, and tetanus, have been effectively controlled through childhood vaccination with the DPT toxoid vaccine. We also have vaccines available for at-risk personnel for several other toxin-mediated diseases, including cholera, anthrax, ricin, and botulism (Alouf and Popoff 2006; Arnon et al. 2006; Hu et al. 2012). The approach used for management of most other toxin-mediated diseases, however, is passive immunization, usually in the form of horse antisera or in some cases humanized immunoglobulins (Arnon et al. 2001; Binder et al. 2003; Arnon et al. 2006; Calvete et al. 2009; Hu et al. 2012). Serum sickness has been a serious concern for treatment with antisera; however, this problem has largely been overcome with recent efforts focused on development of humanized or despeciated antibodies (Arnon et al. 2006; Calvete et al. 2009; Hu et al. 2012; Hill et al. 2013). For example, the plant-derived ricin is a modular protein toxin deemed a high risk as a potential bioterror agent due to its high potency, stability, and ease in production (Schep et al. 2009). However, like for most biotoxins, there is no drug therapy or vaccine available for the general population, and consequently the only available countermeasure for ricin poisoning is neutralizing antitoxin, which has recently been humanized (Hu et al. 2012).

In the absence of vaccination, one of the greatest challenges of developing postexposure treatments for any toxin-mediated disease is the short therapeutic window available for administration of antitoxin (antisera, antivenom, or antidote) after symptoms appear. Prompt administration of neutralizing antisera only slows progression and reduces severity of the disease, but does not reverse toxin effects once exposure has occurred and symptoms are manifested. For toxins such as those found in venoms that circulate in the bloodstream and mediate their toxicity through binding to host cell surfaces, antidotes (usually in the form of antivenoms) are the most effective countermeasures since they directly compete with the toxin binding to the cell receptor and thereby neutralize toxin activity (Calvete et al. 2009). Antibody complexed with the toxin then removes the toxin from the circulation. However, there are still significant issues with regard to the ability of antivenoms to reverse venom-mediated downstream effects that have already

occurred before antivenom treatment, including blood clotting, hemorrhaging, and toxic shock (Calvete et al. 2009). This concern is particularly true for larger modular protein toxins that act intracellularly after internalization of the toxin into the host cells, where antibodies or drugs that work only in the bloodstream no longer neutralize toxin activity. Recovery in this case requires the removal or inhibition of the catalytic portion of the intracellularly located toxin.

For example, the only treatment for botulism currently is the use of antitoxin immunoglobulins (Arnon et al. 2006; Hill et al. 2013). All BoNTs are modular proteins that mediate the cellular delivery of their catalytic module, which is a Zn^{2+}-dependent protease that cleaves their cognate target SNARE protein substrates (Aktories 2013). SNARE proteins are essential for synaptic vesicle fusion and neurotransmitter release at the neuromuscular junction. BoNTs thereby block neurotransmitter release to cause flaccid paralysis. Antitoxin therapy using despeciated or native horse-derived antisera or humanized immunoglobulins is necessary for prevention of further intoxication, rapid clearance of toxins from the circulation, and lessening the severity of the disease (Arnon et al. 2001; Arnon et al. 2006; Hill et al. 2013). BoNTs constitute a highly diverse group of bacterial toxins; BoNT serotypes A, B, E, and F are associated with human botulism (with serotypes A and B being most prevalent), while serotypes C and D cause mostly bird and animal botulism (Popoff 2013). Because of this high diversity, antibodies derived against one serotype are not protective against the other serotypes, leading to the concern that one of the less prevalent serotypes might be utilized in a bioterror attack. Some of this concern was mitigated by the development of antisera against seven of the serotypes BoNT/A–G (Arnon et al. 2001; Arnon et al. 2006). However, the recent discovery of an eighth serotype of BoNT (serotype H) from a clinical case of infant botulism (Barash and Arnon 2013; Dover et al. 2013) has sparked concerns regarding the dual-use nature of this diverse group of toxins. On the one hand, BoNT/H adds to the biomedical repertoire of potential therapeutic agents (Popoff 2013); but on the other hand, BoNT/H provides an additional tool that could be used for harmful intent and for which there are currently no neutralizing antisera available (Relman 2013).

Moreover, even with antitoxin administration, recovery is prolonged, lasting days to months (Arnon et al. 2001; Aktories 2013), since antitoxin is ineffective against toxin that has already entered neurons. During this time patients are still paralyzed and require respiratory and other mechanical support until the toxin present in the nerves at neuromuscular junctions has been removed completely. It also takes much longer for full physical recovery from botulism (Arnon et al. 2001; Aktories 2013). Clearly, there is an urgent need for post-intoxication therapeutics that could neutralize the toxin molecules that are already inside the neurons and thereby shorten the recovery time. For this treatment strategy to succeed, the therapeutic agent must be targeted to the correct cells critical since it would not only be inefficient but also could cause adverse side effects if delivered to the wrong cells. This could be critical in the event of mass exposure to BoNTs, where it is likely to overburden the current medical infrastructure, which is only designed for the rare annual occurrence, particularly in terms of the number of ventilators available for long-term use.

Conclusion and Future Directions

A roadmap for biotoxin research must be generated in response to biosecurity concerns. Understanding the complete spectrum of functions associated with virulence will require a more thorough functional characterization of modular proteins with predicted toxin-like activities. It is imperative that we support further biotoxin research aimed at exploring the vast number of available genomes for additional and/or new toxin-like modules. But importantly, there is a critical need for more functional characterization and annotation of genes within genomes that are predicted to encode toxin-like modules. This research could not only provide valuable insight into the biochemical activity and function of the component toxin-like domains of uncharacterized proteins and their potential roles in pathogenesis, but also could yield alternative targets for therapeutic development and could advance strategies for intervention.

For fungal or algal toxins that are commonly found in high concentrations as a result of natural contamination, such as by fungal growth or algal blooms, perhaps one of the best ways to address this problem is to be able to detect and monitor the presence of these toxins, particularly in the environment, and to know when concentrations are above normal, and hence, of concern (Paterson 2006). The presence of toxins at unusual sites would trigger a response indicating possible deliberate intent. For naturally occurring biotoxins, environmental surveillance through wildlife monitoring is essential for identifying new sources of toxins and new potential exposure routes. Wildlife also have the potential to serve as sentinels for early detection of new toxin threats.

The dual-use research debate that has ensued recently with the discovery of BoNT/H emphasizes the need for enhanced vigilance in monitoring discoveries for their potential biothreat implications (Relman 2013), but also exemplifies the urgency for development of effective postexposure therapies against a variety of toxin-mediated diseases (Ho et al. 2011; Edupuganti et al. 2012). This example points to the need for considering antitoxin strategies along with the development of new dual-use agents for research or biomedical applications. In addition, it is vital that we develop better biotoxin inventory documentation and detection protocols to ensure safety and to monitor where biotoxin work is being conducted and what the nature of that work is.

Cross-References

- ▶ Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment
- ▶ Aflatoxins and Their Management
- ▶ Antidotes to Botulinum Neurotoxin
- ▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
- ▶ Biotoxins and Food Safety

- ▶ Botulinum Toxin: Present Knowledge and Threats
- ▶ Cell-Based Assays for Neurotoxins
- ▶ Challenges in Developing Biotoxin Inhibitors
- ▶ Counterfeit Botulinum Medical Products and the Risk of Bioterrorism
- ▶ Current Insights into Mycotoxins
- ▶ Immunoneutralization of Abrin
- ▶ Marine Biotoxins in History: Misuse and Mayhem
- ▶ Structure, Genetics, and Mode of Disease of Cholera Toxin
- ▶ The Biowarfare Agent Ricin
- ▶ The Public Health Response to Potential Bioterrorism by Toxin Attack

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Biotoxins and Food Safety

9

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Abstract

Foodborne illness in humans is an important global public health issue. This is primarily caused by pathogenic microorganisms (e.g., bacteria, fungi, parasites,

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P. Gopalakrishnakone et al. (eds.), *Biological Toxins and Bioterrorism, Toxinology*,
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and viruses) and toxins from natural source and microorganisms. Toxins are lethal to humans and vary in severity based on their interaction to target sites. They can be either non-proteinaceous small molecule or peptides and proteins. Some important toxins derived from bacteria (e.g., botulinum neurotoxins, staphylococcal enterotoxins), plants (e.g., ricin and abrin), and seafood toxins (e.g., saxitoxins, tetrodotoxins, brevetoxins, ciguatera, domoic acid, okadaic acid, azaspiracids, and palytoxins) causing foodborne illness in humans are of major concern. The annual incidence of foodborne illness has been estimated to affect 5–10 % of the world population in the developed countries, and the incidence rate is even higher in the developing countries. Among the majority of foodborne illness, a limited number of cases are attributed to toxin poisoning. The prevention of foodborne illness relies on good practices of food safety programs; proper handling procedure, good sanitation, and hygiene; and implication of standard food analysis techniques at each step of food processing. Workers in food industries and public services are key players in safe food supply. Government regulations and recommendations are designed and managed by a number of government agencies to ensure the composition and quality of food is safe for consumers.

Introduction

Toxins can be non-proteinaceous small molecules, peptides, or proteins derived from bacteria, plants, and natural sources. They can cause disease on contact with or absorption by body tissues interacting with biological macromolecules (e.g., enzymes or receptors). Toxins vary greatly in their severity ranging from minor, acute, to highly poisonous to humans. Both Gram-positive and Gram-negative bacteria produce soluble protein toxins. The cell-associated toxins are referred as endotoxins which are located mainly in the cell. The other types of extracellular diffusible toxins are referred as exotoxins which are secreted by bacteria during exponential growth. Exotoxins are also known based on their target sites (e.g., enterotoxin, neurotoxin, leukocidin, or hemolysin). Bacterial toxins are strongly antigenic which retain their antigenicity even though they lost their toxicity.

Toxins are responsible for foodborne illness in humans. Toxins can be used as biothreat agents (BTAs) which pose a potential threat to human health. Food safety is very important for food industry, regulators, and consumers because of increasing incidence of foodborne illness. For more related info, readers are advised to consult *Bad Bug Book* – US FDA (http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/), USDA fact sheet-Foodborne Illness and Disease (http://www.fns.usda.gov/fdd/usda-foods-fact-sheets), and food safety on foodborne illness – Centers for Disease control and Prevention (http://www.cdc.gov/foodsafety/diseases/).

This chapter provides an overview on a few important toxins (e.g., botulinum neurotoxin, streptococcal enterotoxins, ricin, abrin, and seafood toxins) lethal to humans and general food safety practices.

Botulinum Neurotoxin (BoNT)

Is listed as a highest-risk (category A) threat agents by the CDC because of the poisonous nature of toxin (http://emergency.cdc.gov/bioterrorism). Seven serologically distinct BoNTs (A–G) are produced by a rod-shaped anaerobic bacteria *Clostridium botulinum*. BoNT is synthesized by *C. botulinum* in the form of a single-chain polypeptide of ~150 kDa that undergoes proteolytic cleavage to yield an active holotoxin, a dichain polypeptide linked by a disulfide bridge. The dichain form of toxin consists of a ~50 kDa light chain (LC) with endoprotease activity and a ~100 kDa heavy chain (HC) bearing a translocation domain (~50 kDa) at amino terminal and a receptor binding domain (~50 kDa) at carboxyl terminal of the HC. Receptor binding and translocation domains of the HC are responsible for binding and internalization of BoNT at the neuromuscular junction. The mechanism of BoNT intoxication is a multistep process involving toxin binding, internalization, membrane translocation, intracellular trafficking, and degradation of target proteins (e.g., synaptic-associated proteins of 25 kDa (SNAP-25), synaptobrevin, and syntaxin) by a proteolytic action of the LC. Cleavage of these polypeptide substrates causes blockage of neurotransmitter release at the neuromuscular junction resulting in muscle weakness and paralysis (Singh et al. 2013). The crystal structure of BoNT is shown in Fig. 1.

BoNT is responsible for a life-threatening neuroparalytic illness, botulism in humans. BoNT serotypes A, B, and E are mainly responsible for human botulism. There are three forms of human botulism: foodborne (most common), wound, and infant or intestinal botulism. Severe botulism leads to paralysis of respiratory muscles and respiratory failure. Botulism patients need emergency hospital care since treatment involves the use of ventilator plus intensive medical and nursing care. The occurrence of the disease is low with high mortality rate in untreated cases. Outbreaks of botulism can occur worldwide. Foodborne botulism has often been from home-canned foods with low acid (>pH 4.6) content, such as green beans, beets, and corn. However, outbreaks of botulism have resulted from more unusual sources such as chopped garlic in oil, chili peppers, tomatoes, carrot juice, improperly handled baked potatoes, and home-canned or fermented fish. Honey has been found as a source of infant botulism.

Botulism can be tentatively diagnosed by the clinical signs and the exclusions of other neurologic diseases. The definitive diagnosis relies on identifying the toxin in clinical and food samples. The toxin can be identified by the mouse bioassay, enzyme-linked immunosorbent assays, and electrochemiluminescent assays. BoNTs can be typed by neutralization assay in mice and the assay takes up to 48 h. *C. botulinum* can also be cultured from clinical or food samples but it requires 5–7 days.

Food Analysis

Foodborne botulism occurs due to ingestion of BoNT contaminating foods. During outbreak investigations, it is recommended that foods be tested for preformed

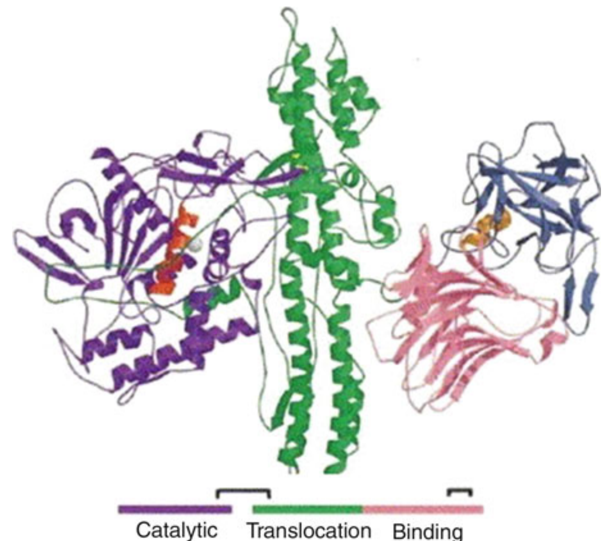

Fig. 1 Structure of BoNT (Adopted from Lacy and Stevens 1999)

neurotoxin and to determine neurotoxigenicity of isolates. Enrichment isolation and direct plating methods are used for detection and enumeration of *C. botulinum* in foods. Conventional methods are also used in the absence of viable bacteria. Suspect food products are typically analyzed by using traditional culture techniques followed by identification of neurotoxigenic *C. botulinum* strains. Food extracts positive for bacteria may then be evaluated for the presence of preformed BoNT which can be detected by a variety of techniques as described in a recent review article (Singh et al. 2013). A limited number of assays (e.g., mouse bioassay [MBA], enzyme-linked immunosorbent assay [ELISA], electrochemiluminescent assay [ECL], immunochromatography assays) have been implicated in food analysis and are briefly described below:

Mouse bioassay – The mouse bioassay (MBA) is considered as the "gold standard method" for detecting all seven BoNT serotypes (Solomon and Lilly 2001). The MBA is based on the mouse LD_{50} assay which involves dosing mice with dilutions of the sample being tested and determining the dilution at which 50 % of the mice die. The LD_{50} assay is accepted as the method of choice for confirming BoNT and also for potency testing by international regulatory agencies. The MBA is the current method of choice for detecting BoNT in foods and environmental samples and for assessing the potency of the therapeutic drug products (Ferreira et al. 2004).

ELISA– This is the most widely applied technique for serotyping and toxin detection and is based on an antigen-antibody interaction (Ferreira 2001; Lindstrom and Korkeala 2006). The sandwich immunoassay is a common format for toxin detection (Stanker et al. 2008; Volland et al. 2008; Scotcher et al. 2010) and uses two toxin-specific antibodies, one antibody as a capture antibody and the second antibody as a detection antibody. The detection antibody is conjugated usually to an

enzyme that cleaves a specific substrate generating a colorimetric or luminescent signal or to biotin with subsequent detection with a streptavidin conjugate. The intensity of the signal is generally proportional to the amount of BoNT present in the sample. Currently, there are several modifications of this technique (viz., amplified ELISA, sd-ELISA, peptide ELISA, and liposome ELISA) resulting in improved sensitivity of BoNT detection (Goldman et al. 2010; Han et al. 2007; Mason et al. 2006; Stenmark et al. 2008). The digoxigenin ELISA (DIG-ELISA) is an example of an amplified ELISA. This assay was developed by the US Food and Drug Administration (US FDA) and has been used to detect BoNT/A, /B, /E, and /F in food samples (Sharma et al. 2006). The assay uses polyclonal antibodies specific to each toxin serotype as both capture and detector reagents and DIG-labeled tertiary antibody conjugated with HRP for signal amplification and BoNT detection. The DIG-ELISA is a sensitive assay with a detection limit ranging from 1 to 10 mouse lethal doses (MLD) depending on serotypes (Sharma et al. 2006).

ECL assay – The ECL microplates contain carbon electrode surfaces and ruthenium-labeled (SULFO-TAG) BoNT-specific antibodies that emit light only when brought into close contact of the electrodes coated with BoNT-specific biotinylated antibodies as capture reagents. A luminescent signal is generated by an electron cycling of ruthenium label and measured by an ECL instrument. The ECL assay can detect samples in a wide linear response range. This assay is used for detecting and identifying BoNT in food and clinical samples and sensitivity of the assay ranges from 5 pg to 50 ng/mL (Sachdeva et al. 2013; Cheng and Stanker 2013).

Lateral flow assay – Lateral flow assays (LFAs) use a nitrocellulose strip which contains BoNT-specific capture and detector antibody and reagent pad. Application of a toxin sample allowed its binding with detector and capture antibodies yielded a colored line. LFAs are self-contained and are ideal for field situations. LFAs have been validated to test for BoNT in a wide variety of food matrices with detection limits ranging from 5 to 50 ng/mL depending on serotypes (Attree et al. 2007; Chiao et al. 2008; Gessler et al. 2007; Sharma et al. 2005).

Immunoaffinity chromatography assay – Immunoaffinity chromatography assay (ICA) uses a column preloaded with matrix coupled to the capture antibody. Toxin sample is then loaded on to the column, followed by washing of the column and addition of detection antibody for color development. The intensity of the color is directly proportional to the toxin concentration in the sample (Brunt et al. 2010). The ICAs have also been validated using a variety of food matrices with a reported detection limit up to 100 pg/mL for BoNT serotype A; furthermore, the sensitivity of the assay was improved to 20 pg/mL (Attree et al. 2007).

Staphylococcal Enterotoxins (SEs)

The staphylococcal enterotoxins A through E are identified as category B biothreat agents by the Centers for Disease control and Prevention (CDC) and regulated as an HHS select agent (http://emergency.cdc.gov/bioterrorism). The staphylococcal

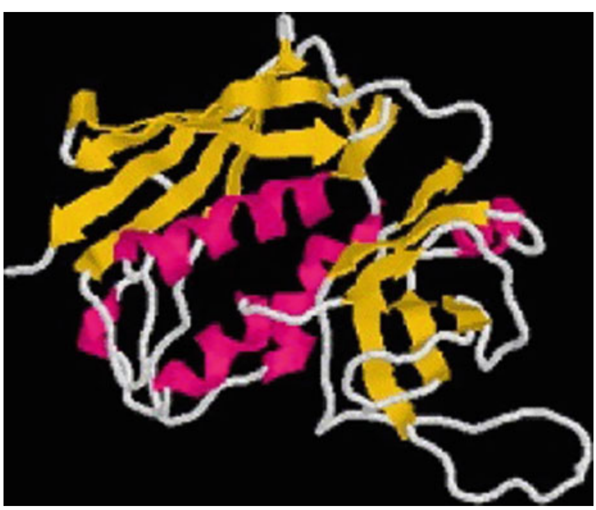

Fig. 2 Structure of SEB (Adopted from Ler et al. 2006)

enterotoxins (SEs) are produced by enterotoxigenic strains of *Staphylococcus aureus* and are recognized to be etiologic agents of gastrointestinal food poisoning in man. SEs is also known as superantigens, which can lead to systemic febrile responses and shock due to the induction of proinflammatory cytokines induced by the strong stimulation of SE-reactive T cells.

SEs is a large family of Staphylococcal pyrogenic exotoxins. They are denoted alphabetically as SEA through SEU and many identified recently by molecular techniques. SEs are relatively small 22–30 kDa single-chain protein molecules that exhibit similarities in secondary and tertiary structure (Fig. 2) and are encoded on plasmids, mobile genetic elements, and bacteriophages. Interestingly, while there is some degree diversity in sequence, the SE demonstrates a remarkable preservation of the three-dimensional structure that enables them to bind to their target receptor sites. Virulence of SEs as food toxins is attributable to their relative resistance to heat and proteolytic digestion.

SE is an etiologic agent predominantly associated with foodborne illness (gastroenteritis) in humans. SE poisoning naturally occurs after ingesting processed meats or dairy products previously contaminated by improper handling and storage. Such conditions favor growth of *S. aureus* which can release SEs into the tainted food. Ingestion of purified preparations of enterotoxin has been shown to induce illness in humans. Microgram quantities of consumed toxin are sufficient to cause emesis and diarrhea within approximately 4 h of ingestion, and one may still experience a general malaise 24–72 h later. As food poisoning by SEs is nonfatal and of short duration, supportive care is needed including use of over-the-counter medication for symptomatic relief of gastrointestinal discomfort. Foodborne SE poisoning is rarely fatal for healthy humans but exceptions are the very young and elderly who are at high risk. As a biological warfare agent, inhalation of nanogram of SEB can be fatal and induce headache, myalgia, fever, dyspnea, coughing, nausea, vomiting, and conjunctival irritation within 2 h. The effective incapacitating

dose for 50 % of exposed individuals has been reported to be 0.4 ng/kg, while the LD_{50} has been calculated to be 0.02 μg/kg for the inhalation route (Ulrich et al. 1997). SEB also acts as a superantigen and possesses the ability to bind directly to the α chain of major histocompatibility complex (MHC) class II glycoprotein outside the peptide-binding groove of antigen presenting cells, thus effectively bypassing normal antigen processing and presenting mechanisms. This event results in the stimulation of a large proportion of $CD4^+$ and $CD8^+$ T cells (5–30 %) without regard to antigenic specificity or antigenic repertoire. The activated T cells proliferate and release large quantities of proinflammatory cytokines that can lead to the development of clinical symptoms such as fever, swollen lymph nodes, and shock. SEB significantly upregulates mRNA expression of IL-2 and IFN-γ. This reaction then leads to a recessive tolerance as a result of the induction of apoptotic cell death.

S. aureus is involved in various food poisoning episodes around the world. SEA is the most common enterotoxin associated with the food poisoning outbreaks (Hennekinne et al. 2010), followed by SED and SEB (Casman 1965). Recently, food poisoning after ingestion of food tainted with SEE, SEG, SEI, and more notably SEH has been recognized (Chen et al. 2004; Ikeda et al. 2005; Morris et al. 1972). The infective ingested dose required to induce illness in humans has not been determined but is thought to vary with individual sensitivity. Early studies estimated that 50 ng SE/kg of body weight is sufficient to induce emetic disease in humans (Raj and Bergdoll 1969). Estimates provided following food outbreaks have determined that ingestion of 94–184 ng of staphylococcal enterotoxin A (SEA) in chocolate milk (Evenson et al. 1988), 20–100 ng of SEA in yogurt and milk products (Asao et al. 2003), and 71 ng SE in 4 oz of mushrooms (Levine et al. 1996) is sufficient to induce disease in a general consumer population. The usual incubation period of staphylococcal food poisoning is between 2 and 6 h, depending on the amount of toxin ingested. Typical disease is characterized by cramping abdominal pain, nausea, vomiting, and diarrhea and can also include shock and fever. Physical examination may reveal dehydration and hypotension if fluid loss has been significant, and the severity of illness can also be attributable to the health status of the affected individual. Nevertheless, the incidence of SEB-mediated food illness is often thought to be underreported, since the illness often recedes in the affected individual (Balaban and Rasooly 2000; Hennekinne et al. 2012). Mortality is rare as demonstrated by a study on 7,126 patients where the case fatality rate was as low as 0.03 % and the death was exclusively seen in the elderly group (Holmberg and Blake 1984).

Identification of SEs as the causative agent of a food outbreak is often the result of a traceback investigation where microbiologic and molecular techniques are used to assay clinical samples and contaminated food samples collected from food poisoning incidents. While these techniques identify the bacterium involved, or confirm the presence of the microorganism through its genetic makeup, biologic assays have classically confirmed the presence of biologically active toxin. SEs has been detected based on the emetic activity in kittens and primates (Balaban and Rasooly 2000; Bergdoll 1979; Surgalla et al. 1953).

Food Analysis

Enrichment isolation and direct plating methods are frequently used for detection and enumeration of *S. aureus* in foods. Conventional methods are also used in the absence of viable bacteria. Suspect food products are typically analyzed by using traditional culture techniques followed by identification of enterotoxigenic staphylococcal strains. During outbreak investigations, it is recommended that foods be tested for preformed enterotoxin and to determine enterotoxigenicity of isolates. Food extracts positive for bacteria may then be evaluated for the presence of preformed SEB. A number of serological methods have been developed for detection of preformed enterotoxin in foods. The most commonly used assays for SEB are summarized as below:

ELISA –The most common technology used for the assay of foods is the ELISA. The oldest technology combines the use of the antibody sandwich technique with a colorimetric readout, which requires the use of a modified spectrophotometer. The specificity of the immunoassay is dependent upon the specificity of the detection antibodies utilized in the assay, and the assay's sensitivity is dependent upon the antibody's specificity and affinity. While the specificity of the antibody is mandated by the original immunogen, it is also critically dependent upon immunization routes and strategies. Antibodies can be chemically modified and conjugated with any number of reporter molecules including enzymes, radioisotopes, fluorescent or chemiluminescent probes, and colloidal metals. The intensity of color or fluorescence is proportional to the amount of toxin present in the sample. Fluorescence-based immunoassays are four times more rapid than classical ELISA and capable to detect SEB in a range of 0.01–0.1 ng/mL. Several commercially available test kits are accepted for use for the detection of SE in foods. One commercially available ELISA assays for the detection of SEB in foods, the Tecra SET VIA (Tecra International, Ltd., New South Wales, Australia), has received approval for use by the AOAC International (Bennett and McClure 1994). Other commercially available systems include the VIDAS SET2 (bioMérieux) for which validation results have been reported (Jechorek and Johnson 2008) and the RIDASCREEN SET Total (R-Biopharm) for the detection of staphylococcal enterotoxins (SEA to SEE) in dairy and nondairy food matrices. Additional newer modifications and technologies have used magnetic particles coupled to the detection antibody. This approach permits entry of the conjugated antibody within less fluidic matrices and affords a greater degree of detection. Nevertheless, the sensitivity and reliability of this assay is severely compromised when working within food matrices. For instance, a solid food must first be physically, and sometimes chemically, modified before antibody can enter the matrix to detect its antigen. Often the chemical treatment may change the antigen's conformation making it unrecognizable to the antibody. Other problems arise with acidic fluids. The extreme pH of carbonated soft drinks and orange juice compromises the ability of antibody to bind its antigen, contributing to the development of false readings in which the color development for the negative control is the same as that of the positive control (Principato et al. 2010). This was demonstrated in a series of experiments utilizing a purified

cytokine, IL-2, as an example of a target protein antigen within a variety of food matrices. Finally, the strategy with which food is treated in order to permit the use of antibody in a food-based ELISA assay is critical and will also affect outcome. Depending on the chemical treatment or treatment inherent to food processing, it is very likely that the antigenic epitope recognized by an antibody will be damaged in such a way that it avoids detection of toxin by the antibody-based techniques (Principato et al. 2009), and one has to rely on measuring the superantigenicity (biologic activity) of toxin.

ECL assay – ECL method can significantly enhance the sensitivity of immunoassay but often requires expensive and complex detectors. ECL utilizes a thiolated antibody immobilized on a gold electrode. A secondary antibody tagged with alkaline phosphatase (AP) was used to cleave α-naphthyl phosphate (α-NP) into α-naphthol and developed color. The sensitivity of ECL assay was also improved by replacing thiolated antibody with biotinylated antibody, prebound to streptavidin-coated paramagnetic beads for the binding of antigen. A detector antibody labeled with ruthenium (II) tris-bipyridal chelate was added which bound to the bead immune complex. Light is generated in the presence of an excess of tripropylamine and recoded by the detector. For improved detection of SEB in food, ECL also utilized a simple cooled charge-coupled device (CCD) detector combined with carbon nanotubes (CNTs) for primary antibody immobilization. The sensitivity of ECL assays for SEB ranges from 0.01 to 5 ng/mL depending on food matrices.

Immunoaffinity chromatography/lateral flow assay – The immunochromatographic test (ICT) is also known as dipstick assay or lateral flow assays (LFAs) which gained most popularity because of its simplicity and quick method. LFA uses similar device except for analyte-specific capture and detector antibodies tailored to detect analyte (SEB). Chicken immunoglobulin (IgY) antibody and sulforhodamine B encapsulated into immunoliposomes have been also used for higher detection sensitivity of LFA. This assay has been successfully adopted for the detection of SEB in foods and sensitivity of the assay ranges from 0.02 to 10 ng/mL.

Ricin and Abrin

Ricin and abrin are two of the most readily available biothreat agents due in part to the ubiquitous presence in the subtropics of the plants from which the toxins are derived, castor beans and rosary peas, and their ornamental use. This section will briefly describe the salient features of the toxins, the role played by these two toxins in the development of immunology, and the application of detection technologies to food safety.

Research on ricin and abrin goes back to the late 1800s and the central role they played in our understanding of immunology. Paul Ehrlich's groundbreaking studies on immunity were performed using ricin and abrin. Mice gradually exposed to ricin developed immunity to the toxin but not toward abrin. Similarly, mice gradually exposed to abrin developed a resistance to its intoxication but not to that of ricin

Fig. 3 Structure of ricin (Adopted from Ler et al. 2006)

(Ehrlich 1891a, b). This research has been repeated by others and used to develop a biological assay to distinguish between the two toxins (Griffiths et al. 1995; Clarke and Humphreys 1971).

Despite the early interest in ricin and abrin, it was not until 80 years later that it was demonstrated that the toxins inhibited protein synthesis (Lin et al. 1969). Purification was achieved in the 1960s (Craig et al. 1962; Lin et al. 1969), and affinity chromatography subsequently (Wei et al. 1974; Hegde et al. 1991) enabled detailed structural characterization based on x-ray crystallography and molecular modeling of the catalytic activity (Tahirov et al. 1995; Tanaka et al. 2001; Zhanpeisov and Leszczynski 2001). Ricin and abrin are class II ribosome-inhibiting proteins (RIPII), and each consists of two subunits (A chain and B chain) linked together by a disulfide bond (Figs. 3 and 4): a ~30 kDa deadenylase moiety referred to as the A chain and a ~32 kDa lectin moiety referred to as the B chain. The B chain is a glycoprotein that recognizes galactosyl groups on cell surfaces and facilitates uptake of the toxin. Once inside the cell, the A chain catalyzes the deadenylation of adenine 4,324 of the sarcin hairpin loop of the 28S rRNA of the eukaryotic 60S ribosomal subunit, a property common to many RIPs and the target of RNAlyases (Endo and Tsurugi 1987).

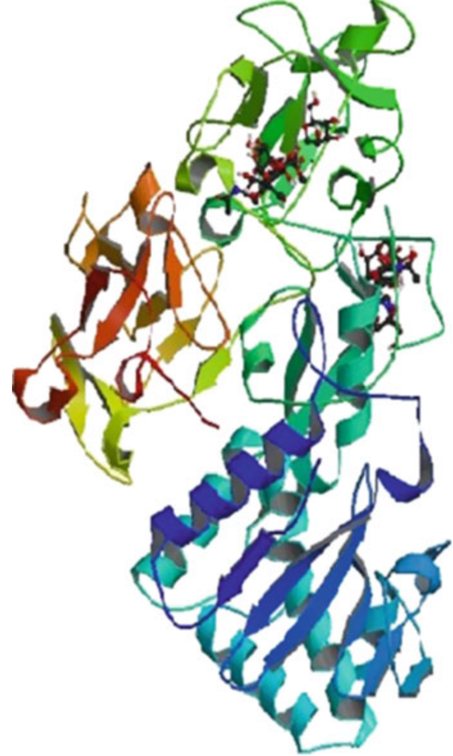

Fig. 4 Structure of abrin A (Adopted from Tahirov et al. 1995)

Ricin and abrin are homologous toxins that exist as multiple isoforms with variable glycosylation that may affect toxicity (Sehgal et al. 2011). The intraperitoneal (i.p.) LD_{50} values for mice are 20 μg/kg body weight for ricin and 22, 2.4, and 10 μg/kg body weight for abrin fractions I, II, and III, respectively (Hegde et al. 1991). The inhalation toxicities of the proteins are comparable to those of i.p. administration, provided the particle size is consistent with reaching the inner lung for uptake into the bloodstream. The oral toxicities of the toxins are a 1,000-fold less than i.p. or i.v. administration with insufficient information available to support the assignment of an LD50 for transdermal exposure (Franz and Jaax 1997; Garber 2008). The minimum time for the onset of death is 10 h, consistent with toxicity resulting from the inhibition of protein synthesis.

No antidote exists for ricin or abrin. Passive resistance, by administration of anti-RIP antibodies or through immunization, has been successively applied to intoxicated animals (Yermakova and Mantis 2011; Han et al. 2011). The administration of galactosyl sugars to reduce the effectiveness of B chain-mediated uptake has been reported to reduce ricin toxicity but only at low concentrations of ricin with no statistically significant effect on ricin toxicity at concentrations $\geq$10 ng/mL (Rasooly et al. 2012). Indeed, the ability of galactosyl sugars and carbohydrates

to stabilize ricin and abrin from thermal denaturation (Krupakar et al. 1999; Zhang et al. 2013) may complicate such approaches.

Also present in castor beans and rosary peas are agglutinins that contain A chains and B chains. The agglutinins and toxins are highly homologous at 67 % and 80 % for the *Abrus precatorius* A and B chains, respectively, and 93 % and 84 % for the *Ricinus communis* chains (Roberts et al. 1985; Liu et al. 2000; Cawley et al. 1978; Funatsu et al. 1991; Wood et al. 1991). However, the agglutinins are considerably less toxic with i.p. LD50 values >1 mg/kg body weight (Lin et al. 1981; Lin and Liu 1986; Zhan and Zhou 2003; Hegde et al. 1991) and less active in *in vitro* assays (Barbieri et al. 2004). Interestingly, it has been reported that the purified A chains from ricin and castor bean agglutinin display similar activity in a cellular assay (Sha et al. 2010). The developments of antibodies and ELISAs that distinguish between the toxins and related agglutinins have been reported (Goldman et al. 2011; Yi 2008).

The famed toxicities of ricin and abrin are primarily due to their activities when administered intravenously. As exemplified by the Georgi Markov poisoning in 1978, delivery of ricin into the bloodstream is not easily achieved (Crompton and Gall 1980). Instead, oral administration is often chosen but is not usually effective (Carus 2002) with a fatality rate of 46 % for ricin intoxication when administered by injection versus a 0 % fatality rate for oral intoxication (Worbs et al. 2011). Despite these and other limitations, acts of terrorism involving the use of ricin have occurred necessitating the development of rapid methods for its detection.

Food Analysis

Analytical methods for the detection of ricin and abrin can be divided among those that detect a unique biomarker derived from *Ricinus communis* or *Abrus precatorius*, those that detect a physical property of the toxins, and those that detect the enzymatic-biological activity of the toxins. Unique biomarkers include DNA, detected by PCR, and the alkaloids ricinine and abrine, detected by liquid chromatography-mass spectrometry (LC-MS) (He et al. 2007; Felder et al. 2012; Johnson et al. 2005; Owens and Koester 2008; Johnson et al. 2009).

The identification of physical properties has focused primarily on the presence of unique amino acid sequences detected by mass spectrometry (Brinkworth et al., 2010; McGrath et al. 2011; Mol et al. 2011; Zhao et al. 2012) or by complexation to a capture agent. Capture agents used to detect ricin and abrin include antibodies, aptamers, and carbohydrates (Gao et al. 2012; Tang et al. 2007; Mok and Li 2008; Kanamori-Kataoka et al. 2011; Zhou et al. 2012; Huebner et al. 2013). The use of a secondary detector to form a sandwich complex increases the specificity of these capture assays. Further, the use of high signal-to-noise markers improves the limit of detection (Garber and O'Brien (2008); Garber et al. 2008; He et al. 2010; Yang et al. 2011). Commercially, the most popular analytical devices are immunodiagnostic assays such as ELISAs and lateral flow devices (LFDs). LFDs provide a field-deployable, rapid alternative to the various instrumental approaches and have been recently validated to reliably detect less than 25 ng/mL (Hodge et al. 2013).

Activity-based assays can be *in vitro* or *in vivo*. Commonly used *in vitro* assays include the detection of deadenylation. The release of adenine has been detected by fluorescence, mass spectrometry, and PCR (Zamboni et al. 1989; Hines et al. 2004; Melchior and Tolleson 2010). The generation of deadenylated hairpin loops that are sensitive to chemical cleavage formed the basis of a novel electrochemiluminescent assay capable of distinguishing RIPs by comparing the activity with different synthetic oligos (Keener et al. 2006, 2008; Cho et al. 2009). The inhibition of protein synthesis (e.g., by rabbit reticulocyte lysate, Olsnes and Pihl 1973) has also been used extensively to study ricin and abrin. *In vivo* assays include cell culture and the use of animals. Cell culture incorporates both B chain-mediated toxin uptake and A chain inhibition of protein translation. However, cell culture does not incorporate the complexity of uptake from the gastrointestinal tract or inhalation (Griffiths 2011). Further complicating *in vivo* studies are species-specific differences in the toxicity of ricin and abrin (Fodstad et al. 1979).

During recent years a significant effort has been made to develop multiplex methods that simultaneously detect multiple toxins. Ricin and abrin are often included in the repertoire (Kim et al. 2009; Pauly et al. 2009; Garber et al. 2010). The ability of these methods to safeguard society depends on the sensitivity and reliability of the assay to detect the various analytes under conditions that are not necessarily optimized but instead represent a compromise for the various target analytes. Fortunately, unless ricin is administered directly into the bloodstream or weaponized to reach the inner lung, its toxicity is not exceptional (Schep et al. 2009). As a result, the sensitivity necessary to detect ricin and abrin to avoid a lethal event, the oral human lethal dose (HLDo), is sufficiently high that most methods safeguard the public. Indeed, it is possible to accommodate less than optimum assay conditions while still detecting less than the HLDo. It is also possible to incorporate sample preparation protocols (e.g., dilution) whereby unique features of food matrices are nullified. This has been successfully applied to various analytical methods. One approach involves diluting the samples into buffers containing diluted milk. Though a slight loss in sensitivity has sometimes been observed, the LOD values are still less than the HLDo (Garber 2008; Dayan-Kenigsberg et al. 2008; Garber et al. 2010).

Seafood Toxins

The substances referred to as "seafood toxins" are nonprotein compounds, with molecular weights ranging from about 300 to 3,500. Unlike bacterial food contaminants and their proteinaceous toxins, seafood toxins are preformed and are relatively unaffected by thermal processing. The seafood toxins can be accumulated at a dangerous rate prior to harvest, but their net concentration does not increase following harvest. They tend to occur as families of structurally related compounds. Within these families of compounds, some members can transform to others. In some cases, these transformations are accompanied by large increases in toxicity so

that while concentration does not increase, toxicity may and one must consider "potential toxicity" in assessing food safety risk.

Most seafood toxins are encountered in fish and shellfish that are normally wholesome, but which may accumulate the toxins through their diet. The sources of the toxins are mostly single cell marine microorganisms (e.g., planktonic and benthic /photosynthetic and heterotrophic) that are eaten by filter feeders or surface grazers and can then be passed up successive trophic levels. The populations of such microorganisms vary greatly with time and location, so the toxicity in the accumulating organisms is similarly variable. In contrast, some seafood appears to be intrinsically toxic, although this may be due to toxic symbionts.

Since the 1920s, it has been common practice to assay seafood for toxicity by injecting mice intraperitoneally with seafood extracts. Such mouse assay is being replaced by methods that do not require live animals, but has served well for decades for detecting paralytic shellfish poison (PSP), and has been the most practical tool for detecting several other kinds of seafood toxicity. However, it is human oral potency that is of concern for the safety of consumers, not mouse intraperitoneal potency. Many compounds have been encountered in seafood extracts that have high mouse intraperitoneal potency but appear to have no significant human oral potency.

The following are some of the major toxin families most relevant to food safety:

Saxitoxins (paralytic shellfish poisoning) – The saxitoxins (Hall et al. 1990; Wiese et al. 2010) are produced by various dinoflagellates (*Alexandrium* spp., *Pyrodinium bahamense*, and *Gymnodinium catenatum*) and cyanobacteria (*Aphanizomenon flos-aquae*, *Anabaena circinalis*, etc.), accumulate in filter feeders such as bivalve molluscs and planktivorous fish, and may be passed to higher trophic levels. Bivalves are the best known vector to human consumers, thus the name of the syndrome, paralytic shellfish poisoning (PSP). The saxitoxins bind to a site on the extracellular surface of the voltage-activated sodium channel, blocking the passive influx of sodium ions that is essential for propagation of the action potential along nerve and muscle membranes. When in vivo concentrations of the saxitoxins are high enough, victims experience symptoms of peripheral nerve blockage, including numbness, paresthesia, and paralysis. For consumers of toxic seafood, onset is rapid and can lead to death due to respiratory paralysis in less than 30 min. However, the binding is entirely reversible at the molecular level and the saxitoxins are flushed rapidly from the body. Even with a potentially lethal exposure, respiratory support generally ensures survival; recovery is prompt and complete.

The saxitoxins are tricyclic compounds in which a ring containing a carbonyl group is flanked by two rings containing guanidinium groups. In aqueous solutions, the ketone/ketone hydrate equilibrium of the carbonyl group favors the ketone hydrate (gemdiol) because of the electron-withdrawing tendencies of the guanidinium groups. The gemdiol is essential for strong binding to the receptor site. Reduction of the ketone of saxitoxin leads to a single hydroxyl in either alpha or beta orientation instead of the gemdiol. In the case of the alpha hydroxyl, which tends to predominate under most reaction conditions, the potency of saxitoxin is

decreased more than 100-fold. This is entirely due to the shortening of its dwell time on the binding site; the on-rate is unaffected.

Many of the saxitoxins bear sulfate or sulfo substituents. Those with a sulfo group on the end of the carbamate side chain have substantially lower toxicity, due partly to a decrease in on-rate but more significantly to a decrease in dwell time. The toxicity can be increased (up to 70-fold) by removal of the sulfo group from the side chain to form the corresponding carbamate or by removal of the entire side chain to form the corresponding decarbamoyl saxitoxin. Since both transformations could occur during processing, storage, or preparation, this constitutes a reservoir of potential toxicity that needs to be considered in assessing food safety.

The saxitoxins are strongly polar and tend to be highly water soluble, although some congeners are now known with substituents that make them somewhat lipophilic. The saxitoxins are relatively stable at pH 4 and below. Susceptibility to oxidation at the bond between the ketone/gemdiol and the ring juncture increases with increasing pH, as the C-8 guanidinium is less fully protonated and the predominance of the gemdiol diminishes in the ketone/gemdiol equilibrium. The oxidation products tend to be purines, devoid of toxicity but strongly fluorescent. The oxidation to fluorescent degradation products is the basis for several detection methods. Saxitoxins bearing an N-1-OH substituent are more readily oxidized than those with N-1-H and, if conditions are too vigorous, may fail to yield fluorescent products.

Tetrodotoxins (puffer fish poisoning) – Though best known from and originally identified with puffer fish (fugu), tetrodotoxins are widespread among both marine and terrestrial animals, including various salamanders and frogs. They are employed in the venom of the blue-ringed octopus and some marine worms. In many cases they appear to be produced by symbiont bacteria. They have an adamantane-like cage skeleton with a single guanidinium group. Their pharmacology is virtually indistinguishable from that of the saxitoxins, binding to the same site on the external surface of the voltage-activated sodium channel in nerve and muscle membranes, interrupting the passive influx of sodium ions that is essential for propagation of impulses, and causing varying degrees of numbness and paralysis, which can be lethal. Treatment of victims involves primarily evacuation of remaining food and respiratory support as needed.

The tetrodotoxins in puffer fish tend to be more concentrated in the skin and viscera, allowing the carefully prepared flesh to be consumed with relatively low risk. In marked contrast, normally nontoxic puffer fish in a lagoon in Florida accumulated saxitoxins from their diet and retained relatively high levels in their flesh. Normal cleaning therefore did not protect consumers and several illnesses resulted.

Brevetoxins (neurotoxic shellfish poisoning) – The brevetoxins are linear cyclic polyethers based on two slightly different backbones. They are strongly lipophilic and bind to a site on the side of the voltage-activated sodium channel, causing the channel to remain open while they are bound. Their initial effects are therefore to cause hyperactivity of the nerve, although this sustained activation can eventually lead to depolarization and blockage. Symptoms include hypersensitivity to normal

stimuli and perceived reversal of hot and cold sensations. The principal source of the toxins is an unarmored dinoflagellate originally called *Gymnodinium breve*, then *Ptychodiscus brevis*, and most recently *Karenia brevis*. The brevetoxins can accumulate in shellfish and in some cases are metabolized to adduct that are also active. Lacking a protective covering, the cells are easily broken. When fish swim through blooms of the organism, the cells lyse as they pass through the fish gills, resulting in large fish kills. Cells in blooms near shore can be broken by wave action, taken up in sea spray, and blown ashore causing respiratory irritation in people on the beach. Fish kills and respiratory irritation are useful indicators of the presence of a bloom. Blooms are most common in the Gulf of Mexico, but significant events have occurred in New Zealand and along the mid-Atlantic coast of the USA. Symptoms tend not to be life-threatening and generally resolve within a couple of days (Watkins et al. 2008).

Ciguatera – Ciguatera is a syndrome that occurs in tropical fish, most notably in top carnivores like barracuda, with the risk being highest in larger, older fish. It is similar to NSP but more severe and more persistent. Symptoms, including itching and reversal of hot/cold temperature perception, last for weeks or more and often reoccur for months. It can be fatal. While various lipophilic toxins may be involved, the principal ciguatoxins resemble the brevetoxins and bind with much higher affinity to the same site on the voltage-activated sodium channel. The source organisms are benthic dinoflagellates, principally *Gambierdiscus toxicus*, which associates with small seaweed and coral surfaces. These are eaten by surface grazers and the toxins passed to successively higher trophic levels.

Because of its broad distribution through the tropics of the world and its debilitating, persistent symptoms, ciguatera has a very large socioeconomic impact. Because of its benthic origin, it is to some extent associated with particular locations, which can be avoided (Friedman et al. 2008).

Domoic acid (amnesic shellfish poisoning) – Domoic acid, like kainic acid, was originally isolated as the active ingredient in seaweed used traditionally as a vermifuge. Again like kainic acid, its structure is based on that of the secondary amino acid proline, with additional side chains terminating in carboxyl groups. It was not recognized to be a potent neurotoxin until a large number of people fell ill in 1987 after eating mussels from Prince Edward Island, Canada. The source was found to be pennate diatoms assigned to the genus Pseudo-nitzschia. Years later domoic acid was recognized as the cause of seabird and marine mammal deaths along the Pacific coast of North America. Monitoring has found it to be widespread in molluscs, crustaceans, and planktivorous fish.

In the majority of consumers, domoic acid causes brief GI distress and is rapidly eliminated without further effect. However, in consumers with compromised excretory function, it can pass through the blood/brain barrier and cause permanent CNS damage. It has a high affinity for glutamate receptors in the brain, particularly those in the hippocampus. Glutamate functions effectively as a neurotransmitter because it has a moderate affinity for its receptors and there are enzymes present that destroy it, maintaining a low concentration in the vicinity of the receptor sites. Domoic acid has a much higher affinity for the glutamate receptor sites and there

are no enzymes present to destroy it. Thus, the cells to which the glutamate receptors are attached suffer prolonged, eventually destructive stimulation from domoic acid molecules that are strongly bound and not cleared away. One of the consequences is permanent loss of short-term memory.

While there are many isomers of domoic acid, only the parent compound has significant activity. This, coupled with its UV chromophore, makes analysis relatively simple.

Okadaic acid (diarrhetic shellfish poisoning) – Okadaic acid, originally isolated from a sponge, was found to be a cause of diarrhetic shellfish poisoning (DSP) and to be produced by dinoflagellates from the genera *Dinophysis* and *Prorocentrum*. The DSP toxins include congeners of okadaic acid, some of which are fatty acid esters and have very low toxicity. These esters can be hydrolyzed *in vivo* so, like the 21-sulfo saxitoxins, constitute a reservoir of potential toxicity. The DSP toxins inhibit the action of protein phosphatase. The primary symptoms are severe diarrhea and other gastrointestinal distress. Tracking the occurrence of DSP is complicated by the similarity of the symptoms to viral and bacterial illnesses due to poor shellfish sanitation (Aune and Yndstad 1993).

Azaspiracids – The azaspiracids are a large family of lipophilic toxins. They were discovered fairly recently as the cause of severe diarrhea in consumers of shellfish from Ireland which contained no significant levels of okadaic acid or its congeners. Through extensive survey, isolation, and culture, the source has been found to be a small, armored dinoflagellate, previously unknown, and now named *Azadinium spinosum* (Tillmann et al. 2009). In an inversion of the history of okadaic acid and palytoxin, an azaspiracid has also been isolated from a marine sponge. The mechanism of action of the azaspiracids is not yet clear, although effects on a number of cellular systems have been demonstrated and there are some histological indications of grave damage in test animals (Twiner et al. 2008).

Palytoxins – Palytoxin was originally isolated from soft corals (zoanthids) of the genus *Palythoa*. More recently, palytoxins have been found in dinoflagellates of the genus *Ostreopsis*. Palytoxins may be encountered in seafood when organisms have grazed on *Palythoa* spp. or have filtered *Ostreopsis* spp. People are also at risk when they handle soft corals or breath mists formed from dense blooms of *Ostreopsis* spp. When palytoxins are injected, or when dispersed as a mist and inhaled, they are among the most toxic substances known, acting on the sodium/potassium pumps to form a nonselective ion channel and destroy the transmembrane potential. Taken orally, the palytoxins are much less potent, but still sufficiently toxic to cause illness or death. They are hemolytic and can be detected by the attenuation of hemolysis by antibodies specific for palytoxin (Deeds and Schwartz 2010).

Food Analysis

Detection methods for toxin monitoring may be considered to be of two types: assays and analyses. An assay provides a single number for a sample, the combined titer of all the toxins that it detects. An analysis separates the various toxic

components more or less completely, allowing each to be individually quantified. An assay requires only a single standard, while an analysis generally requires a standard for each toxin to be analyzed. Most modern analyses for seafood toxins are based on HPLC, often coupled with mass spectrometry for detection (LC-MS) since few of the seafood toxins have chromophores. The main exception is domoic acid, which absorbs well in the UV thanks to a conjugated double bond and is therefore easily analyzed by traditional HPLC with UV detection. Most of the saxitoxins can be oxidized to highly fluorescent products, so HPLC with pre- or post-column oxidation and fluorescent detection has proven very useful (Sullivan and Iwaoka 1983; Lawrence et al. 2005; van de Riet et al. 2011). Okadaic acid and some other toxins can be derivatized with fluorogenic reagents and then quantified by HPLC (Lee et al. 1987). However, the availability of fairly rugged benchtop MS detectors for HPLC has made LC-MS the method of choice for the analysis of most seafood toxins, particularly those that are lipophilic (McNabb et al. 2005).

Most assays employ selective binding to a receptor, followed by transduction of the selective event into a signal that can be measured. In the mouse bioassay, binding of toxin to receptors is reflected in the response of the mouse. In a typical receptor binding assay, binding of toxin to a preparation containing receptors, such as mammalian brain, is detected as the reduction of the amount of a radiolabeled reagent toxin bound. A receptor binding assay (rba) for the saxitoxins, using tritiated saxitoxin as the reagent toxin, is now an AOAC Official Method of Analysis (Van Dolah et al. 2012). Support by the IAEA is facilitating implementation of the rba in many countries. Cell cultures can also be used for assays. The advantage of all of these is that the response of the assay is based on the affinity of the toxins for the native receptors, so it is likely to reflect the relative toxicity of the various congeners in a toxin family. Immunoassays, on the other hand, employ synthetic receptors (antibodies) which often have very different spectra of responses to the various congeners. The charm of immunoassays is the relative ease of transducing the binding event into a detectable signal with coupled enzymes (ELISA). The challenge with immunoassays is to produce antibodies that have useful response spectra to the toxins of concern.

Food Safety

Food safety has emerged as a global challenge because it has an impact on daily life of our society. In spite of the recent advancement in the area of food science and technology and a growing concern raised by various national and international working groups on food safety, prevalence of foodborne illness/ food poisoning still remains a substantial cause of morbidity and preventable death. People can get sick when they eat food contaminated with bacteria, viruses, parasites, chemicals, and also toxins derived from bacteria, animals, and plants. Some foods are more likely than others to support growth of bacteria and their derived toxins when they are kept in the range of temperatures between 41 °F and 135 °F (danger zone). Food, moisture, temperature, and time are important factors

for bacterial growth. Bacteria and viruses are not usually killed by freezing, and they can survive and grow again under favorable conditions (e.g., temperature and time). It is important to understand that the contaminated food usually smells fine, looks safe, and tests good but it can still make someone very sick. Workers in food industries and public services are key players in safe food supply and prevent foodborne illness and its associated financial burden on public and to assure a government.

The food safety focuses mainly on practice of ensuring that foods cause no harm to the consumer and can be divided into three basic categories: (i) protection of the food supply from harmful contamination, (ii) prevention of the development and spread of harmful contamination, and (iii) effective removal of contamination and contaminants. Most food safety procedures fall into one or more than one of these categories. Food analysis is a key factor for food safety and ensures that food products are safe for consumers. ***Good food- hygiene practice is concerned with the protection of food against contamination by implementation of good manufacturing practices including personal hygiene and cleanliness, monitoring of temperature control and time, and applicable standard analytical techniques for food analysis for contaminants throughout processing, packaging, and shipping of food.*** Pasteurization is also used to destroy contaminants.

Government regulations and recommendations are designed to maintain the general quality of the food supply. There are a number of government departments responsible for regulating the composition and quality of foods including the US Food and Drug Administration (US FDA), the US Department of Agriculture (USDA), the National Marine Fisheries Service (NMFS), and the Environmental Protection Agency (EPA). Each of these government agencies is responsible for regulating particular sectors of the food industry and publishes documents that contain detailed information about the regulations and recommendations pertaining to the foods assigned by that agency.

Conclusions and Future Directions

Protection of the food supply requires that special attention be paid to the possibility of contamination of food with microbes and toxins derived from microbes, plants, and natural sources during food processing or resulting from intentional adulteration. Analytical methodology (detection methods) is important for surveillance monitoring as well as for responses to incidents of contamination. Despite advances made in the past decades, the detection of microbes and their toxins in foods remains challenging tasks and is relatively a new aspect of analytical food safety. The rapid, reliable, sensitive, and high-throughput methods are essential for prevention or assessment of a threat or toxin-related outbreak. A number of detection methods (e.g., RT-PCR, ELISA, ECL, LFA, ICA, and LC-MS) have been used for detecting toxins in food matrices, and their detection sensitivities vary in different food matrices. Another most important aspect of food protection is the proper implication of food safety programs. In order to be effective, food safety practice

must be informed about the nature of these contaminants, and food safety procedures must be science based. Government regulations and recommendations are designed to ensure the composition and quality of food is safe for consumers. Routinely monitoring of food safety practices throughout food processing by workers in food industries should be strictly enforced by regulatory agencies for safe food supply to consumers. This will be helpful to reduce the incidence of foodborne illness and its associated financial burden on the country.

Cross-References

- ▶ Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment
- ▶ Botulinum Toxin: Present Knowledge and Threats
- ▶ The Biowarfare Agent Ricin

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Impacts on Human Health Caused by Zoonoses

10

Chao-Nan Lin

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Abstract

Zoonosis is an infectious disease and a potential bioterrorism agent. Bioterrorism aimed at a society, government, and/or its citizens is meant to cause destabilization, fear, anxiety, illness, and death in people, animals, or plants using biological agents. A bioterrorism attack is the intentional release of biological agents such as viruses, bacteria, fungi, rickettsial or chlamydial organisms, toxins, or other harmful agents. This chapter focuses on the induction, monitoring, and prevention of some zoonotic diseases that have potential as

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bioterrorism agents. The etiology, clinical manifestations, transmission routes, and treatment of these zoonotic agents are briefly discussed.

Introduction

Zoonosis is an infectious disease that is transmitted between animals and humans. Zoonosis comes from the Greek words "zoon" and "osis," which represent "animal" and "ill," respectively. In a systematic review of 1,415 species of pathogens known to infect humans, 868 (61 %) were zoonotic. Unfortunately, the majority of emerging infectious diseases over the last three decades have been zoonotic (Taylor et al. 2001). Most zoonoses are often previously unrecognized diseases or have increased virulence in populations lacking immunity, such as henipavirus (Marsh and Wang 2012), severe acute respiratory syndrome (SARS), and influenza virus (swine-origin H1N1 or avian influenza H5N1) (Tseng 2007). The major factor influencing the appearance of novel zoonotic diseases in the human population is increased contact between humans and wildlife, such as (i) encroachment of human activity into wilderness areas and (ii) movement of wild animals into areas of human activity (Daszak et al. 2001).

Zoonoses are potential bioterrorism agents (Ryan 2008). Terrorist attacks using conventional weapons cause fear, havoc, illness, and death. Bioterrorism agents include bacteria, viruses, fungal, rickettsial or chlamydial organisms, and toxins, i.e., they can be transmitted between animals and humans (Spencer 2007). The potential for a bioterrorist attack is no longer a debate of "if" but "when" one will occur. It is impossible to predict when, where, or how bioterrorism will occur (Ippolito et al. 2006). Therefore, the control and prevention of these diseases in animals can also be accomplished to reduce disease transmission between humans and other animals.

This chapter documents the history, agents, routes of exposure, detection, monitoring, and prevention of zoonotic pathogens.

Zoonoses Likely to Be Used in Bioterrorism

Bioterrorism aimed at a society, government, and/or its citizens is meant to cause destabilization, fear, anxiety, illness, and death in people, animals, or plants (Balali-Mood et al. 2013). According to the US Centers for Disease Control and Prevention (CDC), a bioterrorism attack is the intentional release of biological agents such as viruses, bacteria, fungi, rickettsial or chlamydial organisms, toxins, or other harmful agents that cause illness or death in people, animals, or plants (Balali-Mood et al. 2013). These biological agents can be spread through the air, water, or food. The intended use of biological agents might target humans directly or might be used to disrupt an economy. The disease caused by anthrax was directed at animal populations as early as World War I. Glanders, a virulent disease in horses and mules, was used in the 1910s. Typhoid was reported in a water supply in the 1970s.

In September and October 2001, several cases of anthrax occurred in the United States. Letters laced with infectious anthrax were concurrently delivered to the US Congress and news media offices (Spencer 2007).

Zoonotic Pathogens

The US CDC categorizes biological toxins and bioterrorism agents as A, B, and C. Category A includes high-priority agents that pose a risk to national security because they (i) can be easily transmitted and disseminated from person to person, (ii) cause high mortality and have potentially major public health impacts, (iii) may cause public panic and social disruption, and (iv) require special action for public health preparedness. Category A agents include anthrax, plague, tularemia, botulism, filovirus, and smallpox. Category B, the second highest priority agents, includes pathogens that (i) are moderately easy to disseminate, (ii) cause moderate morbidity and low mortality rates, and (iii) require specific enhancements to the CDC's diagnostic capacity and disease surveillance ability. Category B agents include brucellosis, epsilon toxin, glanders, melioidosis, psittacosis, Q fever, ricin toxin, food safety threats, staphylococcal enterotoxin B, typhus fever, viral encephalitis, and water safety threats. Category C, the third highest priority agents, includes emerging pathogens that may be engineered for mass dissemination in the future because of (i) their availability, (ii) ease of production and dissemination, and (iii) potential for high morbidity and mortality rates and ability to cause major health effects. Category C agents include Nipah virus and hantavirus. Other zoonotic pathogens, such as rabies, West Nile virus, and *Streptococcus suis* type II, will also be discussed briefly. Table 1 summarizes the organisms and their classification by the US CDC.

Zoonotic Pathogens Listed in CDC Category A

Anthrax

Anthrax is one of the oldest known animal diseases and has been encountered since antiquity. The first documented used of anthrax as a weapon was in the 1910s during World War I. The most recent event of bioterrorism was in the United States in 2001, which resulted in five deaths (Spencer 2007). The causative pathogen, *Bacillus anthracis*, is an aerobic Gram-positive rod bacterium. It produces highly resistant spores that are stable when the vegetative form of the organism circulates in the blood during disease. Anthrax can be found naturally in soil and commonly affects wild and domesticated herbivorous animals that ingest or inhale the spores while grazing. The most common natural infection route in humans is cutaneous. However, spores may cause disease through inhalation or through the ingestion of contaminated animal products. The most common clinical manifestation is cutaneous anthrax, followed by respiratory anthrax and gastrointestinal anthrax (Balali-Mood et al. 2013; Waterer and Robertson 2009). The neurological symptoms of

Table 1 Zoonotic pathogens for potential use in bioterrorism

Disease	Pathogen	Previously used by terrorists	Clinical signs	US CDC category
Anthrax	*Bacillus anthracis*	Yes	Respiratory, gastrointestinal, or neurological symptoms	A
Plague	*Yersinia pestis*	Yes	Respiratory and gastrointestinal symptoms	A
Tularemia	*Francisella tularensis*	Yes	Respiratory symptoms	A
Viral hemorrhagic fevers	Ebola virus and Marburg virus (filovirus)	No	Neurological and respiratory symptoms	A
Brucellosis	*Brucella* spp.	No	Arthralgia, myalgia, and undulant fever	B
Salmonellosis	*Salmonella* spp.	Yes	Gastrointestinal symptoms	B
Colibacillosis	*Escherichia coli* O157:H7	No	Gastrointestinal symptoms	B
Shigellosis	*Shigella dysenteriae*	Yes	Gastrointestinal symptoms	B
Glanders	*Burkholderia mallei*	Yes	Respiratory symptoms	B
Melioidosis	*Burkholderia pseudomallei*	No	Respiratory symptoms	B
Psittacosis	*Chlamydophila psittaci*	No	Respiratory and gastrointestinal symptoms	B
Q fever	*Coxiella burnetii*	Yes	Respiratory symptoms	B
Typhus fever	*Rickettsia prowazekii*	No	Neurological and respiratory symptoms	B
Viral encephalitis	*Alphavirus*	No	Neurological symptoms	B
Cholera	*Vibrio cholerae*	Yes	Gastrointestinal symptoms	B
Cryptosporidiosis	*Cryptosporidium parvum*	No	Gastrointestinal symptoms	B
Nipah virus encephalitis	Nipah virus	No	Neurological and respiratory symptoms	C
Hantavirus pulmonary syndrome (HPS) and hemorrhagic fever with renal syndrome (HFRS)	Hantavirus	No	Renal and respiratory symptoms	C

(*continued*)

Table 1 (continued)

Disease	Pathogen	Previously used by terrorists	Clinical signs	US CDC category
Severe acute respiratory syndrome	SARS coronavirus	No	Respiratory and gastrointestinal symptoms	None
Influenza	Swine influenza and avian influenza virus	No	Respiratory symptoms	None
Rabies	Rabies virus	No	Neurological symptoms	None
West Nile encephalitis	West Nile virus	No	Neurological symptoms	None
Streptococcal toxic shock syndrome	*Streptococcus suis*	No	Neurological symptoms	None

anthrax infection are usually at a later stage and are part of an established systemic disease (Donaghy 2006). There are no risks of person-to-person spread. For people exposed to anthrax, 60 days of an oral antibiotic is recommended to prevent development of the disease (Waterer and Robertson 2009). Vaccination against anthrax is available and appears to be safe. However, the overall efficacy for preventing anthrax in stockpersons is 92.5 % (Inglesby et al. 2002).

Plague

Plague is an enzootic disease in rodents (Stenseth et al. 2008). The first documented use of plague as a weapon was in the fourteenth century. More recent bioterrorism using plague occurred in World War II (Spencer 2007). The causative pathogen, *Yersinia pestis*, is a Gram-negative, facultatively anaerobic, nonmotile, coccobacilli, nonspore-forming bacterium that was discovered by Yersin in 1984 to be transmitted by rat fleas (*Xenopsylla cheopis*) (Balali-Mood et al. 2013; Stenseth et al. 2008). There are three syndromes of human plague: bubonic, pneumonic, and septicemic plague. Most cases are in the bubonic form, which is a rapidly progressing, serious illness with a mortality rate of 40–70 %. *Y. pestis* is highly contagious from person to person and is transmitted by droplets of respiratory secretions. Plague is endemic in many regions such as the Americas, Asia, and Africa. Currently, more than 90 % of all plague cases are reported in Africa (Stenseth et al. 2008). Early antibiotic therapy is essential to reduce the risk of complications and death. Patients with pneumonic plague must be isolated to avoid aerosol transmission. Fortunately, unlike anthrax, *Y. pestis* does not form spores and does not survive well outside the body of the host (Waterer and Robertson 2009).

Tularemia

Francisella tularensis is a small, rod or coccoid, nonmotile, strictly aerobic Gram-negative, intracellular bacterium that causes tularemia (Waterer and Robertson 2009). Arthropod vectors transmit *F. tularensis* among animal reservoirs such as

rodents, hares, deer, beavers, and squirrels. Humans are occasionally infected via inhalation, direct contact with infected animals or their products, insect bites, or the ingestion of contaminated food or water. There are no risks of person-to-person spread. The incubation period of human tularemia is approximately 2–10 days, and it has a variety of clinical manifestations, such as asymptomatic, rapidly progressive, fulminant, and fetal disease. Antibiotics such as streptomycin and gentamicin are the drugs of choice. A live attenuated vaccine is recommended for laboratory workers who are routinely exposed to *F. tularensis* (Balali-Mood et al. 2013). There have been no confirmed cases of tularemia used in bioterrorism.

Viral Hemorrhagic Fevers

Ebola virus and Marburg virus are the causative agents of viral hemorrhagic fever (Leroy et al. 2011). Both viruses belong to the family *Filoviridae*, which causes severe disease in humans and nonhuman primates. The *Filoviridae* family is classified within the order Mononegavirales, together with the *Bornaviridae*, *Rhabdoviridae*, and *Paramyxoviridae* families. The genome of all viruses under the order Mononegavirales consists of a linear, non-segmented, single-strand RNA molecule. Filovirus hemorrhagic fevers are typical zoonotic diseases that are transmitted accidentally through direct contact with infected animals. The role of wildlife species in the human epidemiology of filovirus is only partly understood. Several Zaire Ebola virus outbreaks occurred when hunters handled the infected carcasses of nonhuman primates and duiker (Colebunders and Borchert 2000); outbreaks may also be associated with exposure to fruit bats (Calisher et al. 2006; Leroy et al. 2011). The fruit bats *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata* represent possible Ebola reservoirs in Africa (Leroy et al. 2011). A high risk of transmission to people has been reported as contact with the patient in the later disease stages. The clinical manifestations for filovirus infection are epistaxis, melena, hematemesis, petechiae, ecchymosis, and bleeding at needle puncture sites. Patients may also develop neurological symptoms such as delirium, coma, and convulsions (Colebunders and Borchert 2000). There is no specific treatment for filovirus hemorrhagic fever. Supportive care is required for the treatment. Blood transfusions from convalescent patients were reported to help in the Kikwit Ebola epidemic. Several antiviral compounds have been shown to inhibit the viral replication in vitro and prevent death in animal models (Leroy et al. 2011).

Zoonotic Pathogens Listed in CDC Category B

Brucellosis

Brucellosis is a zoonotic disease that commonly causes reproductive failure and undulant fever in domestic animals and humans, respectively (Doganay and Doganay 2013). The causative pathogen, a *Brucella* species, is a small, nonmotile, non-sporulating, Gram-negative coccobacilli bacterium. The most common transmission route of brucellosis is contact with infected animals and their products such as milk, butter, cream, cheese, ice cream, urine, blood, carcasses, and abortion

products. Brucella organisms can survive for up to 2 days, 3 weeks, and 3 months in milk (at 8 °C), frozen meat, and goat cheese, respectively. Brucellosis is rarely transmitted between humans. Aerosol transmission and dissemination is considered the most effective route of delivery in a bioterrorism scenario. The treatment of brucellosis is based on the administration of doxycycline or streptomycin (Ariza et al. 2007). Currently, there is no safe and effective human brucellosis vaccine. Only live and attenuated vaccines have been used effectively in livestock (Yumuk and O'Callaghan 2012).

Food Safety Threats

Several foodborne diseases, including *Salmonella* species, *Escherichia coli* O157:H7, and *Shigella*, should be considered (Chang et al. 2012; Franz and van Bruggen 2008; Lim et al. 2010). Salmonellosis represents a major foodborne and waterborne disease. *Salmonella* species are rod-shaped, Gram-negative, nonspore-forming, facultative anaerobic, motile enterobacteria. Salmonellosis is a zoonotic disease, and various agricultural animals, such as cattle, pigs and chickens, can form a reservoir for this bacterium. Additionally, vegetable contamination has been reported to be an important route of *Salmonella* species infection (Franz and van Bruggen 2008). The disease presents with two clinical manifestations including typhoid and typhoid-like fever and gastroenteritis. The most common infection route of salmonellosis is through the ingestion of contaminated food. Therefore, the establishment of human salmonella infection depends on the ability to survive the environment of the digestive system (such as gastric acid) and to attach and enter intestinal cells.

Escherichia coli (*E. coli*) is a Gram-negative, facultative anaerobic bacterium. Enterohemorrhagic *E. coli* (EHEC) is one of the main pathogroups of *E. coli* and includes a definite zoonotic pathogen. The prototype EHEC strain, *E. coli* O157:H7, was first identified in 1982 and causes bloody diarrhea and hemolytic uremic syndrome in humans (Lim et al. 2010). Cattle is considered the primary reservoir of *E. coli* O157:H7 (Etcheverria and Padola 2013), but the pathogen has been isolated from a variety of animals such as sheep, pigs, horses, chickens, and wildlife (Lim et al. 2010). The most common infection route of *E. coli* O157:H7 is contact with contaminated food, water, animal feces, or an infected animal. Several disease outbreaks have been associated with the consumption of contaminated beef (Lim et al. 2010). Shiga toxin is a potent cytotoxin and the critical virulence factor in EHEC diseases (Bergan et al. 2012).

Shigellosis is caused by *Shigella*, which is a Gram-negative, nonspore-forming, and rod-shaped bacterium. *Shigella* species are related to *E. coli* and should be classified as a distinctive species in the genus *Escherichia*. The two bacteria evolved from the same ancestor (van den Beld and Reubsaet 2012). Shigella is a human-specific causative agent of bacillary dysentery; the clinical manifestations are abdominal cramps, nausea, fever, and bloody and mucoid in diarrhea. This disease is primarily transmitted via the fecal-oral route.

To treat patients with severe diarrhea and dehydration, it is necessary to use IV fluids immediately to replace the fluids and salts that are lost. If the patients can

drink, oral rehydration therapy is satisfactory (Dekate et al. 2013). The decision to treat with antimicrobial therapy should be made on a case-by-case basis. Antimicrobials can be administered for acute diarrhea when the pathogen is known. Additionally, anti-induction strategies to prevent toxin production and the use of anti-Shiga toxin antibodies have been proposed for the treatment of *E. coli* O157: H7 (Tzipori et al. 2004).

Glanders

Burkholderia mallei is the causative agent of glanders (Whitlock et al. 2007). *B. mallei* is a Gram-negative, nonmotile, facultative intracellular bacterium. This disease primarily affects equids (such as horses, donkeys, and mules) and causes glanders and farcy (known as the cutaneous form) depending on the route of infection. The transmission route of this disease is through inhalation, percutaneous inoculation, or ingestion. Acute infection causes high fever, emaciation, and ulceration of the nasal septum with mucopurulent to hemorrhagic discharge. Glanders can cause an acute or chronic lung infection and nodules on internal organs such as the liver and spleen. *B. mallei* is highly infectious as an aerosol, and infection requires only a few bacteria. Fortunately, human glanders is rare, and human-to-human transmission is extremely rare. The clinical presentation of human glanders includes ulcerative necrosis of the upper and lower respiratory tract, extensive pneumonia, cervical or mediastinal lymphadenopathy, pustules, and abscesses. Antibiotics such as tetracycline, ceftazidime, cefotaxime, amoxicillin-clavulanate, piperacillin-tazobactam, imipenem, trimethoprim-sulfamethoxazole, and streptomycin are the drugs of choice. No vaccine is currently available (Choh et al. 2013).

Melioidosis

Melioidosis is caused by *Burkholderia pseudomallei*, a Gram-negative bacillus. This organism is most commonly found in soil and water in melioidosis-endemic areas. Transmission in humans occurs following bacterial inhalation, inoculation, and ingestion. This disease was described as a “glanders-like” disease. The development of melioidosis symptoms is related to several factors such as the bacterial strain, the host immune response, and the route of transmission. Acute infection causes severe pneumonia or rapid septicemia. Chronic stages are associated with the formation of abscesses in multiple organs or even an asymptomatic infection. Currently, prolonged antibiotic therapy is the only option for controlling the melioidosis infection. The antibiotic susceptibility pattern of *B. pseudomallei* is generally similar to that of *B. mallei*. At present, there are no effective vaccines for the prevention of melioidosis (Choh et al. 2013).

Psittacosis

Psittacosis is a zoonotic disease caused by *Chlamydophila psittaci*, which is a Gram-negative, obligate intracellular bacterium. The disease is also known as parrot fever. The clinical manifestation of human psittacosis includes flu-like syndromes such as fever, headache, exhaustion, arthralgia, and loss of appetite. Atypical pneumonia is usually detected on X-rays. In avian chlamydiosis, bird

psittacosis is characterized by clinical signs such as anorexia, depression, respiratory distress, or diarrhea. The most common transmission route for human psittacosis is via the inhalation of contaminated material such as dried feces or nasal discharge. Human psittacosis is most frequently treated with antibiotics such as doxycycline, tetracycline, or chloramphenicol. Chlamydiae can develop persistent forms, which can lead to chronic clinical courses (Magnino et al. 2009).

Q Fever

Q fever is caused by *Coxiella burnetii*, which is a Gram-negative, pleomorphic coccobacilli, and obligate intracellular bacterium. Q fever is a zoonotic disease. Sheep, cattle, goats, birds, dogs, and cats are natural reservoirs for *C. burnetii*. This rickettsia is highly sensitive to environmental stresses such as ultraviolet light, osmotic pressure, and high temperature. Disease transmission can be tick-borne between animals; infection in humans is caused by inhaling droplets containing a few *C. burnetii* bacteria. The foodborne transmission of human Q fever rarely occurs. Q fever in animals commonly causes reproductive disease, especially abortion, infertility, and retained placenta. The clinical manifestations of Q fever are chronic (such as endocarditis, chronic granulomatous hepatitis, aseptic meningitis, and encephalitis) or acutely symptomatic (pneumonia or hepatitis), while some cases are asymptomatic. Doxycycline is recommended as the first-line treatment for Q fever (Balali-Mood et al. 2013).

Typhus Fever

Typhus fever, also known as epidemic typhus, is caused by *Rickettsia prowazekii*, which is a nonmotile, Gram-negative, and obligate intracellular bacterium (Bechah et al. 2008). Four *Rickettsia* species frequently cause incapacitating and life-threatening illness. Lice are the most important reservoir of *R. prowazekii*. In the United States, flying squirrels (*Glaucomys volans volans*) have also been identified as potential reservoirs. The transmission route of epidemic typhus has been suggested to occur through the inhalation of aerosolized infected feces from lice. Disease outbreaks have generally been related to war, famine, refugee camps, cold weather, or gaps in public health management. The clinical manifestations of typhus fever include rashes, neurological syndrome, respiratory syndrome, and shock. Tetracycline and chloramphenicol have been recommended for the treatment of epidemic typhus (Badiaga and Brouqui 2012).

Viral Encephalitis

Several viral encephalitis varieties should be considered (Donaghy 2006), including eastern equine encephalitis, Venezuelan equine encephalitis, and western equine encephalitis. The pathogens are arthropod-borne RNA viruses of the genus *Alphavirus* under the family *Togaviridae* and are highly pathogenic in equines and humans. The most common transmission route of alphaviruses is thought to be an arthropod vector such as mosquitoes (Go et al. 2014). The survival of alphaviruses depends on the presence of mosquitoes and vertebrate hosts. The human infection is characterized by fever, headache, lymphopenia, myalgia, malaise, and severe

neurological signs such as fatal encephalitis. There are no effective antiviral drugs to treat the viral encephalitis. Supportive care is recommended to reduce brain swelling and seizures. Vaccines against the alphavirus are currently at various stages of development (Zacks and Paessler 2010).

Water Safety Threats

Vibrio cholerae and *Cryptosporidium parvum* should be considered water safety threats (Austin 2010; Fayer 2004) and are listed in category B by the US CDC. *V. cholerae* is a Gram-negative, rod-shaped, motile bacterium. Several cholera pandemics have been related to O1 and O139, which are structures of the O antigen in its lipopolysaccharide. The cholera toxin (CT) is the primary toxin produced by *V. cholerae* O1 and O139. CT is important for the clinical manifestations of cholera. This disease causes the patient to hypersecrete electrolytes and water, sometimes causing death. The most common clinical presentations of this disease are watery diarrhea, which may be associated with vomiting, muscle cramps, and complications related to dehydration and metabolic acidosis. There are two routes of transmission of *V. cholerae*: aquatic reservoirs in the environment (primary transmission) and already-infected individuals (secondary transmission), which initiate outbreaks and epidemics in the endemic areas, respectively. Rehydration is the first recommendation for cholera treatment, but antibiotics have also been shown to be important therapeutics in both severe cases and epidemic situations. Cholera is most frequently treated with antibiotics such as doxycycline and tetracycline. Furazolidone, erythromycin, trimethoprim-sulfamethoxazole, ampicillin, and chloramphenicol are effective against severe cholera in young children and pregnant women. For multidrug-resistant cholera, ciprofloxacin is an important substitute drug of choice. The currently available killed injectable vaccine has been shown to be less effective.

Cryptosporidiosis is the most prevalent waterborne parasitic disease caused by *C. parvum* (Fayer 2004). This medically and veterinarily important disease causes gastroenteritis in a variety of vertebrate hosts. Cryptosporidiosis is characterized by watery diarrhea; other clinical features include abdominal discomfort, nausea, vomiting, and low-grade fever. Cryptosporidium infection can also cause atypical manifestations, such as gastrointestinal, biliary, or respiratory syndromes and pancreatitis, in immunocompromised patients. Cryptosporidium transmission is waterborne and is contracted directly from infected hosts. There are no consistently effective treatments for cryptosporidiosis in animals or humans. Paromomycin provided prophylaxis in an animal model but was inconsistently efficacious in humans. No immunotherapeutics or vaccines are currently available for preventing or treating cryptosporidiosis in animals or humans.

Zoonotic Pathogens Listed in CDC Category C

Nipah Virus

Nipah virus (NiV) first emerged in the 1990s in an outbreak of neurological and respiratory disease that infected pigs and humans and caused 110 human deaths in

Malaysia and Singapore (Marsh and Wang 2012). NiV is a single-stranded negative RNA virus that, together with the Hendra virus, is a variety of *Henipavirus* under the family *Paramyxoviridae*. *Pteropus* species fruit bats were identified as natural reservoir hosts of henipavirus (Calisher et al. 2006). Several NiV outbreaks appeared after human contact with excreta such as the saliva, urine, and feces of diseased pigs or bats. Person-to-person transmission has also been reported. The treatment of human patients with NiV infection remains dependent on supportive care. Humanized monoclonal antibodies have been used successfully to treat NiV infections in animal models (Zhu et al. 2006).

Hantavirus

Hantavirus is an enveloped, single-stranded, negative-sense RNA virus. Hantaviruses cause the most widely distributed zoonotic disease (Klempa et al. 2013). There are two important syndromes: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (Macneil et al. 2011); the occurrence of these syndromes depends on the infecting virus species. HPS is also referred to as hantavirus cardiopulmonary syndrome (HCPS). The Old World hantaviruses were identified during the outbreak of HFRS in Asia and Europe. The New World hantaviruses include species that cause HPS in North and South America. The epidemiology and geographical distribution of hantaviruses are related to their rodent reservoirs. The infected rodents exhibited asymptomatic and persistent infections. Additionally, several non-rodent reservoirs, such as crocidurines, shrews, moles, and bats, have been discovered. The most common transmission route of hantaviruses is via contact with virus-contaminated rodent feces, urine, or saliva. Human-to-human transmission is minimal, with the exception of the Andes virus outbreak in Argentina. There are no effective antiviral therapeutics available for hantavirus infections. Supportive care and therapy are the best treatments to inhibit the progression toward life-threatening symptoms. The most common prevention tactic is avoiding exposure to rodent excreta (Klempa et al. 2013).

Other Zoonotic Diseases

SARS and Influenza

The outbreak of severe acute respiratory syndrome (SARS) and influenza virus (swine-origin H1N1 or avian influenza H5N1) represents the new face of pandemic disease (Tseng 2007). These pathogens share some common characteristics: they have animal origins, are highly contagious, are fatal viruses, and lack vaccines. Therefore, these zoonotic diseases are proposed as threats in a bioterrorist attack. The outbreak of SARS in 2002–2003 had approximately 8,000 probable cases in 29 countries and a fatality rate of approximately 10 %. Due to the speed and reach of international air travel, SARS spread globally within weeks; additionally, transmission was amplified within hospitals. In 2009, an outbreak of a swine-origin H1N1 demonstrated worldwide expansion. These diseases are most effectively spread as aerosols. Therefore, aerosol sprays would be the most common route in a potential bioterrorism attack (Tseng 2007).

Fig. 1 A ferret-badger suffering from rabies virus in Taiwan (From Dr. Chen-Chih Chen, National Pingtung University of Science and Technology, Taiwan)

Rabies

Rabies virus is an enveloped, single-stranded, negative-sense, bullet-shaped RNA virus that belongs to genus *Lyssavirus*, under the family *Rhabdoviridae*. Rabies virus is transmitted between mammals (Calisher et al. 2006). The most common transmission route of rabies virus is by the bite of an infected animal that has rabies virus in its saliva. More than 55,000 cases of human rabies are reported yearly in the world, most in tropical countries of Asia and Africa. Rabies in enzootic areas appears to be cyclic and spreads into unexposed and susceptible wildlife populations. These wildlife species serve as maintenance hosts for virus transmission to domestic animals. The wildlife species include foxes, raccoon dogs, jackals, mongooses, wolves, manuls, and ferret-badgers (Fig. 1).

There are two important syndromes of rabies virus infection: furious and paralytic. Not all infected animals progress through all the clinical stages. There is no effective treatment for animals and humans with this fatal encephalitis. Vaccination is recommended to control rabies in dog and cat populations. Oral ingestion of an attenuated strain of rabies virus has been used in feral and wild animals. These baited vaccines may be effective in wildlife. For preventing human rabies, three doses of an FDA-approved vaccine are recommended in preexposure. Preexposure prophylaxis is warranted in humans with a high vocational risk of encountering rabid animals, such as veterinarians, animal health technicians, animal control officers, wildlife biologists, bat handlers, laboratory workers, and spelunkers. For previously unvaccinated patients, postexposure prophylaxis is 100 % effective against rabies.

West Nile Virus

West Nile virus (WNV) is an enveloped, single-stranded, positive-sense RNA virus that, together with the Japanese encephalitis virus and dengue virus, is a variety of *Flavivirus* under the family *Flaviviridae* (Go et al. 2014). The main transmission route of flavivirus is thought to be an arthropod vector such as mosquitoes. *Culex* mosquitoes are the main vector of these viruses. WNV was first isolated in West

Fig. 2 Pig, such as the one pictured here, is affected by *Streptococcus suis*. The neurological symptoms such as meningitis, paddling, opisthotonus, convulsions, and nystagmus are the most striking feature of this disease (From Dr. Ming-Tang Chiou, National Pingtung University of Science and Technology, Taiwan)

Nile District, Uganda, in 1937. Before its appearance in New York, WNV caused sporadic outbreaks in Africa, the Middle East, Asia, and Australia. WNV has spread rapidly across the United States, Canada, and Central and South America and is currently one of the most common causes of epidemic encephalitis in the United States. The most common of the WNV infections in humans are subclinical, but some patients develop clinical signs and symptoms such as biphasic fever, malaise, headache, nausea, anorexia, vomiting, myalgia, and arthralgia. The inactivated WNV vaccine produced by Fort Dodge Animal Health received a full licensed from the US Department of Agriculture in 2003.

Streptococcus suis Type II

Streptococcus suis is a peanut-shaped, Gram-positive bacterium and an opportunistic pathogen in swine (Fig. 2). *S. suis* is also an emerging zoonotic pathogen among humans (Gottschalk et al. 2010). It is the leading cause of human acute bacterial meningitis in several countries, including Vietnam and China (Mai et al. 2008; Tang et al. 2006). The transmission route of this disease in humans is associated with direct exposure to infected pigs or infected raw or undercooked pork products. Penicillin is recommended as the first-line treatment for *S. suis* infections.

Detection and Early Identification of Zoonotic Pathogens

Syndromic Surveillance

Zoonotic pathogens, such as those in a bioterrorism event, must be recognized in a timely manner; however, this reaction time is dependent on sufficient funding, training, equipment, and personnel. An intimate understanding of the natural ecology, geographic distribution, clinical syndromes, and lesions of a given disease

is essential for early recognition and control (Bravata et al. 2004). Early diagnosis and prompt, effective control measures are critical determinants of the eventual impact of any infectious disease emergency. Syndromic surveillance is the gathering of data for public health purposes before laboratory-confirmed information is available. Nurses and veterinarians are important resources in collecting and interpreting surveillance data. Many modern diseases are zoonotic diseases; therefore, veterinarian and medical staff face an enormous challenge in the early recognition, reporting, treatment, and prevention of zoonotic diseases. After an acute outbreak event, the active surveillance of wild or domestic animal populations may help identify many ongoing exposure risks.

Molecular Approaches

Bacteria and viruses are the most problematic zoonotic pathogens. Therefore, rapidly and accurately identifying the pathogens in a disease outbreak is a key factor in any biological defense strategy. Detection and identification methods based on real-time PCR assays are currently of the greatest use for zoonotic pathogens because of their rapidity, sensitivity, and reproducibility (Ivnitski et al. 2003). Microarray-based (gene chips) technologies offer great potential for environmental monitoring; this approach includes improved accuracy, lower power and sample consumption, disposability, and automation (Ivnitski et al. 2003). Additionally, hybrid technologies represent a stand-alone system for the rapid, continuous monitoring of multiple biological agents in a given environment (Ivnitski et al. 2003). This system has several key advantages over competing technologies, including (i) the ability to detect up to 100 different agents, (ii) the flexibility and ease with which new bead-based assays can be developed and integrated into the system, (iii) low false-positive and false-negative rates, (iv) the ability to use the same basic system components for multiple deployment architectures, and (v) the relatively low cost per assay and minimal consumables. Therefore, hybridization-based approaches will be extended for detecting bioterrorism agents in the near future (Ivnitski et al. 2003).

Next-generation sequencing (NGS) can be an attractive tool for broad-based pathogen discovery. The technique, also known as massively parallel to deep sequencing, has emerged as one of the most promising strategies for discovering novel infectious agents in clinical specimens. NGS approaches have also been successful in the identification of novel animal viruses. There are two main parameters for the choice of NGS platforms for pathogen discovery: read length and rad depth. Therefore, currently available technologies such as NGS can survey the full breadth of undiscovered pathogens (Chiu 2013).

Immunological Methods

Immunoassays are regularly used to confirm a clinical diagnosis. Immunological methods include immunochromatographic lateral flow assays,

electrochemiluminescence assays, ELISA, time-resolved fluorescence assays, immunofluorescence assays, and immunohistochemistry. The primary disadvantage of polyclonal antibodies of immunoassays is the lack of the specificity required for useful detection and definitive identification, due to the cross-reactive properties of certain antigens of various pathogenic species.

Biosensors

Biosensors represent a combination of biological receptor compounds (such as enzymes, antibodies, microorganisms, and DNA) in proximity to the signal transducer that can provide a reagent-free sensing system that is specific for a target analysis (Deisingh and Thompson 2004). The first biosensor was the “enzyme electrode,” which was used to describe glucose oxidase in 1962. The biosensor enables a broad spectrum of analyses in complex sample matrices (such as blood, serum, urine, or food) and the real-time monitoring of specific biological agents. Biosensors have shown great promise in areas such as clinical diagnostics, food analysis, bioprocess, and environmental monitoring (Leonard et al. 2003). However, several disadvantages of biosensors have been observed, including high costs, hazards, and disposal problems in radiolabeled probes (Ivnitski et al. 1999); a long incubation time and insufficient sensitivity in flow immunosensors (Ivnitski et al. 1999); a long incubation time in piezoelectric biosensors (Ivnitski et al. 1999); a long assay time and lack of sensitivity in bioluminescence sensors (Ivnitski et al. 1999); high cost and flow cell stability in surface plasmon resonance-based biosensors (Leonard et al. 2003); and antigen-binding sites in phage-antibody technology (Dover et al. 2009).

Prevention and Preparedness Against Zoonoses

A zoonotic outbreak has the potential for mass destruction and may cause significant economic losses. The majority of emerging infectious diseases are zoonotic in origin (Taylor et al. 2001). A zoonotic disease outbreak in human populations would likely pose a health risk to animal populations in the target area. Therefore, communication between veterinary and human public health officials is essential. There are several steps related to preparedness for zoonotic diseases: (i) Improved communication is necessary between veterinarian and human health professionals, as is surveillance of animal populations (such as wildlife and companion animals) and thus the control and prevention of zoonotic disease in animals. Domestic and wildlife animals are exposed to infectious agents and environmental contaminants in the air, soil, water, and food; therefore, animals serve as disease sentinels or an early warning system (Rabinowitz et al. 2006). (ii) There should be adequate planning, such as diagnostic tools, for the zoonotic diseases. (iii) Antibiotics should be stockpiled. (iv) Training should be provided to medical personnel and government officials. (v) Personal protective equipment (such as eye protection,

gowns, gloves, and masks) would facilitate reducing the likelihood of infection through either direct contact or respiratory droplets. (vi) Vaccinations should be planned.

Conclusion and Future Directions

The majority of emerging infectious diseases are zoonotic in origin. Medical and veterinary communities should work closely in clinical, public health, and research settings. It is impossible to predict when, where, or how zoonotic disease will occur. All countries should continue to carefully monitor the events associated with zoonotic disease and should work to develop defense strategies.

Cross-References

▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
▶ Biotoxins and Food Safety
▶ Botulinum Toxin: Present Knowledge and Threats
▶ Immunosensors: Using Antibodies to Develop Biosensors for Detecting Pathogens and Their Toxins
▶ Structure, Genetics, and Mode of Disease of Cholera Toxin
▶ The Public Health Response to Potential Bioterrorism by Toxin Attack
▶ Yesterday, Today, and Tomorrow: A Selective View of Toxins in Weapons and Medicine

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Counterfeit Botulinum Medical Products and the Risk of Bioterrorism

11

Andy Pickett

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Abstract

Botulinum toxin has established a strong, worldwide position for the treatment of a wide range of clinical and aesthetic conditions. The products have brought considerable benefits to patients, from children with cerebral palsy to older patients with stroke-related conditions. Aesthetic use alone is probably over 10 million treatments per year and rising rapidly. The latest licensed applications in pain treatment and urology are amongst a range of new applications

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also under study. With this overall rise in clinical use, an increase in the availability of counterfeit and fake products has occurred. These products are readily available from Internet sources and may be purchased by doctors or patients. Their quality is, however, of considerable concern, in particular their content of excess amounts of active botulinum toxin in some cases and their lack of assurance of sterility for an injectable product. The potential for the sources of such products to become providers for bioterrorist activities is therefore of key importance. Can botulinum toxin from medical sources become a terrorist weapon? The background to this situation is discussed in detail, and the potential for such medical product use for bioterrorism acts is considered, using case studies.

Introduction

The use of botulinum toxin (BoNT) for both therapeutic and aesthetic applications has grown continually in the last 25 years since the first products were licensed in the United States (Oculinum®, now Botox®) and the United Kingdom (Dysport®) (Berry and Stanek 2012; Persaud et al. 2013). In addition to these main uses, there is a myriad of other equally important uses now being pursued in pain, dermatology, and a range of specific, often rare diseases (Feily et al. 2011). There is no decrease in use for products that should, by now, be past their peak of applications with the emergence of newer products. But nothing available currently or in development has the overall qualities of BoNT – exceptional potency (low-dose administration), long-term benefit (up to 6 months from a single injection is commonplace), and virtually guaranteed success in the treatment of many clinical conditions.

Two key aspects about BoNT have kept the products in the forefront of clinical uses and development. Firstly, the exceptional potency of the molecule, where doses are typically in nanogram quantities, has yet to be surpassed. Newer-generation, hybrid molecules are showing activities only at higher-protein doses when compared to the original parent molecules (e.g., μM compared to pM – Stancombe et al. 2012). Secondly, new ways BoNT can be used are being discovered as different sites of action and mechanisms of action are identified. Given that new neurotransmitters are still being discovered (D'Aniello et al. 2011) and that BoNT is showing varying affinities to various organelles other than nerves, in, for example, the skin (sebaceous gland, eccrine gland, activity in regulating cell growth and collagen formation) (Pacini et al. 2007; Xiao and Qu 2012; Oh et al. 2012; Li et al. 2013), there is a clear way forward for new applications to be identified in the foreseeable future.

With a market size now valued in billions of dollars, the attraction for some to try and take part of the market by illegal means is significant. Counterfeit medicines are now big business across the world. The Pharmaceutical Security Institute (PSI), established in 2002 as an initiative of 14 companies to combat counterfeit medicines, has recorded some 2000 incidents of these illicit products every year for the last 4 years (http://www.psi-inc.org/incidentTrends.cfm. Accessed 10 Nov 2013). Nearly 1000 of these were linked to Asia. Although by no means the largest

commercial biological product, BoNT is attractive particularly for the aesthetic applications and, so many believe, the relative ease with which the products can be directly linked or even sold to the patient directly. Coupled with ease of access to counterfeit products through the Internet, the attraction of BoNT as a means of illicit gains is clear.

The subject of bioterrorism is of much concern to many countries and their agencies worldwide. Many books have been written on the subject (see, e.g., Morse 2012; Khardori 2006). One encyclopedia is published relating to the entire subject area (Pilch and Zilinskas 2011), and there is at least one journal dedicated to the topic (Biosecurity and Bioterrorism; Biodefense Strategy, Practice, and Science: Mary-Ann Liebert, Inc., publ.).

Within the bioterror category, BoNT has received detailed attention since both the neurotoxin and the pathogenic organism are potential agents for misuse. Governments and their agencies have planned strategies if either or both are ever employed in the field (Bossi et al. 2004; Knutsson 2011). With the significant clinical use of BoNT now, the potential for this to be used as a bioterror weapon needs to be examined and monitored, especially in relation to the availability of the products, fake and counterfeit versions, and their availability for use in certain circumstances which may cause harm to animals or humans or both.

Here, the current situation regarding the potential for use of BoNT as a bioterrorism agent, originating from counterfeit botulinum medical products, as of the end of 2013 will be examined and discussed. Ease of access to these illegal products, with examples, will be described. In particular, the relevance of ready availability of these products to potential risks of bioterrorism will be explored.

What Is a Counterfeit Botulinum Toxin Product?

In general, the term “counterfeit” has been used loosely to describe different types of illegal (i.e., copied, unlicensed, and/or falsely manufactured) medicinal products. The term as defined by PSI (http://www.psi-inc.org/counterfeitSituation.cfm. Accessed 10 Nov 2013) is as follows:

> Counterfeit medicines are products deliberately and fraudulently produced and/or mislabeled with respect to identity and/or source to make it appear to be a genuine product.

This is a strong definition which means, in practice, any direct attempt to illegally copy an original, branded product is considered a counterfeit. This will mean use of the original trademark in a product format (vial) and packaging (box/carton) that closely resembles the original in most, if not all, respects.

There is, however, another category of BoNT product which is equally important and which is also considered here, namely, “fake” products (previously called “look-alike” products). A suitable definition of these products would be:

> A fake BoNT product is one that has a misleading or false appearance, with the purpose to look real or valuable and hence to deceive the purchaser/clinician/patient. (Pickett 2011)

Typically, a fake BoNT product is sold via the Internet or from undetermined sources of origin, with product names intended to convey the quality of the product or convince the observer of the product potential to achieve certain results if used. Names of such products are *not* the same as the branded originals but are often manipulated to closely resemble those. Examples of fake BoNT products are Beauteous, Amazing, Refinex, Excellent, ßotoxinA (beta zero toxin A), Beautox, Biotox, MedBotox.

Background

There is no specific date that can be identified when counterfeit and fake BoNT products (CFBP) appeared on the market. Detailed observation of the Internet and other sources for the last 7 years has shown that their presence, persistence, and availability are quite constant, regardless of publications, publicity, and warnings about their nature in learned publications, trade magazines, and daily newspapers.

The concept that BoNT could be used as a weapon of mass destruction (WMD) or by a bioterrorist organization has existed for many years, certainly back to the original days when activities such as the US Army Biological Warfare Laboratories, Camp Detrick, now Fort Detrick, were first established in the 1940s. Equivalent sites were set up in other countries (e.g., at Porton Down in the United Kingdom) and generally owned by the armed forces of each country, at least initially. But other institutes, notably the Center for Nonproliferation Studies at the Monterey Institute of International Studies (MIIS) in California, USA, have maintained an independent assessment of the biological WMD situation, country by country and agent by agent, in addition to the governmental activities.

The issues for BoNT as, firstly, a pharmaceutical product and a highly researched biological protein, coupled with the WMD and bioterrorism potential, were probably first voiced by Jonathan Tucker of MIIS in 2001, during a testimony before the US Senate on Governmental Affairs, Subcommittee on International Security, Proliferation, and Federal Services on 7 November 2001 (http://cns.miis.edu/archive/cbw/ttuck2.htm. Accessed 2 Sept 2013). This presentation on the dual-use dilemma (i.e., something with both military and civilian uses) was just over a decade after the BoNT products first became commercially available in 1989, but at a time when usage was still low around the world. The situation is very different now.

The First Example of an Issue with Illegal BoNT Products

In 2004, an extraordinary event occurred which showed the world very clearly how illegal BoNT products could cause significant harm to humans (Chertow et al. 2006).

On 23 November 2004, a clinic in Florida received a shipment of research-grade, purified BoNT. The product was bought from a legitimate research reagent supplier and was certainly not intended for human use, meeting none of the requirements for such an injectable biological product. The product was incorrectly diluted by the nurses in the clinic for use in aesthetic treatment of patients (Chertow et al. 2006).

A clinician at the clinic, who previously had his license to practice revoked, injected himself and his partner. He also injected two friends a day later. All were injected in the facial muscles for aesthetic relief of wrinkles. Two days later, the physician and his partner left for New Jersey, where they sought medical treatment on 26 and 27 November at a hospital; the friends went to a different hospital on 26 November. Because the symptoms of the patients after diagnosis were typical of botulism, three were administered an antitoxin reserved by the US Center for Disease Control and Prevention (CDC) for such emergency cases. Antitoxin administration was delayed by some days to the fourth patient, who consequently suffered considerable and long-lasting paralytic disabilities. Investigations subsequently showed that the patients had probably been given 20–40 times the estimated human lethal dose of BoNT. Estimates of cost for the incident have also been published, though not substantiated, at $350,000 per patient for the first 2 weeks (Wein and Liu 2005). Even if somewhat inaccurate, the total cost of discovery, identification, treatment, and care is likely to have run into millions of dollars, just for these four patients.

Two subsequent events took place.

Firstly, the hospitalized patient with delayed administration of antitoxin became a highly studied case, receiving the best care that could be provided (Souayah et al. 2006; Marcus 2009). The main clinician involved, who saw the patient through many months of issues, subsequently published a detailed account of the incident, including the recollections of the patient herself (Marcus 2009). The story is exceptionally interesting to those involved with BoNT clinically.

Secondly, the Food and Drug Administration in the United States, in conjunction with the US Department of Justice, conducted a detailed investigation of the event. They immediately uncovered another supply of a second, research-grade BoNT that had been given to another group of patients from the same clinic. Follow-up of these patients revealed no side effects. But this led to the discovery of a comprehensive web of clinics throughout the United States who had bought illegal BoNT and routinely "sold" this to patients as just another version of the branded product.

FDA reported their first findings in October 2009 (http://www.fda.gov/downloads/forconsumers/comsumerupdates/ucm143721.pdf. Accessed 10 Nov 2013). Their Office of Criminal Investigations had tracked down, from the offending company's records, over 200 clinicians who had purchased the illegal material. Some 29 had been convicted and given jail sentences and restitution fines. The small "incident" was found to have large, nationwide implications.

Subsequent reports on the arrest, conviction, and jailing of clinicians and clinical practices have appeared from the Department of Justice on a regular basis since the original incident (see, e.g., Press Release US Department of Justice, Northern District of New York, 11 August 2009, *Albany Plastic Surgery group pleads guilty*

in connection with use of unlicensed "Botox" substitute on unsuspecting patients). These indictments continued through until 2010, 6 years after the original incident (see, e.g., Press Release US Department of Justice, Southern District of Texas, 16 April 2010, *Local physician sentenced for injecting patients with fake Botox*). Debarment notices have also appeared in the Federal Register for the clinicians involved (see, e.g., Federal Register 77(90), 27235–8).

The First Publicity: 2006

On 22 May 2006, the Chinese web site www.foqoo.com (hosted by an organization called the China AB Network, an industry portal) published an article in Chinese about the investigation of sale of counterfeit BoNT by the State Food and Drug Administration (SFDA: now the China Food and Drug Administration, CFDA). SFDA had announced on 19 May 2006 about the seizure of counterfeit products from ten beauty salons and outlets. This information went entirely unnoticed in the rest of the world and only partially surfaced when, in September 2006, a newsletter was published by the International C-BTXA Association. This Association is a cover for a promotional activity related to the legitimate, licensed Chinese BoNT-A product BTXA™ manufactured by Lanzhou Institute of Biological Products and distributed worldwide by Hugh Source (International), based in Hong Kong.

Unfortunately, while the Association's newsletter was very interesting and contained several examples, with photographs, of illegal BoNT products, this also went unnoticed by the rest of the world. The newsletter has a very limited circulation and only to registrants of the Association. Nevertheless, this marked the first publicity that any illegal BoNT products had received, and many of the illustrated products are still available from the Internet today.

The Situation Unfolds: 2008

Following the publication of the Florida incident details in 2006 (Wein and Liu 2005; Hunt and Clarke 2008), the companies who were manufacturing and distributing licensed BoNT products increased their awareness of which illegal products were circulating in the world.

In March 2008, Terry Hunt and Ken Clarke from Allergan, the manufacturer of the American product Botox®, published their findings in relation to a Chinese illegal BoNT-A product they had identified (Hunt and Clarke 2008). They reported that the product had over four times the labelled potency when tested in their own assay method. They also reported that the manufacturer had been shut down by the Chinese State Food and Drug Administration but, even today, this supplier is still advertising their product for sale via the Internet.

In June 2008, at the International Toxins meeting held in Baveno, Italy, Andy Pickett and Martin Mewies from Ipsen Biopharm, the manufacturer of the European product Dysport®, published their findings of a detailed investigation into illegal

Table 1 Characteristics of counterfeit and fake botulinum toxin products available from Internet sources or illegal suppliers (Reprinted from Journal of the American Academy of Dermatology 61 (1), Pickett, A. & Mewies, M. Serious issues relating to the clinical use of unlicensed botulinum toxin products, 149–150, © 2009 with permission from Elsevier)

	Country where product is found	Possible country of origin	Labelled potency (u/vial)	Actual measured potency (u/vial)[a]	Comments
Counterfeit products					
Dysport counterfeit (Spain Tox)	Brazil	Unknown	500	~1.5	Insert contains the following statement: "Training: Ipsen can facilitate training"
Dysport counterfeit	Iran	China	500	No toxin detected (<20 u/vial)	Poor quality replica (cap, seal, stopper, dark particles, and incorrect excipients)
Dysport counterfeit	Russia	China	500	71	Contains excipients found in toxins of Chinese origin
Look-alike products					
Lanzhou CBTX-A (Prosigne in Brazil, Quick Star in the United States)	Hong Kong	China	100	145	Rebranded as other products; licensed in China and Brazil
Estetox-A	Iran	China	100	No toxin detected (<2 u/vial)	From Lanzhou Institute of Biological Products
Refinex/Amazing	Iran	China	100	~125	May be rebranded as other products
Novotox Ultra	Iran	China	150	~35	Indicated of Canadian origin
Canitox	Iran	China	100	~98	Indicated of Canadian origin
Linurase	Iran	China	50	~233	Indicated of Canadian origin

[a]Determined either by standardized potency assay or activity assay, expressed as Dysport-equivalent units

BoNT-A products found worldwide (Pickett and Mewies 2008). Their findings were subsequently published (Pickett and Mewies 2009b; Pickett 2010) and presented at several other international meetings (Pickett and Mewies 2009a) in order to publicize to the clinical communities, as far as possible, the serious issues. A summary of their main findings is given in Table 1.

Pickett and Mewies found extraordinary information. Several illegal, fake products were found in Iran as well as counterfeit products of their own Dysport, mainly

from Russia. Some counterfeits were very good copies, needing expertise from the manufacturer to identify them completely. Other counterfeits were very poor, not even using the same size vial. The illegal products from Iran had adopted names such as Canitox to try and brand them as of Canadian origin. One product even had a Canadian symbolic maple leaf embossed into the plastic removable flip-top! The attempts to legitimize such products by misleading the unsuspecting purchaser into thinking they were from a highly reputable Western country were almost unbelievable.

Pickett and Mewies also identified that while several of the products they had tested had no (or virtually no) BoNT activity, some had several times the labelled potency (Table 1). This made them highly dangerous with the potential risk of serious overdose for patients. This risk was, of course, *in addition* to any risk they might have for poor quality or non-sterility, neither of which could be tested further from the limited samples available.

These reports did have an impact. For example, the president of the main Iranian Society of Dermatology at that time, Dr. Yahya Dowlati, went onto the regular morning television show twice and presented the findings, also showing vials of the illegal products found in their country. He warned about their use and their illegal status.

The Monterey Report, 2009

The 2008 publications and data attracted interest from several sources, but in particular from MIIS. Ken Coleman and Ray Zilinskas, experts in the field of chemical and biological warfare and WMD, decided to undertake their own threat assessment of the potential for these rogue products to be used for bioterrorism. Their findings were published in a report, issued finally in 2011 (Coleman et al. 2011) but which is actually dated January 2009. The main contents of the report were published in 2010 (Coleman and Zilinskas 2010).

This comprehensive report examined, in detail, the potential threat from commercial BoNT. All companies known at that time were surveyed, and their role in countering the threat, if any, was assessed. They identified that no agency or organization, other than two of the producers of existing products, was collecting information about the situation. They wanted to organize an immediate meeting that brought together various agencies, with a view to achieving two actions:

- Establishment of an interagency committee with a focus on the situation, using the main companies as advisors
- Establishment of a reporting site (essentially, an Internet portal) to which information could be readily added by individuals and institutions, building a database of illegal production and products

Their recommendations also went further. Firstly, they proposed the establishment by MIIS and the FBI of a Botulinum Toxin Nonproliferation Network

(BotNet), to act as an independent information clearing house with analytical capabilities. BotNet would enable the sensitive information available from legal manufacturers to be appropriately handled. Secondly, a proposal was made for the Defense Threat Reduction Agency (DTRA), the sponsor of their investigations and report, to establish a Botulinum Toxin Investigative Team (BoNTIT), which would be a close-knit activity, in conjunction with other interested US and foreign agencies, with an analytical capability to collect samples, analyze, and essentially "fingerprint" illegal manufacturers.

The Monterey report was intended and was labelled as a Phase I activity, "Scoping out the Problem." In the event, nothing further emerged. None of the recommendations, to the current author's knowledge, was ever implemented. No agency demonstrated sufficient interest to go further. Whether this was because the agencies believed it was an issue for the legal manufacturers to deal with (http://money.cnn.com/2010/06/08/news/companies/allergan_botox_counterfeits.fortune/index.htm. Accessed 2 Sept 2013), or the Food and Drug Administration to handle (with its abilities to deal with counterfeit pharmaceuticals) or indeed something else, has not been revealed. The situation on monitoring, challenging, and dealing with the illegal BoNT producers today is unchanged for the last several years.

One follow-up meeting on the subject was held by Global Green USA, the US affiliate of Green Cross International (founded by President Gorbachev to foster a global value shift to a sustainable and secure future), in November 2010. Ray Zilinskas spoke at the meeting, repeating his earlier published comments and requests, but there has been no follow-up reported (http://www.gcint.org/news/terrorism-profit-illicitly-produced-andor-distributed-botulinum-neurotoxin. Accessed 2 Sept 2013).

As the years have passed since the Monterey report, there have been occasional articles to keep the subject alive, namely, the use of CFBP and their sources as potential bioterrorist threats (http://www.washingtonpost.com/wp-dyn/content/article/2010/01/24/AR2010012403013.html. Accessed 2 Sept 2013). Liang and colleagues have directly reviewed the situation most recently (Liang et al. 2012). They have called for BoNT to be classified as a controlled substance, which would enable track-and-trace control to be applied together with other regulation. This has not yet occurred, even though control legislation around use of BoNT has tightened recently.

Quality or Quantity?

One of the issues that need to be considered under the present subject is whether any BoNT pharmaceutical product, legal or illegal, could ever become a bioterror threat.

Pharmaceutical-grade BoNT is of the highest quality. The legal products are made to the highest standards, equivalent to any other sterile injectable pharmaceutical product available. The same cannot be stated or confirmed for the illegal products. Their quality is entirely unknown. As such, they represent a significant danger to any individual that receives such a product. Their sterility cannot be

guaranteed and their BoNT content, as demonstrated in Table 1, can be highly variable, nonexistent, or even superpotent.

There is actually no requirement whatsoever for any illegal BoNT supplier to make their "product" to any recognized safety or quality standard. What purpose would this serve? Their interest is to try and sell, in as short a time as possible, as much of their product to unsuspecting direct purchasers from the Internet or other illegal sources. They have no need for standards. In fact, they have no real need for BoNT to be included in their product. Their deception does not have to go so far as to include such a potent active material in their false products. So, as expected, many contain no detectable BoNT (Table 1). Only when there is some evidence of BoNT present or, far worse, when the quantity of BoNT is significantly higher than the labelled potency do such products represent a double risk of poor quality and significant danger to the recipient. Presumably, these suppliers are hoping to attract repeat business from purchasers if their product can be shown to have some activity.

There are two sources for any active BoNT that such products may contain.

Firstly, research-grade reagent could be purchased and then heavily diluted to give the minute amounts per vial needed for clinical uses. The supply of such materials is, however, significantly controlled since the Florida incident and distribution regulations have tightened regularly since then. Now, potential recipients usually have to show appropriate research need in order to obtain the products. This type of supply is therefore impractical and inefficient to the illegal producer, ultimately being traceable upon investigation – as the Florida incident demonstrated quite clearly.

Secondly, illegal production of the active BoNT from an institute or a small, owned laboratory would be a more likely source. There is no requirement for any sort of purity of such an illegal material, crude, unrefined BoNT being more than adequate for the purposes. Existing bacterial (clostridial) strains could already be in the possession of such institutes or isolation of a production strain from a natural source can be achieved: there are many openly available publications on this subject. Growth and production of the BoNT only requires a suitable medium which is again well documented. In general, the bacterial strains producing BoNT are highly productive, and only small cultures will be required to obtain an amount able to be used illegally. A culture may typically yield 10^5 or 10^6 LD_{50} units per milliliter of culture (Schantz and Johnson 1992; Rao et al. 2007). In consideration that each vial of product might contain only 100 LD_{50} units, a typical dosage form, the potential for a very high yield of vials using crude BoNT from small cultures is demonstrable simply by calculation.

Therefore, quality is not relevant in this discussion. Quantity is achievable using known technology that is accessible. But any individual or organization undertaking such an exercise without suitable safety equipment would be taking an extraordinary risk of contaminating either themselves, their colleagues, their facility, or the environment with the material they were handling. BoNT is highly potent when inhaled (Park and Simpson 2003; Sanford et al. 2010; Al-Saleem et al. 2012) and suitable precautions must be taken under all circumstances to separate people from product and product from the environment. There are no "almost adequate" precautions that can be taken.

So could a final product in vials be used as a bioterror weapon? To make any estimate of this, the lethal dose for a human needs to be estimated, since no data exist to provide a confirmed value.

In 1982, D. Michael Gill from Tufts University published a now famous table of bacterial toxin lethalities, drawing on a wide range of information sources. The range of BoNT toxicities was from sub-nanogram up to approximately 2.5 nanograms for different species. Only one value was given for human lethality of BoNT serotype A (the most common clinically used serotype), about 1 nanogram. However, these data are very old from the 1950s and almost certainly refer to impure, complexed BoNT since knowledge of the composition of BoNT produced by bacteria as a toxin complex was inexistent at that time. We now know from normal therapeutic clinical use that a patient may be injected with perhaps 15 nanograms or more during a single treatment session (for, typically, large muscle spasm treatment such as in poststroke spasticity) without suffering major side effects. The old Gill data are therefore unreliable.

A more recent estimation of the LD_{50} dose was derived by Alan Scott and David Suzuki in a unique study that has never been repeated since, using macaque primates (Scott and Suzuki 1988). They used Oculinum®, the predecessor to Botox®. Their finding was an intramuscular LD_{50} of about 39 Botox® units/kg body weight. For an average weight individual now of about 75 kg, this represents approximately 3,000 LD_{50} units or some 30 vials of Botox®. By the oral route, BoNT is known to be much less toxic (Crawford et al. 2012), perhaps 1,000 times. The potential to cause widespread intoxication by using vials of clinical product is therefore highly improbable; only a very local effect could be achieved and then only by much patience in reconstituting the vials and preparing them in such a way that the dose can be administered. Intoxication by injection seems even more unlikely and the oral route is inefficient. Therefore, the only practical way that an illegal source of BoNT might be used for bioterrorism purposes is by utilization of a crude, bulk quantity of material, not a clinical dosage form, with the associated inherent safety risks, as described above.

The Potential Bioterrorist Use of BoNT: Food Contamination

The potential that BoNT could be used as a bioterrorist weapon, a WMD, has long existed as discussed earlier. The early interest of, for example, the US Army and their associated publications is well documented (Abrams et al. 1946; Lamanna et al. 1946). Coleman and Zilinskas (Coleman and Zilinskas 2010) highlighted the previous failed attempts of the Japanese cult Aum Shinrikyo to test BoNT in Tokyo. But several publications have directly examined the potential for BoNT to be used in today's society, by introduction of the material into the food chain.

In 2005, Lawrence Wein and Yifan Liu published a detailed analysis of the potential use of BoNT in the milk supply to give widespread contamination that would be directly accessible to humans (Wein and Liu 2005). Their work was based upon a mathematical simulation and, as a consequence, included a number of

assumptions which must be borne in mind in the assessment of risk involved. For example, details such as the actual LD_{50} or ID_{50} for humans could only be estimated, as discussed above. They concluded that while they could obtain reasonably accurate estimates of how many people might be poisoned by such an incident, they only had a poor estimate of how much BoNT would be required to cause such an outbreak.

Their paper caused an outcry in the United States, and attempts were even made at the highest level of the Department of Health and Human Services to prevent publication, labelling the paper "a road map for terrorists" (Letter from Stewart Simonson, Assistant Secretary of HHS, to Dr. Bruce Alberts, National Academy of Sciences, 27 May 2005). But the publication was heavily defended and still went ahead (Alberts 2005). The journal *Nature* reported in an editorial on this somewhat historic and difficult situation but supported publication (Risks and benefits of dual-use research Nature 2005). Wein published his comments and background to the work in an article to the New York Times in May 2005 (http://www.nytimes.com/2005/05/30/opinion/30wein.html?pagewanted=all&_r=0. Accessed 18 Sept 2013) which was again heavily criticized (http://www.fas.org/sgp/eprint/milk.html. Accessed 2 Nov 2008).

This controversy about the potential contamination of the milk supply with BoNT took no account of the numerous other publications that have examined BoNT and milk over the decades, since milk has always been identified as a potential source of BoNT contamination as a normal food of natural origin (Graham et al. 1922). Indeed, more recent studies have demonstrated that BoNT can be especially resistant to milk pasteurization, (for example, Rasooly and Do 2010; Weingart et al. 2010), although certain of these studies are contradictory in their findings. None of these subsequent publications have raised issues at all in the same way that the Wein and Liu work produced (2005).

However, taking into account the issues related to availability of pharmaceutical-grade BoNT in vials, as discussed earlier, and the actual content of BoNT within those vials, the likelihood that such products might be used to cause any outbreak of food-related botulism is both extremely impractical and extremely remote.

Case Study: Iran and Botulinum Toxin

For many decades, Iran has been considered a sponsor of terrorism in the world, including the suspicion that Iran has provided WMD technology to terrorist groups. This is especially the position of the US State Department (http://www.cfr.org/iran/state-sponsors-iran/p9362. Accessed 17 Nov 2013) and US presidents have made clear their views over the years (http://millercenter.org/president/speeches/detail/4540. Accessed 17 Nov 2013). A brief examination of the role and position of Iran within the world of BoNT is therefore warranted in the present context.

In 2012, Iran announced to the world that it had successfully produced a BoNT product for clinical use (Announcement by Islamic Republic of Iran Broadcasting 2012). The name of this product is Masport®, produced and distributed by the

Iranian company Masoondarou Biopharmaceutical Co. together with the Research Deputy of Ministry of Health. Iran has always been a significant market for BoNT, which is perhaps reflected by the name given to their own product in relation to one of the commercial branded products, with an implication of association and similarity. In fact, the vial formulations of the Iranian product and the branded product are very similar. Indeed, their program to produce a national product had been announced 2 years previously and so the final result could be no surprise to the world (http://www.iran-daily.com/1389/9/22/MainPaper/3845/Page/8/MainPaper_3845_8.pdf. Accessed 17 Nov 2013).

Of interest is that one of the main manufacturers of BoNT products was investigated in 2009 for apparent links to Iranian institutions (http://www.washingtontimes.com/news/2009/apr/23/the-toxin-trade-foreign-firms-can-pick-the-mullahs/. Accessed 17 Nov 2013). The concept that one of the original manufacturers of BoNT clinical products was selling "raw botulinum toxin" to Iran was beyond belief. This accusation was vehemently denied and entirely erroneous. But what was interesting was the timing of the accusations – immediately prior, within a few days, to FDA granting a license for the company's BoNT product to be used in the United States. This timing, and the involvement of US congressman in the accusations, would make a fascinating story in its own rights!

Iranian clinicians have actively published in the area of BoNT clinical use for over a decade. Their work has covered a wide range of uses, from therapeutic to aesthetic applications and including a number of novel applications still unlicensed anywhere in the world. Simultaneously, Iranian scientists have reported significant work on the fundamental science of BoNT. In particular, their work deals with the cloning and expression of BoNT subunits, associated proteins, production of antibodies, and, in addition, vaccines. On review, their scientific work has perhaps a main theme toward the production of materials of value for vaccination. But what is certain is that the Iranian scientists have significant knowledge and science in the area of BoNT. One review of interest to the current reader may be the work of Balali-Mood and colleagues (Balali-Mood et al. 2013), reviewing the medical aspects of bioterrorism!

So has Iran become a supplier of botulinum toxin to bioterror organizations? There is no evidence known to the author to support such a claim. No source has emerged to make this statement, even since Iran announced its own BoNT product. And if this were by chance the case, then there has been no reported incident which implicates BoNT in a bioterrorism incident anywhere in the world to date.

Conclusion and Future Directions

Little has changed in the intervening years since the investigations and publications relating to the issues surrounding CFBP. The subject is raised at international aesthetic conferences on a regular basis by key speakers and is maintained in the clinicians' eyes as a consequence (Pickett 2013). This is a positive activity that serves to inform and warn. But much more is needed to limit or, better, eliminate the issue.

As a consequence, future activities need to be coordinated between the manufacturers and the relevant agencies in a concerted effort to deal with the issues on a global scale. There is currently no cooperation on CFBP between the companies who are the main producers of the therapeutic and aesthetic products. Indeed, there is little apparent agency action, although occasional information is released, giving a little insight into the problems (http://www.fda.gov/Drugs/DrugSafety/ucm349503.htm. Accessed 1 Sept 2013). The future holds the hope that a concerted effort will eliminate the illegal products and especially their sources, within a sensible time frame.

Cross-References

► Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
► Biotoxins and Food Safety
► Botulinum Toxin: Present Knowledge and Threats
► The Biosecurity Threat Posed by Biological Toxins

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Section III

Surveillance Tools for Biotoxins

Cell-Based Assays for Neurotoxins

12

Patrick McNutt, Phillip Beske, and Nagarajan Thirunavukkarasu

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Abstract

The implementation of cell-based assays in basic research, disease modeling, and drug discovery has dramatically increased over the past 10 years. One field which has suffered from the lack of physiologically relevant cell-based models that are compatible with moderate-throughput applications is neurotoxicology. The development of next-generation stem cell-derived neurons for neurotoxicology research has the potential to resolve this limitation; however, derived neurons have to be demonstrated to exhibit the same physiological responses as primary neuron populations. In particular, derived neurons must demonstrate appropriate electrical behaviors, transsynaptic signaling, and network activity to be considered relevant models. This chapter reviews the state of the art in cell-based assays (CBAs) predicated on the use of stem cell-derived neurons, describes the different pluripotent populations that are currently used, discusses advantages and limitations of differentiation methods, and reviews some initial applications. Finally, developing techniques are presented that are anticipated to increase throughput, sensitivity, and relevance. While the transition to moderate-throughput assays is still under way, it is clear that stem cell-derived neurons offer a potent combination of physiological relevance with scalability and genetic tractability, and therefore, CBAs based on derived neurons are poised to revolutionize neurotoxicology research and drug discovery.

Introduction

The Critical Need for Cell-Based Assays in Drug Discovery

The process from drug discovery to commercialization takes an average of 12 years and has been estimated to cost $1–3 billion (Fig. 1; DiMasi et al. 2003). On average, 1 in every 10,000 compounds identified as lead candidates during early drug discovery will be approved by the Food and Drug Administration (FDA). Although several factors contribute to this high failure rate, the two largest are drug efficacy (25 %) and toxicity (31 %) during animal testing and clinical trials (Kola and Landis 2004). The preclinical and clinical studies phases are far more costly than drug discovery; therefore, there is a financial incentive to determine as early as possible whether a drug is ineffective or toxic. In addition to mitigating financial risk, eliminating non-marketable drugs early in the process increases the probability of later successes. Although the discovery phase comprises a small fraction of the total cost of drug approval, the predictive accuracy, efficacy, and efficiency of screening assays are therefore important determinants of the ultimate outcome of

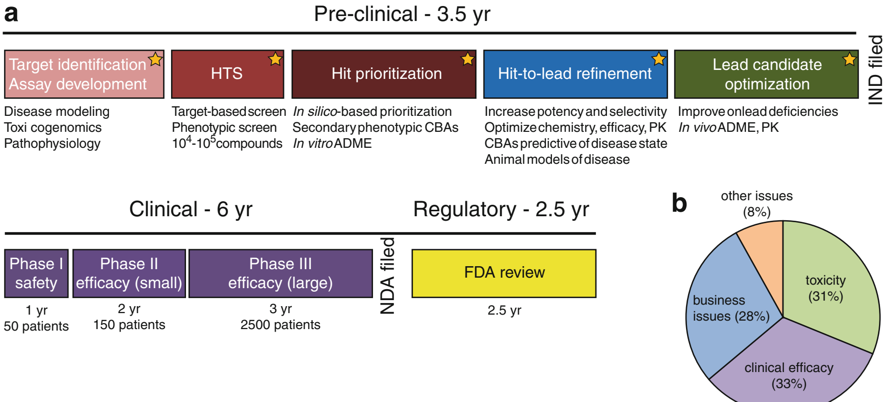

Fig. 1 Overview of importance of cell-based assays to the drug discovery process. (**a**) Representative pipeline of pharmaceutical drug discovery, starting with target identification and assay development and progressing through regulatory approval. *Yellow stars* represent steps where cell-based assays can be used to reduce risk or accelerate development. (**b**) Most common failure modalities for lead drugs during animal- and human-based evaluations

lead candidates (Hrusovsky et al. 2010). Consequently, one of the driving motivations for organizations interested in pharmaceutical discovery and development is the identification of new technologies that can improve early-stage drug identification and reduce risk. One such example is the implementation of cell-based assays (CBAs) during the drug discovery process (Fig. 1).

Cell-based assays can refer to a wide variety of experimental modalities using live cells, typically in a tissue culture format. CBAs exhibit two fundamental advantages over enzyme- or antibody-based assays: (1) drug candidates are evaluated in the context of the complex biochemistry of the intracellular environment, thereby offering a more accurate representation of physiological responses, and (2) CBAs support longitudinal evaluations, facilitating studies testing intracellular kinetics, cytotoxicity, and off-site effects. The reductionist nature of cell models is also well suited for systems biology approaches to identify and characterize functional targets, develop rapid screens for lead candidates, or produce improved models of toxipathogenesis. This capability has been significantly expanded by recent advances in "omics" technologies that facilitate identification of novel potential targets and new informatics systems designed to easily sort and visualize large data sets.

While animal models of disease can provide a wealth of relevant pathophysiologic data for the discovery of new therapeutic agents, animal use is relatively expensive, typically low throughput, ethically complicated, and subject to phenotypic and/or genotypic variability which can complicate data interpretation. To increase the bandwidth of early drug discovery, academic and pharmaceutical labs have transitioned to high-throughput screening (HTS) assays. In HTS, representative models of a targeted enzyme or phenotype are interrogated with large libraries of compounds, and specific readouts are used to select compounds

(hits) for further analysis. The assays used for HTS can be generally divided into target-based screens, which interrogate the effects of a drug on a single molecule using biochemical assays, and phenotypic screens, which seek to identify candidate modulators of a pathway of interest in the context of a cell, tissue, or organism (Korherr et al. 2006). Unlike target-based assays, which only interrogate the function of a specific molecular target, phenotypic screens are often agnostic regarding molecular mechanisms of toxipathogenesis. In such assays, entire pathways of interest can be interrogated, simultaneously probing for multiple modalities that can be therapeutically targeted. The increased relevance and expanded target range of phenotypic assays are balanced by the fact that the readouts tend to be indirect and therefore must be carefully validated.

Although an improved mechanistic understanding of disease targets initially drove a preference for target-based HTS assays, it has become increasingly apparent that biochemical assays produce a large frequency of candidates with a physiologically irrelevant mode of action (Swinney and Anthony 2011). Consequently, modern target-based screens typically employ a CBA as a secondary or tertiary screen. As a result, approximately half of HTS studies included a cell-based component in 2010, and global estimates of market commitment to cell-based screens are predicted to nearly double to $10.7 billion (USD) from 2010 to 2015 (Nagavarapu et al. 2011). This increased utilization is driven by a requirement for more relevant models of target-drug interactions. Unlike biochemical assays, CBAs can simultaneously evaluate the activity of candidate drugs in a cellular environment, determine transmembrane permeability, test for acute cytotoxicity, and evaluate off-site effects. Thus, although CBAs are often less sensitive, difficult to interpret, and more laborious than biochemical assays, these negatives are compensated for by the ability of CBAs to evaluate target-drug interactions in a complex physiological context, including some of the factors that affect the in vivo behaviors of drug and target.

Depending on the desired throughput, the design of next-generation CBAs must balance physiological relevance and cost with throughput. Cells used in CBAs should be amenable to the desired assays, faithfully represent the relevant disease model or system, and express the necessary factors and signaling intermediates. Consequently, the selection of a CBA requires the careful consideration of several assay parameters. The first is to identify the particular need for the assay (small molecule screening, mechanistic discovery, toxin detection, toxin characterization), choice of biological system (primary cell, wild-type or transgenic continuously cultured cell lines), choice of assay (functional, reporter gene, or phenotypic), and assay readout (single readout or multiplexed readouts). Additional factors that can impact the reliability of the CBA are ease of use, throughput, and reproducibility.

The remainder of this chapter will focus on the use of CBAs in neurotoxicology, a research area that has experienced difficulties in establishing physiologically relevant cell models that are compatible with moderate-throughput screening, toxin detection, or target discovery. While the focus of this chapter will be on the development of improved models for drug discovery, it should be noted that CBAs

can also be used to support other aspects of disease management, such as validating the potency and efficacy of pharmaceutical formulations or diagnosing and/or quantifying the presence of an active toxin in clinical or forensic samples (Fernandez-Salas et al. 2012; Pellett 2013).

From Neuronal Structure to Network Activity

A neuron is a component of the nervous system that uses electrical and biochemical impulses to communicate with other cells of the body. This communication is most often chemical in nature and occurs at highly specialized, asymmetric sites of cell-cell contact termed synapses, where electrical signals are converted to chemical information and vice versa. Presynaptic compartments are found within the neuronal axon and contain neurotransmitters enclosed in small, membrane-bound synaptic vesicles. Activation of voltage-dependent Ca^{2+} channels in the presynaptic compartment by a propagating action potential stimulates the release of neurotransmitters (e.g., glutamate, GABA, acetylcholine, serotonin, noradrenalin, substance P, etc.) into the synaptic cleft between the opposing axon and dendrite. Released neurotransmitters bind to receptors on the postsynaptic membrane and alter the electrical characteristics of the postsynaptic cell. In the case of a neuron-neuron synapse, an excitatory signal would make the postsynaptic neuron more likely to stimulate downstream cells, whereas an inhibitory signal would make the postsynaptic neuron less likely to stimulate downstream cells. The functional response of the postsynaptic compartment is determined by the functional expression of neurotransmitter receptors, which in turn is intricately regulated by an elaborate complex of proteins found immediately within the postsynaptic membrane. Depending on the receptor, synaptic activity can elicit ion fluxes that can depolarize or hyperpolarize the postsynaptic compartment, activate second-messenger systems, and initiate phosphorylation or dephosphorylation events within the dendritic spine that have neuron-wide effects. Neuronal axons and dendrites often extend long distances from the soma and can synapse with an estimated 5,000–100,000 other neurons.

Several mechanisms have evolved to allow for the emergence of neuron ensembles from this high degree of synaptic complexity. These mechanisms utilize activity-dependent changes in the kinome, transcriptome, and proteome of the pre- and postsynaptic compartments to “tune” the weight of individual synapses within a network. These behaviors support the self-organization of a nascent neuronal network into a critical state, maximizing entropy and therefore information transmission (Shew et al. 2011). The implication of this self-organized critical state is that a cell model that does not produce functioning synapses is a poor platform for predictive toxicity screens. In contrast, a model that produces a synaptically coupled network that exhibits activity-dependent changes in neuronal behavior is more likely to offer a comprehensive platform to evaluate a broad array of neurotropic neurotoxins, regardless of the specific neuronal compartment that is targeted (Table 1).

Table 1 Representative neurotropic neurotoxins based on functional target

Functional target	Representative toxins
Postsynaptic receptors	Glutamate, lead, TETS, cicutoxin
Presynaptic compartments	Botulinum neurotoxin, tetanus toxin
Synaptic cleft	Organophosphorus nerve agents, anticholinergic agents
Neuronal membranes (pore-forming)	Alpha-latrotoxin, epsilon toxin, ionomycin
Axonal voltage-gated channels	TTX, TEA, omega conotoxin, saxitoxin
Cytoskeleton	Arsenic, ammonia

The Requirement for Neuronal Behaviors in Neurotoxicology Research

The functional topology of the central nervous system is highly complex and therefore difficult to model with in vitro approaches. Not surprisingly, a common criticism of in vitro neuron model systems is that findings often fail to translate to in vivo studies. Consequently, designing a CBA that recapitulates the poorly understood interactions that take place within and among neurons is a formidable challenge. This functional complexity presents a pivotal constraint on CBA selection for neurotoxin research: to be a viable model, cells should replicate the relevant functional and spatiotemporal behaviors exhibited by primary neuron populations. One means to determine the suitability of a neuron model is to test for higher-order emergent phenomena, such as network behavior. The ability of neuron ensembles to conduct impulses in a coordinated manner is an indication that the underlying biochemical, proteomic, transcriptional, and structural features necessary for emergence of neural networks are appropriately expressed. This requirement for neurotypic behaviors in neurotoxicology testing is particularly important for the development of phenotypic screening assays or diagnostic assays, in which disruption of network behavior can be a primary readout. In contrast, a cell model that does not form functioning synapses will not display the complex behaviors responsible for the emergence of a stable network, nor will it be as susceptible to perturbation by agents that modulate network activity.

An example of the need for network behavior in neurotoxicology studies is assays to characterize the effects of lead (Pb^{2+}) exposure on neuronal activity. Pb^{2+} is an environmental neurotoxin that has been shown in vivo to impair cognitive function in regions of the brain responsible for learning and memory (Toscano et al. 2002). Pb^{2+} has been identified as a noncompetitive antagonist of the NMDA receptor (NMDAR) with IC_{50} values ranging from 0.87 to 6.1 μM, depending on the receptor subunit composition (Omelchenko et al. 1996). Hypotheses about the cognitive deficits associated with Pb^{2+} exposure involve the disruption of NMDAR activity-dependent phosphorylation of the cAMP response element-binding protein (CREB) and decreased production of brain-derived neurotrophic factor (BDNF), both of which are important events in synaptogenesis, neurotrophic signaling, and synaptic function (Neal et al. 2010). Since these processes are governed by synaptic communication, neuroblastoma cell lines, which do not

form functioning synapses or undergo synaptic plasticity, are unlikely to be physiologically relevant for the study of Pb^{2+} toxicity. Not surprisingly, the neuroblastoma cell line SH-SY5Y is mildly sensitive to Pb^{2+} concentrations at 500 μM, whereas studies in rat cortical neuron cultures show significantly impaired neuronal viability at 5 μM Pb^{2+} (Chen et al. 2011; Ishida et al. 2013). Consequently, it is doubtful that studies using SH-SY5Y cells are capturing the full range of neuron-toxin interactions that occur in vivo.

This concept applies to CBA selection for a wide range of neurotoxins. Since the central role of neuronal activity is synaptic communication, the biochemical and electrical mechanisms underlying neurotoxic responses in vivo must be replicated in vitro.

Historic Use of CBAs in Neurotoxicology Research

An effective cell-based model system for general neurotoxin detection/screening would combine the biological relevance of primary neurons with the flexibility of continuous cell lines (NIAID 2004). Cells should be neuron based, form functioning synapses that release neurotransmitter, recapitulate the full range of interactions between primary neurons and neurotoxins, and exhibit toxin sensitivities commensurate with the appropriate target neuronal subtypes. They should be genetically tractable, supporting knock-in, knock-down, and knockout experiments. Finally, cell culture should be scalable, enabling moderate-throughput screens for candidate therapeutics and large-scale systems biology approaches. A quick overview of the cell-based models currently used in neurotoxicity research is presented in Table 2 and discussed in detail below.

Until recently, in vitro neuroscience was dominated by two types of cell-based platforms-primary neurons and neurogenic cell lines. While primary neurons offer strong biological relevance, their use is limited by the necessity of dissection and isolation. Furthermore, obtaining homogenous neuronal populations that remain viable for prolonged periods is technically difficult, and resulting cultures are poorly responsive to genetic techniques (although this is improving). While the use of primary neuron cultures and/or organotypic slices slightly reduces animal use, ethical concerns and resource costs still remain. Finally, and perhaps most importantly, primary cultures often experience inter-experimental variability that can confound data interpretation.

In an attempt to increase the throughput and reproducibility of neurotoxin screening, researchers began to utilize continuously cultured cell lines that could be induced to develop neuron-like characteristics. The most common of these “neurogenic” cell lines are rat adrenal pheochromocytoma cells and human and mouse neuroblastomas. Generally speaking, induced neurogenic cells have heterogeneous phenotypes and in most cases form either nonfunctioning or abnormal synapses, making how accurately they model neuronal behaviors questionable. Furthermore, they typically exhibit a poor sensitivity to neurotoxins, suggesting that neuron-toxin interactions are atypical (Hubbard et al. 2012). For example, neurogenic cell lines fail to form functioning synapses, which are the neuronal compartment at which the toxin naturally acts, and are several orders of magnitude

Table 2 Cell-based models used in neurotoxicity research

Cell type	Representative applications	General notes
Primary neurons	Excitotoxicity (Bonfoco et al. 1995)	Fully differentiated with physiological responses
	Botulinum neurotoxin (Foran et al. 2003)	Close approximate native function
	Environmental toxicants (Rush et al. 2010)	Can be isolated from transgenic animals
	Heavy metal neurotoxicity (Neal et al. 2010)	Inter-experimental variability
		Most neuronal preparations contain multiple cell types
		Requires animal use with associated costs
		Low throughput
		Limited ability to employ genetic modification
Immortalized cell lines	Excitotoxicity (Ma et al. 2012)	Inexpensive to maintain
	Botulinum neurotoxin (Purkiss et al. 2001)	Can be maintained in culture indefinitely
	Environmental toxicants (Dingemans et al. 2009)	Genetically tractable
	Developmental neurotoxicity (Laurenza et al. 2013)	Lack in important aspects of native cellular function
	Heavy metal neurotoxicity (Chen et al. 2011)	Not representative of normal cells
		Produce continuum of phenotypes
Embryonic stem cells (ESCs)	Excitotoxicity (Hubbard et al. 2012)	Highly scalable
	Latrotoxin (Mesngon and McNutt 2011)	Tissue culture is not resource intensive (mouse)
	Botulinum neurotoxin (McNutt et al. 2011)	Directed derivation of multiple neuronal subtypes
	Developmental neurotoxicity (Bosnjak 2012)	Genetically tractable
		Close approximation of native function
		Can be maintained in culture indefinitely
		Growth and maintenance is difficult (human)
Human-induced pluripotent stem cells (iPSCs)	Excitotoxicity (Gupta et al. 2013)	Same advantages as ESCs
	$MgCl_2$ on network activity (Ylä-Outinen et al. 2010)	Can be derived from specific diseases
	Botulinum neurotoxin (Whitemarsh et al. 2012)	Tissue culture is resource intensive
	Developmental neurotoxicity (Bai et al. 2013)	Differentiation methods not well optimized
		Difficult to reproducibly derive neurogenic hiPSC lines

(*continued*)

Table 2 (continued)

Cell type	Representative applications	General notes
Primary neural stem cells	Excitotoxicity (Choi et al. 2010)	Relatively new model
	Developmental neurotoxicity (Li et al. 2013)	Differentiative capacity limited to neural cells
	Heavy metal neurotoxicity (Bose et al. 2012)	Close approximation of native function
		Limited lifespan in culture
		Growth and maintenance with high cost
		Isolation is difficult and requires animal use

less sensitive to botulinum neurotoxin (BoNT) than primary neurons, suggesting that they do not undergo neuronal mechanisms of activity-dependent uptake and subsequent synapse intoxication (Pellett 2013; McNutt et al. 2011).

A signature example of the unreliability of neurogenic CBAs is their application to BoNT research. Although BoNT will be discussed in greater detail below, briefly the toxin, which is a zinc-dependent metalloendoprotease, specifically cleaves presynaptic proteins that are essential for neurotransmitter release, thereby paralyzing the synapse. The catalytic target and active sites of several BoNT serotypes were first reported in 1993, raising hopes that the identification of small molecule inhibitors (SMIs) via target-based screens was imminent (Schiavo et al. 1993). Nonetheless, despite extensive effort a clinically viable therapeutic that restores synaptic transmission has not yet been identified (Larsen 2009). While the extraordinary characteristics of BoNT have made this effort more challenging than initially expected, the use of neurogenic cell models as screening platforms has been a major contributory factor. A reported lack of correspondence between animal models and neurogenic CBAs in the efficacy of SMIs of BoNT/A further suggests that neurogenic cell lines do not undergo relevant neuron-toxin interactions (Eubanks et al. 2007). Overall, it has become clear that traditional neurogenic cell lines lack sufficient relevance to justify their continued use as a model for target discovery, HTS, or diagnostic screening for BoNTs, Pb^{2+}, and other neurotoxins.

Stem Cell-Derived Neurons as a Next-Generation Platform for Neurotoxicology Studies

Development of Stem Cell-Derived Neurons

Despite the significant limitations of neurogenic cell models, until recently they were the only alternative to primary neurons for cell-based studies of toxin-host interactions and screening for potential therapeutics. This was particularly true in

labs that lacked the resources to reliably obtain primary neurons and for teams conducting screening assays that required a throughput exceeding the available capacity of primary culture. However, the advent of pluripotent stem cell (PSC) lines capable of differentiating into populations of functionally networked neurons is revolutionizing the use of neuronal models for neurotoxin research. The most common PSCs are mouse and human embryonic stem cells (ESCs), which are isolated from early-stage embryos, and human-induced pluripotent stem cells (hiPSCs), which have been reprogrammed to an embryonic stem cell-like state. ESCs and hiPSCs share the unique ability to differentiate into a wide range of postmitotic cells, including various neuronal subtypes. In some cases, PSC-derived neurons have been shown to be able to form appropriate neuronal morphologies with functioning synapses, exhibit spontaneous and elicited electrical behaviors, develop emergent network activity, and be highly sensitive to a broad range of neurotoxins (Hubbard et al. 2012; Buzanska et al. 2009). This combination of physiological relevance, neuromimetic responses, and scalable expansion suggests that PSC-derived neurons may offer an optimal cell-based platform for neurotoxicology studies. Furthermore, the ability to develop CBAs based on hiPSC-derived neurons exhibiting the genotypic/phenotypic characteristics of clinically diagnosed neurological disorders introduces unprecedented possibilities for improved disease modeling and more relevant therapeutic screens.

The Range and Specificity of Stem Cell-Derived Neuronal Platforms

Early differentiation studies indicated that mouse ESCs could be induced to assume morphological and functional characteristics similar to primary neurons (Bain et al. 1995). However, these efforts generally resulted in heterogeneous cultures comprised of a high percentage of glial cells and multiple neuron subtypes, which made these cultures unsuitable for research objectives requiring inter-experimental consistency, homotypic neuronal populations, or characterization of low-abundant targets (Bibel et al. 2007). Painstaking refinement of these protocols has yielded methods to produce large quantities of neuronal precursors that can, in turn, be efficiently differentiated to specific neuronal subtypes from mouse and human ESCs, hiPSCs, and primary neural stem cells isolated from central nervous system (CNS) populations (Table 3). The potential for in vitro comparative neurotoxicology was recently expanded by the demonstration of efficient neuronal differentiation from nonhuman primate (NHP) ESCs, an exciting development that may facilitate a direct translational path from “bench to bedside” in NHP populations (Wianny et al. 2011). As methods are further refined to improve the yield and purity of specific neuronal subtypes (e.g., by using SMIs to drive neurogenesis (Chambers et al. 2012)), it is anticipated that derived neurons will be suitable for use in a broad range of neurotoxicology research, including diagnostics (Hubbard et al. 2012), phenotypic and target-based screens (Yang et al. 2013; Lee et al. 2012), and disease modeling/target discovery (Xia et al. 2013; Lee et al. 2009).

Table 3 Representative subtype-specific neuronal platforms for next-generation CBAs

Mouse ESCs	
Glutamatergic (vGluT1; cortical)	Bibel et al. 2007
Glutamatergic (vGluT2; thalamus, midbrain, and brainstem)	Hubbard et al. 2012
Motor neurons	Wichterle et al. 2002
GABAergic	Chatzi et al. 2009
Dopaminergic/serotonergic	Chung et al. 2011
Human ESCs	
Glutamatergic	Nat and Dechant 2011
Motor neurons	Wada et al. 2009
GABAergic	Goulburn et al. 2012
Dopaminergic/serotonergic	Cho et al. 2008
Retina	Mellough et al. 2012
Human hiPSCs	
Glutamatergic	Shi et al. 2012
Motor neurons	Hester et al. 2011
GABAergic	Higurashi et al. 2013
Dopaminergic	Swistowski et al. 2010
Sensory	Chambers et al. 2012
Retina	Meyer et al. 2011

Potential Applications of Stem Cell-Derived Neurons for Toxinology

As highlighted in previous discussions, the study of neurotoxins in vitro requires a physiologically relevant model system that recapitulates the full range of toxin-host interactions and cell responses that occur in vivo following neurotoxin exposure. While cultured neuroblastoma cell lines offer advantages in ease of use, scalability, and genetic tractability, these benefits often come at a cost of decreased sensitivity and questionable relevance. Conversely, while cultured primary neurons provide a more physiologically meaningful model, the technical skill, time, high cost, and labor-intensive preparations associated with reproducibly isolating primary neuronal cultures limit their application in moderate-throughput testing. Recently, the use of stem cell-derived neurons has been developed as a "best of both worlds" alternative, combining the ease of culture, scalability, and genetic tractability of continuous cell lines with the sensitivity and physiological relevance of primary neuron cultures. Neurons derived from human and mouse PSCs have been utilized by a handful of research teams for the study of neurotoxicology over the past few years, with initial emphases on the *Clostridium* neurotoxins and excitotoxins, including tetanus, botulinum neurotoxins, glutamate, and alpha-latrotoxin (Hubbard et al. 2012; Whitemarsh et al. 2012; Gut et al. 2013). The following section will discuss the recent application of stem cell-derived neurons for neurotoxin research.

Current Applications of Stem Cell-Derived Neurons in Toxin Research

CBAs for the Study of Clostridial Neurotoxins

The *Clostridium botulinum* neurotoxins (BoNTs) are the most poisonous substances known, with human toxicities estimated to be 1–2 ng/kg. Following internalization into presynaptic termini of neurons, the seven BoNT serotypes act with high specificity and high potency to cleave SNARE proteins, preventing neurotransmitter release. Several serotypes (/A,/B, and/C) have been shown to functionally persist within the presynaptic compartment for weeks to months, resulting in a prolonged paralysis that requires sustained intensive care (Foran et al. 2003). The same characteristics that make BoNTs a significant biological threat agent (extended activity in vivo, exceedingly high potency, ease of use, and ease of production) also render the toxin effective for a broad range of therapeutic and cosmetic uses, resulting in $2 billion in sales in 2011 (Tirrell 2011). Once the toxin has been internalized into the presynaptic compartment of the neuron, therapies to reverse paralysis of intoxicated neuromuscular junctions are not available. Consequently, while postexposure administration of antitoxin can accelerate clearance of BoNT from the plasma, passive immunization is unable to prevent or reverse paralysis once the toxin has entered neurons.

Currently, the mouse lethality assay (MLA) is the gold standard for BoNT detection and potency determination. The MLA measures the amount of intraperitoneally injected toxin sufficient to kill 50 % of mice (reported as the MLD_{50}). The MLA is used for tests that require evaluation of the full range of toxin activity, including determinations of (a) the potency of clinical- and research-grade BoNT preparations, (b) the presence of functional toxin in clinical or environmental samples, and (c) the ability of antitoxin preparations to prevent clinical intoxication. Quantitation and serotype determination of a single sample typically involves 50–100 mice, requires 72–96 h (not including sample preparation time), and can be confounded by the presence of unrelated toxins in the injected suspension. Although the number of animals used in the MLA assays is not publically available, recent estimates of annual usage by the cosmetic sector alone exceed 600,000 mice; these numbers would be significantly higher if academic labs and government agencies were included (Bitz 2010). Obviously, the cost and throughput of the MLA prohibit its use for drug screening, mandating the need for alternative approaches. Furthermore, the duration of the MLA test for the presence of viable toxin complicates clinical management of casualties, indicating that an improved diagnostic approach with greater speed, higher throughput, and comparable sensitivity is badly needed.

Until recently, CBAs for BoNT detection and quantitation have been limited to primary neuron cultures and neuroblastoma cell lines (McNutt et al. 2011). Recently, Allergan® obtained FDA approval for the determination of BoNT/A potency in a 96-well format using the SiMa neuroblastoma cell line, which was first characterized in 1999 (Fernandez-Salas et al. 2012). The Allergan assay uses immunodetection of the cleaved form of SNAP-25 as a molecular readout of

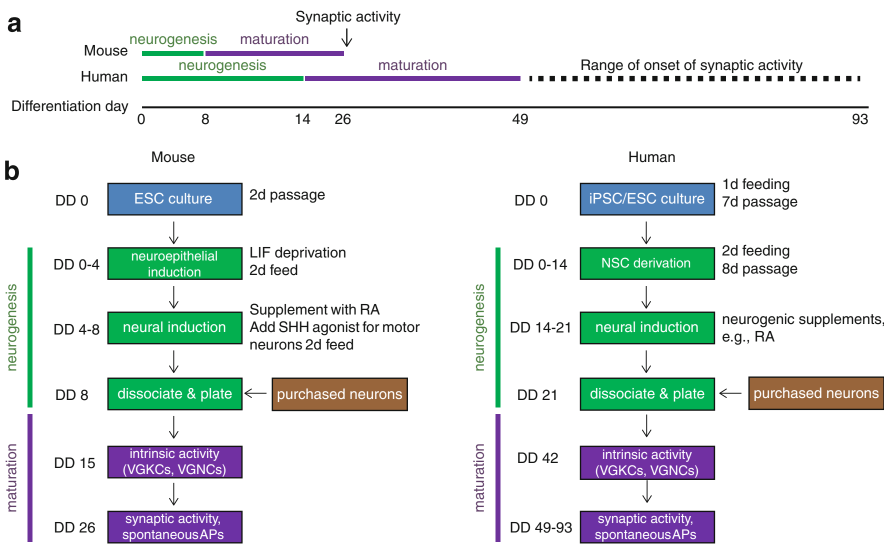

Fig. 2 Comparison of neuronal differentiation between mouse and human pluripotent stem cell populations. (**a**) Comparative timelines of neurogenesis in mouse ES cells versus human ES/iPS cells. (**b**) Breakdown of the temporal development of specific markers of neuronal differentiation, maturation, and network formation

intoxication. Similar to most neuroblastoma lines, SiMa cells do not appear to form functioning synapses, develop activity-dependent synaptic responses, or exhibit neuronal morphologies, making it doubtful that toxin uptake and behavior occur in a fashion equivalent to primary neurons. Furthermore, the lack of transsynaptic signaling, neuronal morphology, and synaptic architecture prevents physiological readouts of intoxication. Despite these drawbacks, SiMa cells received FDA regulatory approval in 2011 as an in vitro method to conduct cell-based potency determination of clinical botulinum formulations.

For the reasons presented above, researchers have sought to identify alternative CBAs for BoNT drug discovery that do not rely on neurogenic models or primary neuron culture. The use of stem cell-derived neurons in BoNT research was first proposed in the fall of 2008, at the 45th Interagency Botulism Research Coordinating Committee meeting, with preliminary data demonstrating that derived neurons were highly sensitive to BoNT/A. Since then, multiple PSC-derived neuron cultures have been demonstrated to be sensitive to BoNT. While the most characterized model is based on the derivation of enriched glutamatergic neurons from suspension-cultured mouse ESCs (ESNs) (Hubbard et al. 2012), other groups have developed differentiation protocols to produce human (Restani et al. 2012) and mouse (Pellett et al. 2011) cholinergic motor neurons from ESCs and pan-neuronal cultures from hiPSCs (Fig. 2) (Whitemarsh et al. 2012). In all cases derived neurons have exhibited sensitivities to BoNT/A that are roughly equivalent to primary fetal spinal cord neurons (measured by SNAP-25 cleavage), suggesting that regardless of the neuronal subtype, BoNT/A uptake, processing, and activation in derived

neurons are of comparable efficiency to primary neurons. Notably, ESNs have been shown to be highly sensitive to all seven classical BoNT serotypes (/A-/G) with the same relative potencies observed in vivo, suggesting that derived mouse neurons are suitable for the study of all BoNTs (Hubbard et al. 2012).

Although all derived neuron populations have been reported to be highly sensitive to BoNTs, each model has strengths and weaknesses in terms of cost, technical methods, and relevance. For example, while cholinergic motor neurons may more closely resemble the neuronal subtype that is intoxicated in vivo, intoxication assays have not been conducted in mixed cultures with functioning neuromuscular junctions (NMJs). Moreover, it is questionable whether a cholinergic motor neuron is capable of undergoing synaptogenesis with non-muscle cells (i.e., other neurons) and, if so, whether such a hybrid synapse is any more relevant to a NMJ than a glutamatergic synapse. Thus until in vitro-derived NMJs composed of cholinergic motor neurons synapsing upon muscle cells can be produced in sufficient quantities for HTS and are shown to exhibit relevant responses to intoxication, it is unclear that cholinergic motor neuron differentiation is worth the additional cost and lower yield.

In contrast, while human PSC-derived neurons offer a greater physiological relevance to human disease modalities than do mouse PSC-derived neurons, they also take significantly longer to progress from the pluripotent stage to a synaptically active neuron population (see comparison in Fig. 2) and require several additional treatment conditions that are correspondingly more expensive. For example, mature neuron populations exhibiting functional synapses, with an excitatory-inhibitory balance and spontaneous miniature excitatory and inhibitory postsynaptic currents, are routinely produced within 18 days from mouse ESCs (Gut et al. 2013). Although a number of reports suggest that differentiation of human PSCs to neuronal populations can generate synaptic activity, many of these studies involved cocultures with active primary neurons, implantation into CNS tissue, or genetic manipulation. Even so, the appearance of synaptic activity in these cultures takes 7–12 weeks, and the network activity and neuron subtype composition of resulting populations are not yet well characterized (Hu et al. 2010; Kim et al. 2011). These facts suggest that considerable optimization is still needed to identify reliable and reproducible methods to generate synaptically mature neuronal networks from human stem cells. Some of the delays associated with differentiation of human neurons from ESCs or hiPSCs can be avoided by purchasing pre-derived neurons (e.g., see Whitemarsh et al. 2012); however, the costs can be prohibitive to small labs. Moreover, as above, commercially available neurons have not yet proven to be able to reliably produce functioning synapses with emergent network behaviors. Given recent examples of accelerated neuronal differentiation of human PSCs into defined lineages using SMIs, resource costs involved in human PSC derivation may soon drop and/or production by small labs may become less daunting, allowing a wider array of methods to be tested to accelerate maturation (Drury-Stewart et al. 2011; Morizane et al. 2011).

In contrast, mouse ESC differentiation is not resource intensive (costs are less than \$40 per 1.25×10^8 neuron precursors, sufficient to plate ~1,000 cm^2 per

differentiation, and eight differentiations per week are not beyond the capabilities of a single technician), and mouse ESC-derived neurons offer the facile validation of presumptive targets in vivo using reverse genetics approaches or exploiting already-existing knock-in/knockout mouse lines. The only recurring complication that has been observed in ESC maintenance and neuron production is the identification of differentiation-compatible serum lots, an issue that occurs every 18–24 months.

Although specialized, the technical expertise required to produce PSC-derived human and mouse neurons is not significantly more demanding than routine tissue culture, and once the appropriate protocols are in place, producing large quantities of neurons for moderate- to high-throughput screens is relatively easy. Labs new to neuronal derivation have access to a variety of resources, and existing labs can be lobbied for specific protocols or may be willing to host researchers for specialized training. While improved methods are always in development, given the temporal and resource limitations described above, a reasonable research paradigm might be to conduct target discovery in mouse ESC-derived neurons, confirm findings in human PSC-derived neurons, and then validate using in vivo models.

Glutamate Toxicity

Glutamate is the primary excitatory neurotransmitter within the mammalian central nervous system (CNS), and synaptic release of glutamate activates a diverse family of glutamate receptors (GluRs). Synaptic receptor targets for glutamate can be subdivided into two groups: (1) ionotropic glutamate receptors (iGluRs), including the NMDA, AMPA, and kainate receptors (Traynelis et al. 2010), and (2) metabotropic glutamate receptors (mGluRs), which are coupled to various G-protein targets important in mediating downstream signaling events (Shigemoto et al. 1997). In addition to key physiological roles in excitatory neurotransmission and CNS plasticity, excessive glutamate can also trigger neurodegeneration through a process termed excitotoxicity. Excitotoxicity is induced following the overstimulation of GluRs by pathologic levels of glutamate, particularly NMDARs, allowing sustained Ca^{2+} influx which, in turn, initiates apoptotic signaling pathways (Arundine and Tymianski 2003). Glutamate-induced toxicity is a shared mechanism of neuronal dysfunction in a number of neurodegenerative states, including Alzheimer's disease, traumatic brain injury, stroke, multiple sclerosis, epilepsy, Parkinson's disease, and chemical nerve agent intoxication (Mehta et al. 2013).

Previous attempts to clearly characterize excitotoxicity using in vitro model systems have been hampered by various technical and biological limitations. Neuroblastoma cell lines exhibit poor sensitivity to glutamate stimulation and fail to form functioning excitatory synapses, while primary neuron cultures exhibit variability in results, depending on origin, homogeneity, handling, and treatment (Singleton and Povlishock 2004). Characterization of two PSC-derived neuron platforms has demonstrated that derived neurons can be highly sensitive to glutamate and express AMPA and NMDA receptors with physiologically consistent current/voltage relationships and that glutamate-induced excitotoxic cell death

(metabolic failure and neurite fragmentation) is mediated through the NMDA receptor in a time- and dose-dependent fashion (Gupta et al. 2013; Gut et al. 2013). Moderate-throughput CBAs have identified several treatments that modulate excitotoxicity, demonstrating the suitability of derived neurons for more intensive screens of compound libraries (Gut et al. 2013). Other stem cell-derived models, including mouse neural stem cells (Choi et al. 2010) and hiPSCs (Gupta et al. 2013) have also been demonstrated to be suitable for glutamate-induced excitotoxicity research. Collectively, stem cell-derived neuron models can provide a well-defined, sensitive, homogenous, functionally relevant platform for the testing of candidate compounds aimed at mitigating the pathogenesis associated with glutamate excitotoxicity in a number of diseased states.

Latrotoxin

While the majority of toxinology research in stem cell-derived neurons has focused on BoNTs and glutamate, derived cultures have been shown to be compatible with the study of other toxins, such as α-latrotoxin (LTX) in mouse stem cell-derived neurons (Hubbard et al. 2012; Mesngon and McNutt 2011). LTX is the active component in black widow spider venom, and clinical symptoms of latrodectism (LTX poisoning) include muscle pain and cramping, tremors, diaphoresis, hypertension, tachypnea, lacrimation, and tachycardia (Jelinek 1997). Molecularly, LTX binds synaptic adhesion molecules, inserts into the neuronal membrane, and assembles into calcium-permeant pores, resulting in the unregulated presynaptic release of neurotransmitter and activation of calcium-sensitive intracellular signaling pathways (Silva et al. 2009). Mouse stem cell-derived neurons have been demonstrated to express transcripts of known LTX receptor targets and, following toxin exposure, display dose- and time-dependent effects of LTX intoxication, including calcium uptake and decreased cellular viability (both measured in a moderate-throughput plating format) (Hubbard et al. 2012). Interestingly, a brief application of LTX was also found to restore non-cleaved SNAP-25 protein levels within 48 h following intoxication of ESNs with BoNT/A (Mesngon and McNutt 2011). These findings were consistent with an in vivo model of BoNT/A-induced muscle paralysis (Duchen et al. 1981; Gomez and Queiroz 1982), thereby recapitulating an in vivo phenomenon.

Developmental Neurotoxicity

While the list of toxins tested in stem cell-derived neurons currently remains relatively short, the applications are not limited to mature neuronal cultures for toxin testing. Derived neurons also enable the high-resolution analysis of developmental neurotoxicity (Visan et al. 2012). Derived neurons are ideally suited for such studies, as the in vitro maturation from immature neurons to a fully mature

synaptically coupled network faithfully simulates known aspects of in vivo neurogenesis (Shi et al. 2012; Muguruma and Sasai 2012). Neuronal differentiations of mouse embryonic stem cells (Visan et al. 2012; Sanchez-Alvarez et al. 2013), human neural stem cells (Buzanska et al. 2009), and rat neural progenitor cell (Singh et al. 2009) have been successfully used as in vitro models to characterize the developmental neurotoxicity of environmental toxicants such as lead acetate, methylmercury, and ethanol. As characterization of neuronal differentiation continues, studies on the effects of neurotoxins on neurogenesis are expected to increase commensurately.

Future Applications of CBAs in Toxinology Research

Activity-Dependent Phenotypic Assays for Toxin Detection

A distinguishing feature of stem cell-derived neuron cultures is the formation of functional synapses and the emergence of network behavior. For example, mouse ESC-derived neurons display network behavior 3 weeks after differentiation, characterized by spontaneous action potentials, miniature excitatory, and inhibitory postsynaptic currents and an excitatory/inhibitory balance (Gut et al. 2013). This capacity renders derived neuron cultures suitable for phenotypic assays based on activity-dependent neuronal responses. Since subtle perturbations in electrical responses or ion currents can have profound effects on network activity, activity-dependent CBAs offer the ability to rapidly capture neuronal responses to toxins that may not be amenable to molecular readouts. Assays designed around neuronal behavior therefore can serve as a sensitive, rapid, and comprehensive platform for target discovery and therapeutic screening while concurrently providing information on the effect of toxins on network behavior. Examples of phenotypic assays based on neuronal activity include fluorescent reporters of ion flux (e.g., Fluo-4 (Ikegaya et al. 2005) and GCaMP (Chen et al. 2012)), changes in neuronal voltages (Jin et al. 2012), and the release of neurotransmitters (GluSnFR (Hires et al. 2008)) as well as electrophysiological measurements of network activity (Potter 2001).

As one example of the increased sensitivity and specificity provided by phenotypic assays, it has been shown using whole-cell patch-clamp electrophysiology that exposure of networked mouse stem cell-derived neurons BoNT/A eliminated miniature excitatory postsynaptic currents within 240 min. Notably, in these cultures only ~5 % of cellular SNAP-25 was cleaved at the same time point (unpublished data). These findings confirm that BoNT intoxication of mature neuronal populations elicits the same pathophysiology that occurs in vivo with clinical botulism. Moreover, they suggest that measurements of synaptic activity can provide a direct readout of the integrity of the subpopulation of SNAP-25 involved for neurotransmitter exocytosis. In contrast, immunoblot assays measure the fraction of cleaved SNAP-25 among the total cellular pool of SNAP-25, a readout with a significantly lower signal-noise ratio.

Though patch-clamp electrophysiology offers exquisite resolution and sensitivity, it is currently not compatible with moderate- to high-throughput testing of differentiated and networked neuronal cultures. Advances in other technologies are beginning to address this capability gap by combining high-content or high-throughput readouts with activity-based assays. For example, multielectrode arrays (MEAs) are planar arrays of substrate-integrated electrodes on which neuronal cultures can be grown. In this format, action potentials in overlying neurons can be simultaneously stimulated and/or recorded in moderate- to high-throughput formats. By correlating spatial and temporal signatures of neuronal activity, changes in network behavior can be correlated to time and toxicant dose. MEAs have been used to detect the presence of BoNT/A in synaptically coupled cultures of cortical neurons (Scarlatos et al. 2008) and to evaluate effects of a broad variety of toxicants on network activity (Zhou et al. 2009; van Vliet et al. 2007; Weiss et al. 2011). Another technology that is expected to support moderate- to high-throughput phenotypic CBAs is fluorescent imaging plate reader (FLIPR) technology. FLIPR uses a rapid imaging camera to enable real-time quantitation of fluorescence in living cells at 6 Hz in 96-well or 1384-well dishes. Although a new technology, FLIPR has been used to evaluate neuronal responses to a variety of toxicant assays, including intoxication-induced changes in the network activity of cultured hippocampal neurons exposed to the $GABA_A$ antagonist tetramethylenedisulfotetramine. Additionally, FLIPR technology has successfully been applied to the identification of small molecule potentiators of AMPARs in mouse ESC-derived neurons and for the pharmacological profiling of metabotropic glutamate receptor agonists (Vetter 2012; McNeish et al. 2010; Sanger et al. 2013).

PSC-Derived Neuromuscular Junctions

Because many toxins utilize the NMJ as a physiological target, the in vitro production of functioning NMJs from PSCs may offer a next-generation platform for toxin testing and therapeutic discovery. While it has been known since the 1970s that primary tissues from the spinal cord and skeletal muscle can assemble into functioning NMJs (Thomson et al. 2012), the ability to produce motor neurons from PSCs has only recently become achievable. Recent studies have demonstrated the ability to derive spinal cord neurons from human ESCs that form morphological and functional evidence of synapse formation with myoblasts and primary skeletal muscle (reviewed in Thomson et al. 2012). However, despite these successful advances using human PSCs, the resultant cocultures remain immature and short-lived compared to cocultures using primary tissue. A recent study has addressed the issue of in vitro NMJ maturity by demonstrating the production of functional in vitro NMJs from mouse ESC-derived neurons that exhibit maturing morphologies through 28 days in vitro (Chipman et al. 2014). While these PSC-derived cocultures are promising, the field is still immature and the technical difficulty associated with in vitro NMJ production may be

prohibitive to higher-throughput platforms. Further advances that resolve these limitations may render in vitro NMJs a suitable cellular model for the study of toxin-NMJ interactions.

Reverse Translational Research

One exciting capability of stem cell-derived platforms is their potential application in reverse translational research, or "bedside-to-bench" methods, which enable the elucidation of molecular mechanisms underlying a previously diagnosed phenotype or behavior. Much of the promise of hiPSCs is derived from this capability. Following clinical diagnosis of a disease, patient-specific stem cell lines can be generated and subsequently differentiated into a variety of cell types, with the anticipation that the differentiated cells will exhibit the phenotype of interest. These cells can then be used for target discovery, disease modeling, or HTS assays for potential therapeutics (reviewed in Cashman et al. 2013).

An analogous approach also has considerable potential to characterize the molecular effects of homozygous knockouts and conditional gene expression in murine ESCs, with the additional experimental power of translational in vivo experiments in the corresponding mouse lines. Derived neurons could then be used to assign functions to genes, dissect genetic pathways, and screen for candidate therapeutics. This technique would be applicable to hemizygous and homozygous progeny derived from stem cell implantation or transgenic mouse lines produced from nuclear injection. It would offer particular value in instances of developmentally lethal crosses, in which viable progeny do not occur, but ESCs can be harvested, and the effect of the specific mutation can be evaluated at various developmental stages of neurogenesis. Finally, ESCs can be isolated from a transgenic mouse line expressing a fluorescent reporter of a neuronal activity, expanded and subsequently differentiated into neurons.

While the reverse translational approach requires greater up-front expenses than does the differentiation of transfected ESCs, it offers several specific benefits for CBAs, including (a) enabling a more direct comparison between behavioral and molecular readouts of toxin-host interaction, since transgene expression would occur from an identical genetic context as in vivo; (b) decreasing noise in HTS assays by ensuring consistent gene dosage effects; and (c) facilitating a high degree of inter-experimental consistency.

Advanced Culture Formats for Complex CBAs

Finally, the construction of 3D cultures, complex multicell models, or even artificial neuronal structures offers exciting capabilities for toxicity testing. While still highly controlled and reductionist, these approaches take advantage of the promise of recent advances in 3D cultures (Li et al. 2012), compartmentalized cultures

(Takayama et al. 2012), micropatterned substrates (Hughes et al. 2013), microstamped surfaces (Branch et al. 1998), and/or microfluidic chambers (Park et al. 2013) to explore mechanisms underlying neuronal behaviors in increasingly complex and physiological environments. Although these approaches are more resource intensive than traditional neuron tissue culture, if they can be made specific, sensitive, and compatible with moderate- to high-throughput assays, they may offer a more comprehensive approach that integrates the behavior of multiple cell types or tissues in a single experiment. Combining these next-generation platforms with derived neurons expressing genetically encoded fluorescent proteins would facilitate rapid, activity-based assays that have the potential to accelerate neurotoxicity research and drug discovery.

Conclusion and Future Directions

Although the use of neurogenic cells in CBAs for neurotoxinology research has expanded in recent decades, such assays are limited by the questionable physiological relevance of non-neuronal cells. Not surprisingly, many neuronal studies conducted in non-neuronal CBAs have failed to properly translate to *in vivo* studies. Recent advances in stem cell-derived neuron techniques have begun to address this limitation by developing cell-based models that combine the scalability and flexibility of continuous cell-lines with the physiologic relevance of primary neuron populations. Compatibility studies conducted in recent years in various stem cell-derived neuron models have yielded promising results for drug discovery and neurotoxicology research. Mouse stem cell-derived neurons have been shown to be amenable to neurotoxicology studies, are easily scalable and their derivation is relatively inexpensive in terms of cost, time and resources. However the translational relevance of mouse neurons to human disease modalities still remains a concern. The recent development of human stem-cell derived neurons has considerable promise for addressing the question of relevance, but is not without its own disadvantages, including high-costs, length of culture, and the general difficulty in producing neurons exhibiting mature characteristics, particularly synaptic activity and network emergence. As these disadvantages are addressed and next-generation, moderate- to high-throughput assays based on functionally mature, derived neurons become widely available, the use of human and mouse CBAs is expected to transform and accelerate neurotoxicology research, disease modeling, and therapeutic drug discovery.

Cross-References

▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
▶ Botulinum Toxin: Present Knowledge and Threats
▶ Challenges in Developing Biotoxin Inhibitors

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13 Immunosensors: Using Antibodies to Develop Biosensors for Detecting Pathogens and Their Toxins

Olivier Laczka

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Abstract

Fast, reliable, and low-cost methods for the screening of pathogens are paramount in areas such as environment, the food industry, healthcare, and defense. The word pathogen defines any disease-producing agent.

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With the constant progress of scientific knowledge, a fast diversification of the detection techniques is occurring, brought about by the appearance of imaginative new concepts within the scientific community. Biosensors are a perfect example of the combination of multidisciplinary knowledge. They encompass many fundamental, technological, and scientific advances in biology, chemistry, and physics.

This chapter includes an overview of different types of antibodies and labels used as recognition elements for the elaboration of immunosensors. The most common ways to immobilize Ab on a transducer surface will be described and a description of some of the most popular transducing techniques will be given.

Introduction

Biosensors have been defined (http://www.biosensors-congress.elsevier.com/about.htm) as "analytical devices incorporating a biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products etc.), a biologically derived material (e.g., recombinant antibodies, engineered proteins, aptamers etc.) or a biomimic (e.g., synthetic catalysts, combinatorial ligands, imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical." They can thus be classified according to their signal transduction method or according to the biological recognition element used.

The field of immunoassay-based methods for pathogen detection is growing very fast as it provides a very powerful analytical tool, compatible with a high diversity of potential targets. The enzyme-linked immunosorbent assay (ELISA) (Crowther 1995) is the most established immunoassay and is also the source of inspiration for many biosensor applications. ELISA combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. There are two main variations on this method: ELISA can be either used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen. The most common type of ELISA is the "sandwich ELISA," coupled to either direct or indirect detection depending on the type of enzyme-labelled antibody used (a primary labelled antibody that recognizes the target directly in the first case, while a secondary labelled antibody in the second case, used to bind to an unmodified primary antibody against the target and thus indirectly detect the target) (Crowther 1995; Li et al. 2001). A sandwich ELISA is a four-step procedure which is carried out in well plates made of polystyrene. The principle of this test is shown in Fig. 1. Each well can be manipulated independently; thus up to 96 samples or more (depending on the plate capacity) can be assayed in parallel. First, the microtiter plate wells are coated with, for example, an antigen-specific antibody and the remaining uncovered space blocked with an appropriate inert protein (often bovine serum albumin). Then the sample containing the target antigen is added to the wells followed by addition of an antibody conjugated to an enzyme. Each step is

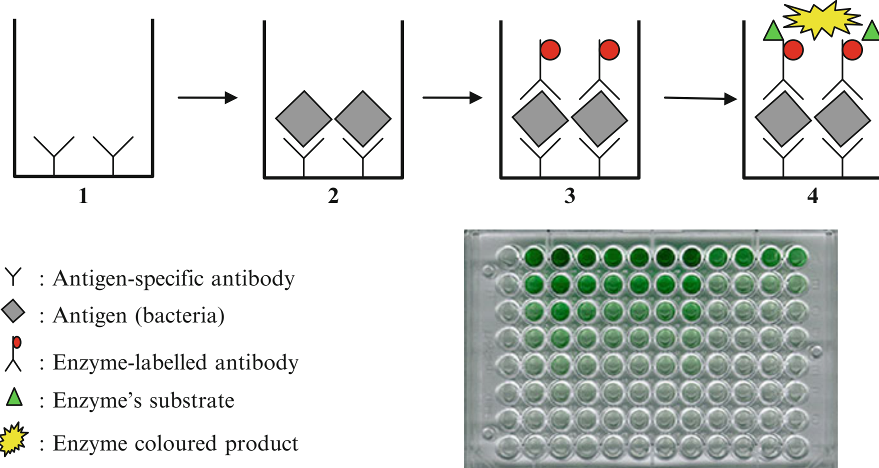

Fig. 1 Schematic representation of a sandwich ELISA and photograph of a plate after the enzymatic reaction. *1* Immobilization of the antigen-specific antibody on the well surface and blocking step; *2* Immuno-capture of the antigen; *3* Addition of the enzyme-labelled antibody; *4* Addition of an appropriate enzyme's substrate and production of the colored product.

followed by extensive washing, often with a detergent-containing buffer, as to ensure desorption of nonspecifically bound molecules. Finally, an ultimate reaction includes the addition of a chromogenic or fluorogenic substrate which is converted by the enzyme into a colored/fluorescent product, indicating a positive reaction and the amount of target present in the sample (Blais et al. 2004).

Immuno-capture can be coupled to a variety of analytical techniques to confirm the pathogen's presence, for example, immunomagnetic separation (IMS) (Gagne et al. 1998), a pretreatment and/or pre-concentration step consisting of introducing specific antibody-coated magnetic beads in the suspension containing the antigen in order to capture the targeted pathogen.

Antibodies are the most widely used biorecognition elements thanks to their proven sensitivity and specificity (Byrne et al. 2009). They were applied to detection for the first time in the 1950s (Yallow and Berson 1959). Depending on their production methodology, they can be polyclonal or monoclonal. The partial enzymatic digestion of Abs can generate different types of antibody fragments (Fab, Fab' and F(ab')2, Fv, sc-Fv, depending on their size and components) that interestingly keep their target-binding ability. The advances in recombinant technology enables the in vitro production, and even selection, of some of them by cloning the gene codifying for the antibody-binding site into an expressing host (transformed *Escherichia coli*, phage-displayed libraries). On the other hand, certain antibodies from both camels and sharks are being studied, as they are smaller in size and more stable than regular antibodies (Holliger and Hudson 2005; Hoogenboom 2005). The use of small antibody variants in sensor development is expected to generate better surface coverage and favor integration and miniaturization.

Even if antibodies can be simply adsorbed to a sensing surface through nonspecific means, many authors defend that directed immobilization, by chemical conjugation or cross-linking of precise functional groups in the antibody (i.e., amines, carboxylates, carbohydrates), helps preserve their integrity and functionality, while promoting more organized structures.

Antibodies

General Description of Antibodies

Antibodies belong to the family of proteins known as immunoglobulins (Ig). They are produced by B lymphocytes which, in mammals, mature in the bone marrow. Their role is related to the immune system of animals. Briefly, antibodies identify intrusive compounds called antigens and bind to them, facilitating their subsequent elimination. Antibodies are especially effective in recognizing other proteins as these have large three-dimensional structures and, consequently, offer different potential binding sites (or epitopes) for particular antibodies (Eryl 1995).

The structure of IgG, the antibody most extensively exploited for analytical purposes, shown in Fig. 2, provides a comprehensive picture of its recognition mechanism. IgGs have an average molecular weight of 150 kDa and are composed by four subunits: two identical light chains and two identical heavy chains. Disulfide bonds and non-covalent interactions stabilize the association of these subunits,

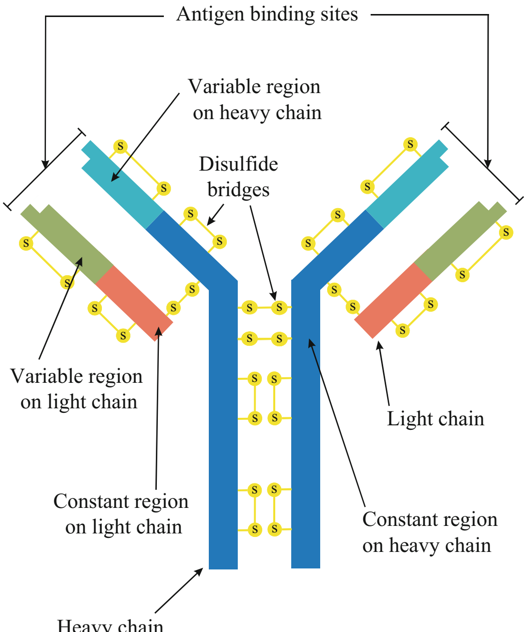

Fig. 2 Schematic representation of an antibody belonging to class G (IgG).

forming a Y-shape symmetric dimer with dimensions of approximately 14.5 nm × 8.5 nm × 4 nm (Silverton et al. 1977). The constant regions are chemically similar among different antibodies whereas the variable regions are chemically unique for each antibody. This diversity enables each antibody to interact with its target molecule with high affinity and specificity.

The Different Types of Antibodies and Their Production

Polyclonal antibodies (PAb) are produced by inoculating an animal with a target protein or component of the protein of interest, so that its immune system produces antibodies in an attempt to neutralize the intruder. Different animals can be used for antibody production: e.g., rabbits, goats, or mice. After a period of time allowing the recognition process and antibody production, the animal is bled and the serum containing the antibodies is extracted. Provided that the target has many epitopes, the resulting serum contains a collection of antibodies that recognize different regions of the analyte. This not only makes the process highly variable due to batch-to-batch differences, but it also makes it difficult to produce antibodies towards small molecules, which may cause little or no immunogenic response. However, PAb are useful in approaches not demanding "excessively high" specificity, such as simultaneous detection of different bacterial serotypes.

The development of hybridomas allowed the production of **monoclonal antibodies (MAb)** in the mid-1970s (Kohler and Milstein 1975). Hybridomas are established by fusing an immortal cell line (a tumor cell line) with a single blood cell precursor isolated from the immunized animal, meaning that each hybridoma produces a single type of antibody against a single epitope. MAb are characterized by their high selectivity. This methodology allows the consistent production of relatively high amounts of MAb, with no significant batch-to-batch differences, and reproducible specificity and sensitivity. It is precisely their highly valued selectivity that can limit their application when a single antibody is to be used in the detection of a series of bacteria serotypes or virus variants. This implies that MAb cocktails should be used instead. On the other hand, MAb are significantly more expensive than PAb. They are often provided in smaller amounts and are not always available against nonpathogenic compounds (i.e., laboratory molecules used as model systems during sensor optimization).

Often used for immunosensing application, **secondary antibodies** are directed towards other antibodies (e.g., goat anti-rabbit antibodies, which have been produced by injecting rabbit antibodies in a goat). Secondary antibodies are usually conjugated to a label molecule that allows their further detection and quantification and, for example, their application to the indirect detection of a target in immunoassays. This way, the target that is captured on the surface is first bound by an unmodified anti-target antibody, followed by detection with a labelled secondary antibody. Indirect detection takes longer than the direct strategies but usually provides better detection limits. Alternatively, secondary antibodies can be used

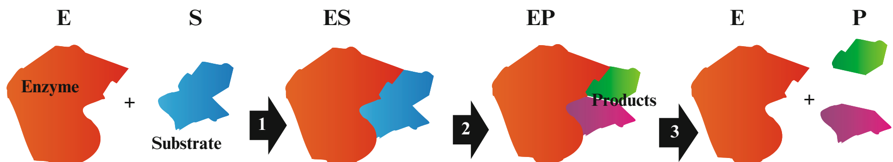

Fig. 3 Schematic representation of an enzymatic reaction with *1* the binding of the enzyme and its specific substrate at the active site of the enzyme, *2* the transformation of the substrate into the products of the reaction, and *3* the release of the products leaving the enzyme free and ready to react again.

by immobilizing on a surface to capture primary antibodies, but they are susceptible to suffer denaturation or inactivation during the functionalization procedure (e.g., MAb).

The Use of Enzyme Labels for Signal Generation and Amplification

Enzymes are proteins that act as biological catalysts, providing *high reaction rates* and *high specificity*. They work under *mild conditions* (moderate pH, temperature, salinity, etc.) and can include *regulation processes*. These four characteristics are the main ones differing when enzymes are compared with ordinary chemical catalysts (Voet and Voet 1995).

The region of the enzymatic molecule where the catalysis occurs is known as the *active site* of the enzyme. The reactants of the enzymes are commonly called substrates. A typical enzymatic reaction is illustrated by Fig. 3. This reaction can be decomposed in three different steps being the recognition and binding of the substrate by the enzyme, the transformation of the substrate into the product of the reactions, and finally the release of this product. The enzyme is then, in general, capable of achieving other substrate transformations.

In pathogen biosensing, however, enzymes are used as labels rather than recognition elements, allowing detection in a sandwich assay format. Enzymes can then be used to label antibodies much in the same manner as they are in classical ELISA. In the case of amperometric (electrochemical) biosensors, the use of labels is critical (Prodromidis and Karayannis 2002). However, other transduction strategies do not depend on the use of labels and give shape to truly reagentless biosensors. This is true for surface plasmon resonance (SPR), piezoelectric or impedimetric biosensors, among others (Guan et al. 2004).

One of the most commonly used enzyme label is horseradish peroxidase (HRP). HRP is a 44,000 Da glycoprotein with four lysine residue for conjugation to a labelled molecule. Its reaction with a substrate produces a color, fluorimetric, or luminescent derivative of the labelled molecule allowing it to be detected and quantified. Its activity can also be detected electrochemically (Laczka et al. 2011). Horseradish peroxidase is ideal in many respects for these applications because it is smaller, more stable, and less expensive than other popular alternatives such as alkaline phosphatase. It also has a high turnover rate that allows generation

of strong signals in a relatively short time span (Veitch 2004). Numerous substrates for the horseradish peroxidase enzyme have been described and commercialized to exploit the desirable features of HRP.

Surface Functionalization with Antibodies

Surface bio-functionalization is critical in the development of any sort of biosensor. It consists of the incorporation of the recognition element of choice, usually on top of the sensing surface (Bae et al. 2004). However, biosensor functionalization can affect the sensitivity of some transduction formats. Consequently, some works report on functionalization of the sensor vicinity (Thévenot et al. 1999) or even modification of an unrelated surface that is approached to the transducer following target capture. The use of paramagnetic particles is a good example (Ivnitski et al. 2000). In any case, bio-functionalization provides the core of the biosensor, ensures its specificity for the target of choice, and gives it its identity. Indeed, by definition (Thévenot et al. 1999), there is no biosensor without an incorporated recognition element of biological nature. On top of this, the selected immuno-functionalization strategy has to provide an immobilized biomolecule that keeps its original functionality as much as possible in order for the biosensor to work. This means that care must be taken so that the recognition sites are not sterically hindered. Another common reason for biosensor failure or underperformance is the chemical inactivation of the active/recognition sites during the immobilization procedure. Due to the inherent complexity of the problem, there is nothing like a universal immobilization method suitable for every application imaginable; thus, when it comes to deciding on which of the existing immobilization methods to use, several important factors need careful consideration, such as the type of transduction used, the nature and composition of the sample, and the possibility of multiple use of the biosensor. Brief descriptions of the three most common approaches follow which are:

- Random physisorption
- The avidin–biotin system
- Covalent bonding onto self-assembled monolayers (SAM)

Random Physisorption

Nonspecific adsorption simply consists of depositing the molecules (antibodies, antigen, etc.) on the sensor surface to interact in a random way. This strategy relies in the fact that proteins consist of a series of hydrophilic/hydrophobic, charged/uncharged segments, arranged in solution as to expose the most thermodynamically stable structure. When the protein approaches a solid–liquid or vapor–liquid interface, where the medium conditions are different from the solution bulk, it interacts with the interface and rearranges itself in search for a new “most stable structure.” Protein adsorption is stabilized by a combination of hydrophobic interactions,

hydrogen bonding, and/or van der Waals forces (Parida et al. 2006). Regardless of the protein isoelectric point (pI), the maximal surface coverage is often attained for most soluble proteins on neutral surfaces at neutral pH and physiological ionic strength (Laczka et al. 2008). When gold surfaces are used, proteins containing free SH_2 groups and/or S–S bonds profit from the strong crossbinding between these functional groups and gold. Generally, monoclonal antibodies show worse performance than polyclonal antibodies following nonspecific adsorption.

Biotin–(Strept)Avidin Affinity Capture

This functionalization strategy is based on the natural strong binding of avidin for the small molecule biotin (also called vitamin H). It is one of the most popular non-covalent conjugation methods and a very useful tool for targeting applications (Hermanson 1996). Avidin is a glycoprotein which is isolated from egg whites among others. It consists of four identical subunits (tetramer) and has a total molecular weight of 66,000 Da. Each one of these subunits contains one binding site for biotin. The dissociation constant of the biotin–avidin complex is 1.3×10^{-15} M, which makes it one of the strongest non-covalent interactions known, while its biospecificity is similar to antibody–antigen or receptor–ligand recognition. Once formed, the complex is extremely stable. Moreover, a functional biotin group can be added to proteins and other molecules, and specific biotinylation agents targeting amine, carboxylates, sulfhydryls, and carbohydrate groups are commercially available.

A very similar protein called streptavidin, isolated from *Streptomyces avidinii*, is preferably used for certain applications. The reason is that streptavidin does not generate the high level of nonspecific binding of negatively charged molecules that characterizes avidin, a consequence of its high carbohydrate content and thus high isoelectric point (10 for avidin versus 5–6 for streptavidin which is not a glycoprotein) (Bayer et al. 1990).

Other modified forms of avidin that are increasingly used are NeutrAvidin™ and ExtrAvidin®, provided by Molecular Probes (Invitrogen) and Sigma, respectively, and do not contain carbohydrates. Their use results in lower levels of background signal than with streptavidin while retaining the high-affinity characteristic of avidin (Chung et al. 2006).

A factor to consider when using this immobilization strategy is that certain proteins naturally possess covalently bound biotin and, when present in the samples under study (e.g., some tissues), they will contribute to increasing the background noise (Wood and Warnke 1981).

Covalent Bonding onto Self-Assembled Monolayers

Self-assembled monolayers (SAM) were first investigated in the 1980s with the study of assemblies formed by the adsorption of organosulfur compounds from solution or the

vapor phase onto metal substrates of gold and silver. They are organic single layers formed by spontaneous organization of thiolated molecules on metal surfaces (Love et al. 2005). The most extensively studied class of SAMs is derived from the adsorption of *n*-alkanethiols on gold, silver, copper, palladium, platinum, and mercury.

SAMs are extensively exploited for immobilization of recognition elements as the functional groups provided by the SAM layer termination can be used to suit any particular requirements using bioconjugation techniques.

In order to obtain a self-assembled monolayer, a metal surface is immersed in a solution containing the chosen SAM-forming molecule in a high-purity solvent (Bain and Whitesides 1989). The majority of studies on the use of SAM in surface functionalization for biosensing applications involve the immersion of gold in an ethanol solution containing disulfides or thiols (Su and Li 2004).

Once the monolayer has been formed, the reactive chemical groups exposed at the free ends of the self-assembled molecules can be used to incorporate a biomolecule of choice. One of the easiest conjugation strategies when it comes to the functionalization of a surface with antibodies involves activation of a carboxyl-exposing SAM using a solution of 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS). The activated surface will react with any free amine groups present in the antibody (Tombelli and Mascini 2000). Parameters such as SAM composition, Ab concentration, or conjugation time have to be carefully optimized in order to ensure optimal coverage and functionality of the surface. The transductor's surface is then rinsed with phosphate buffer solution to remove non-convalently adsorbed antibodies. The binding sites on the SAM that have not reacted with the antibodies then need to be blocked using either bovine serum albumin, BSA (Su and Li 2004), or a small amine (Oh et al. 2003).

Detection Systems Using Antibodies

Optical Biosensors

Optical biosensors offer high selectivity and sensitivity and are the most popular devices for pathogen detection (Lazcka et al. 2007). Numerous optical biosensors have been developed for rapid detection of contaminants (Liao and Ou 2005), toxins or drugs (Bae et al. 2004; Liu et al. 2004), and even pathogenic bacteria (Bokken et al. 2003). Due to their high sensitivity, fluorescence- and surface plasmon resonance (SPR)-based methods are particularly used for biosensing applications.

Fluorescence Detection

Fluorescence occurs for certain molecules when an electron relaxes to its ground state after having been excited by a photon of enough energy. The emitted light is at

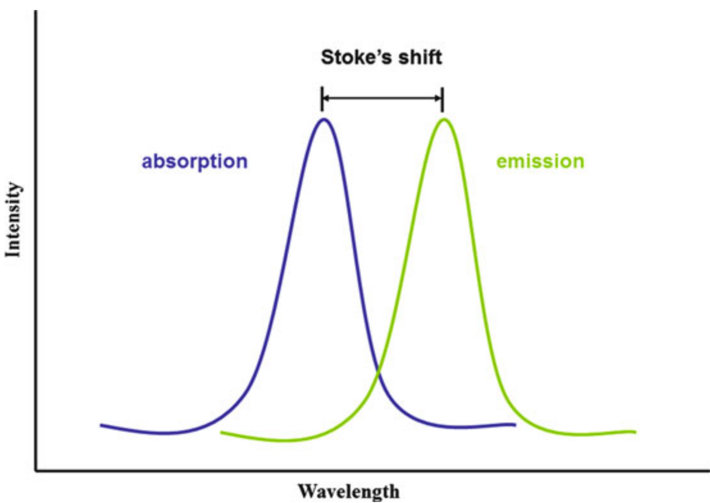

Fig. 4 Example of absorption and emission spectra of an ideal fluorophore or fluorescent dye.

a longer (less energetic) wavelength than the absorbed light due to the loss of energy resulting from vibrations, molecule rotation, or heat. This energy gap is termed Stokes shift as illustrated in Fig. 4. Desirably, the Stokes shift should be large enough to avoid cross talk between excitation and emission signals.

Antibodies can be conjugated to fluorescent dyes, such as fluorescein isothiocyanate (FITC) (Li et al. 2004). There are, however, lots of fluorescent markers commercially available, often modified for their fast and easy conjugation (Selvin 2002).

An interesting example of a fluorescence-based biosensor is given by Grant and Ko in (Ko and Grant 2003). In this work, they make use of a very specific fluorescence mode known as fluorescence resonance energy transfer, FRET. The working principle consists in the transfer of energy from a donor fluorophore to an acceptor fluorophore when the distance between them is very small (<100 Å). This short distance is known as the Försters distance. If the distance between fluorophores is greater than the Försters distance, the excitation of the donor fluorophore results in fluorescence emission only by the donor. When the separation is within the Försters distance, FRET occurs and fluorescence emission will occur from both fluorophores, even if only the donor has been apparently excited (Fig. 5). The biosensor developed by these researchers takes advantage of this principle because the steric conformation of antibodies changes when their antigen binds to them. By attaching both fluorophores to different parts of a *Salmonella*-specific antibody, the authors are able to report whether a food sample contains *Salmonella* down to a detection limit of 2 μg/ml.

Surface Plasmon Resonance

Surface plasmon resonance (SPR)-based biosensors have been drawing increasing attention in the past decade (Bergwerff and Van Knapen 2006; Homola

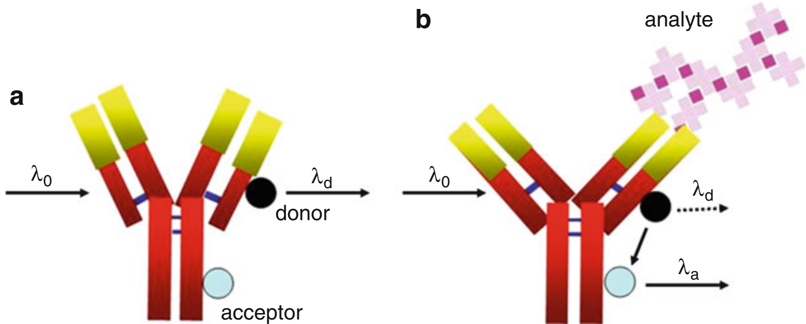

Fig. 5 Diagram of a FRET biosensor. The specific binding of the analyte to the antibody results in a conformation change of the antibody. The distance between the acceptor fluorophore and the donor fluorophore is then shortened, resulting in the emission of the fluorescence signal.

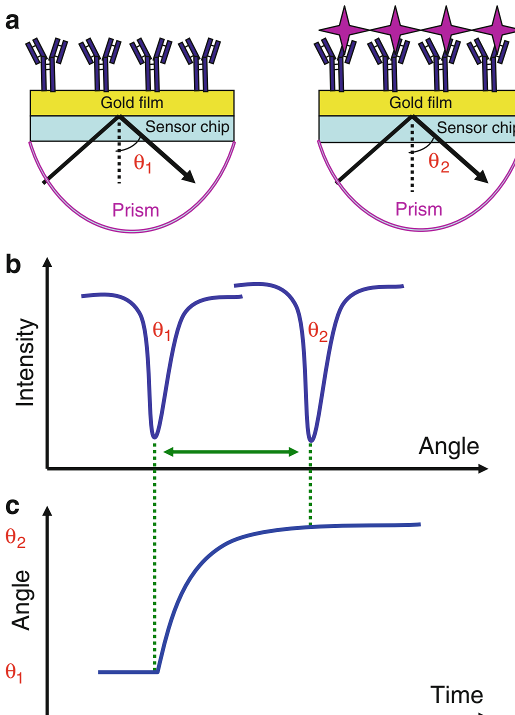

Fig. 6 Diagram of an SPR biosensor. The resonance angle is shifted due to the binding of an antigen to the antibodies and a sensorgram is obtained.

2003). SPR measures the changes in refractive index caused by small changes in the vicinity of a thin-film metal surface. Current instruments are described in Fig. 6. A glass plate covered by a gold thin film is irradiated from the backside by a polarized light (from a laser) at different incidence angles θ via a prism, and light reflection is measured. The plot of θ versus light reflectivity is a curve

showing a narrow dip, known as the SPR minimum. It corresponds to the incidence angle at which light reflectivity is minimal and light dispersion in the shape of surface plasmon is maximal, and it is determined by the properties of the gold-solution interface. When measurements are carried out at this incidence angle (or close to it), any changes occurring at the interface (such as protein adsorption or capture) will affect its refractive index. This will translate into a shift of resonant wavelength or angle. Adsorption phenomena and antigen-antibody reaction kinetics can be monitored in real time using this technique (SPR is often used to determine antigen-antibody affinity constants).

Piezoelectric Biosensors

Piezoelectric sensors comprise the observation of resonance frequency changes following mass changes on the probe/transducer surface (Marx 2003). The most widely used piezoelectric sensing format is the quartz crystal microbalance (QCM). In QCM, the relation between mass and resonant frequency is given by the Sauerbrey equation:

$$\Delta f = \frac{-2.3x10^6 f_0^2 \Delta m}{A} \quad (1)$$

where Δf is the frequency change in Hz, f_0 is the resonant frequency of the crystal in MHz, Δm is the deposited mass in grams, and A is the coated area in cm^2.

As a number of literature reports show (Pathirana et al. 2000; Wang et al. 2012), the use of QCM allows the detection of pathogens and contaminants using sensors modified with immobilized antibodies.

Electrochemical Biosensors

Electrochemical sensing (Palchetti and Mascini 2008; Thévenot et al. 1999) is mainly based on the observation of current or potential changes at the sensor-sample interface, and the relationship between these changes and the concentration of a given analyte in the vicinity of the sensor is studied. Techniques are generally classified according to the observed parameter: current (amperometric), potential (potentiometric), or impedance (impedimetric). Compared to optical methods, electrochemistry allows the analyst to work with turbid samples, and the cost of the equipment is much lower. On the other hand, electrochemical methods present more limited selectivity and sensitivity than their optical counterparts.

Amperometric Biosensors

Amperometric techniques are based on the measurement of the current response to the potential step applied at an electrode. However, different techniques can be

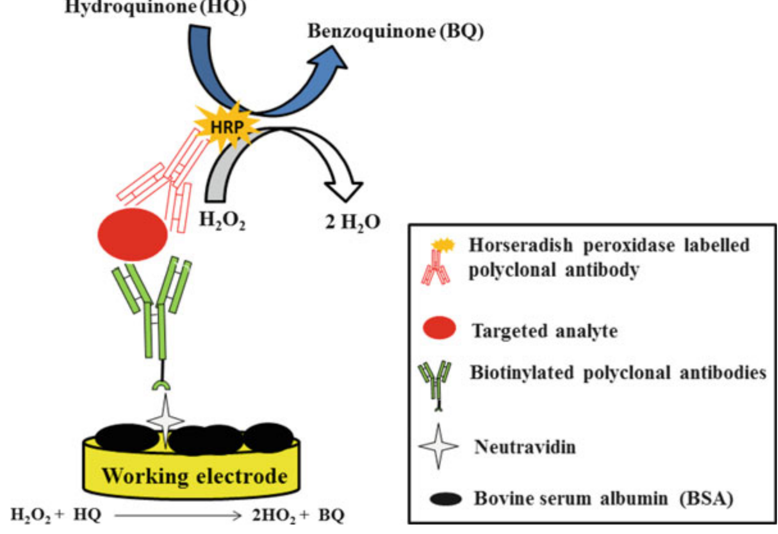

Fig. 7 Schematic representation of the electrochemical detection of a targeted analyte using a sandwich-type capture of the target and the chronoamperometric detection of HRP via the use of hydroquinone as a mediator.

distinguished as a function of the kind of potential function applied, which, for example, can be a ramp or a series of potential steps.

The simplest potential step technique, also known as *chronoamperometry*, is based on the measurement of the current response to a potential jump. The step is usually realized from a potential where no reaction occurs to a potential at which the reaction of interest takes place.

Chronoamperometry is perhaps one of the most common electrochemical detection methods used in biosensing. This method is based on the existing linear relationship between analyte concentration and current. In the case of many electrochemical biosensors, the presence of electroactive species is required to monitor the analyte concentration (Eggins 2002). Redox mediators are small-size compounds able to reversibly exchange electrons between both the sensor and the analyte of choice (e.g., ferricyanide, osmium or ruthenium complexes, etc.). Figure 7 illustrate an example of the chronoamperometric immunosensor. In this case, a biotinylated capture antibody is immobilized onto a gold electrode surface previously covered with NeutrAvidin. The surface was then blocked with BSA and the targeted analyte left to bind with the antibody. An HRP-labelled antibody was then added and left to bind with the target. The detection is based on the electrochemical detection of the labelling enzyme HRP through its catalysis of hydrogen peroxide (H_2O_2) in the presence of the mediator hydroquinone (HQ). The oxidation of HQ, which follows the regeneration of HRP, into benzoquinone (BQ) can be monitored amperometrically along time applying a given potential at which the BQ formed is reduced. An example of a biosensor in which the

chronoamperometric detection of HRP via the use of hydroquinone as a mediator can be found in Laczka et al. 2011.

Many different combinations and strategies are possible and used to build amperometric biosensors, the choice of which depends on constraints imposed by sample matrix, analyte characteristics, or the necessity to reuse the device (Willner et al. 1997).

Amperometric biosensors for bacteria detection, either antigen-antibody capture or DNA probe based, do not differ much from more traditional biosensors (Leonard et al. 2003). A very good example is found in Abdel-Hamid (1999). In this work, the authors propose a flow-through immunofiltration method coupled to amperometric detection of *E. coli*. The experimental setup consists of a flow-through electrochemical cell containing an immunofiltration membrane on top of a working electrode. An immunofiltration membrane is nothing other than a filter with antibodies immobilized on its surface. When a stream containing the target analyte, *E. coli* in this case, is passed through this filter, the bacteria are trapped by the antibodies. A washing step follows which aims to minimize nonspecific binding. After this, a new solution containing peroxidase-labelled antibodies against the already immobilized target is flowed. This configuration is identical to a sandwich scheme for ELISA. Next, iodide and hydrogen peroxide, used as redox and enzyme substrate, respectively, are added. The working electrode measures the current consumed to regenerate the label, which is directly proportional to the concentration of analyte. The authors quote analysis times of 30 min and detection of down to 100–600 cells/ml.

Interdigitated electrodes may be used for amperometric measurements in collector–generator mode to enhance the sensitivity of the assay (Laczka et al. 2010). This model of operation is also known as redox cycling and it consists in operating each of the electrodes at a different potential. One electrode is kept at a potential such that either a reduction or an oxidation process is brought about, while the second electrode is set at a potential where the reverse reaction occurs. This procedure is described schematically in Fig. 8 and it allows the shuttling of the electroactive species between both sets of electrodes, thus enhancing the observed currents.

In amperometric measurements, the electrode which potential is modulated is called the *generator electrode* whereas in all cases the *collector electrode* stays at a constant potential. As depicted in Fig. 8, the signal detected at the generator is higher than if the electrode is used alone. It is also interesting to note that the current recorded at the collector electrode is enhanced as the scan rate at the generator decreases. This is because, as the scan rate gets slower, the product of the oxidation has more time to diffuse towards the collector electrode and back. This increases the collection efficiency and, as a result, the intensity of the current recorded is also enhanced.

The parameters of interest for the characterization of these devices are the collection efficiency, the feedback factor, and the number of cycles (Fosset et al. 1991). The collection efficiency (ϕ_{ss}) is defined as the ratio between collector and generator electrode currents at the steady state (the part of the

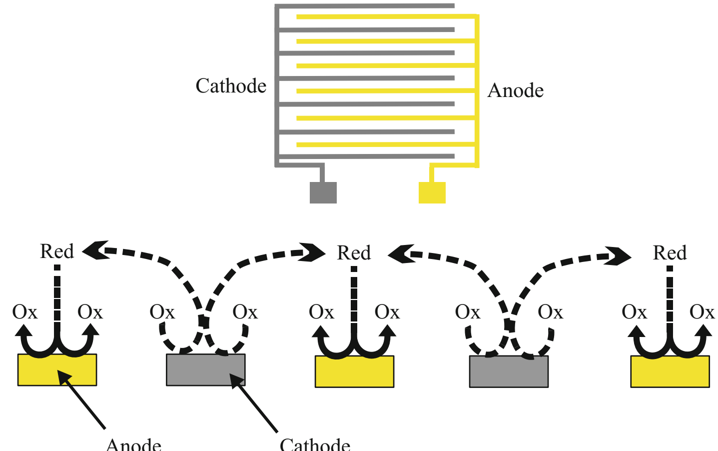

Fig. 8 Redox cycling mechanism at an interdigitated electrode.

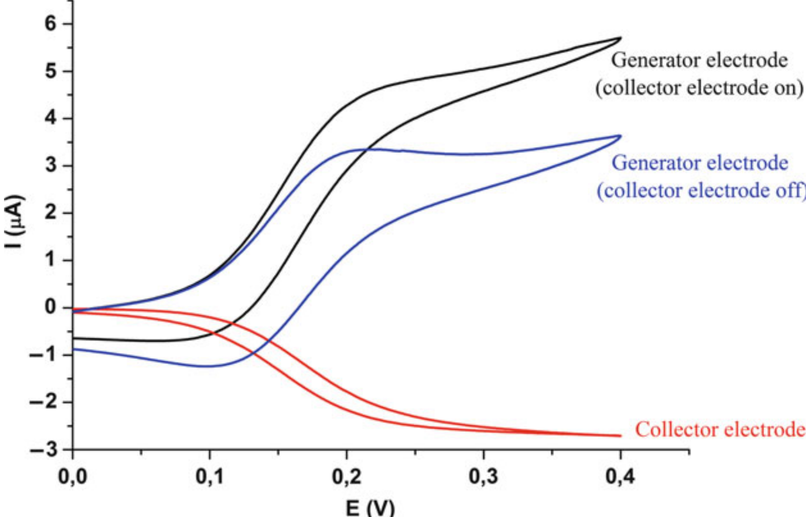

Fig. 9 Experimental redox cycling curves at an interdigitated electrode in a 1 mM ferricyanide solution at a scan rate of 10 mV/s (*black* and *red lines*). Comparison with the voltammogram obtained for the generator electrode used in a single mode under the same conditions (*blue line*).

voltammograms that show a stable current value in Fig. 9) according to the following equation:

$$\phi_{ss} = \frac{|I_c|}{|I_g|} \tag{2}$$

The feedback factor (*FB*) and number of cycles (*Rc*) aim to provide an estimation of the number of times a molecule is able to shuttle back and forth between the electrodes. The next equations depict how they can respectively be calculated.

$$FB = 1 - \frac{I_{g,o}}{I_{g,c}} \tag{3}$$

where $I_{g,o}$ and $I_{g,c}$ are the currents measured at the generator with the collector electrode at open circuit and in the generation-collection modes, respectively:

$$Rc = \frac{1}{1 - {\phi_{ss}}^2} \tag{4}$$

Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) represents a powerful method for the study of conducting materials and interfaces, and its basics can be found in the generalist electrochemistry bibliography (Bagotsky 2006) and dedicated monographs (Orazem and Tribollet 2008).

In contrast to the most commonly used electrochemical techniques, which are based on the application of direct currents, such as the case of *chronoamperometry* or *voltammetry* previously described, EIS is based on the superimposition of a sine-wave potential of small amplitude over a polarization potential of constant value.

The most common way to analyze EIS data consists in defining the independent processes contributing to the system behavior and laying out its equivalent circuit in terms of electrical components, namely, resistors and capacitors. In order to build the equivalent circuit, each phenomenon occurring at the vicinity of the sensing surface must be identified with an equivalent element. This is illustrated in Fig. 10, an example where an electroactive specie is present in solution and thus where faradaic phenomena take place. Once the equivalent circuit is defined, the experimental data is fitted to it using nonlinear least-square fitting techniques.

In the case of EIS-based biosensors, the modification of the electrode brings about important interfacial changes. For a start, it may no longer make sense to talk about double-layer capacitance (Fig. 10) but to leave it all in terms of an overall interfacial capacitance. The presence of a coating (be it a self-assembled monolayer or the biorecognition elements directly adsorbed) on the electrode surface changes both the interfacial capacitance and the electron transfer rate constant. However, depending on the detection strategy chosen for the biosensor, one of these parameters may either be easier to measure or show bigger changes upon detection. In the case of non-faradaic EIS, where no electroactive specie is used and thus the detection is based on the direct recognition of the analyte by the functionalized surface, the study can focus on the changes affecting the interfacial capacitance as no electron transfer takes place.

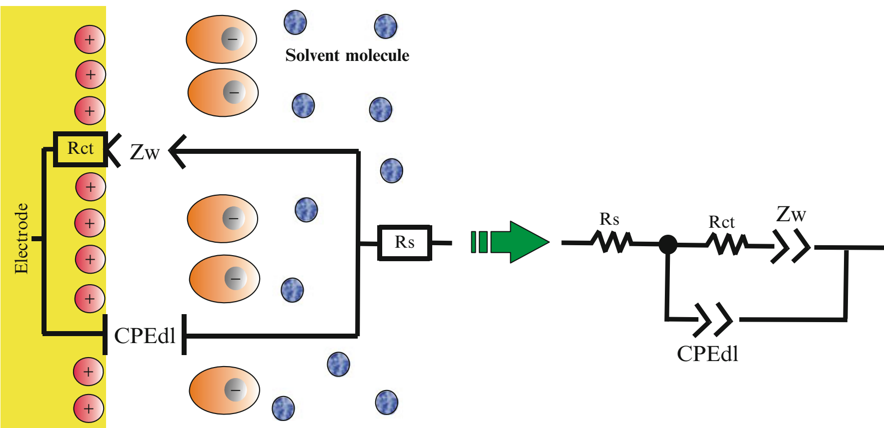

Fig. 10 Diagram showing the construction of an equivalent circuit (Randles circuit in this case) deduced from the behavior of an electrode in a solution containing an electroactive compound. The components are the resistance to electron transfer (*Rct*), the solution resistance (*Rs*), the Warburg impedance due to the diffusion properties of the reduced or oxidized form of the redox couple (*Zw*), and the double-layer capacitance (*CPEdl*).

In Fig. 11a, a typical impedance result is shown where no electroactive species is present in the reaction bulk.

The difference between the curves shown in this figure set is the presence or absence of proteins on the electrode surface.

Figure 11b, c show results of the fitting provided by the software Zplot. Two parameters are considered in order to build our equivalent circuit, the solution resistance (illustrated as R1 in the circuit) and the capacitance CPE. The solution resistance does not change in a significant way which is logical considering that the solution used in both measurements is the same. On the other hand, the capacitance (value indicated as CPE-T) is considerably lower after modification of the electrode as a result of the decrease of the dielectric constant at the electrode-solution interface.

An interesting example of an immunosensor using impedimetric detection is given in a 2010 study carried out by Chai et al. (2010). In this work, an aluminum electrode was immunofunctionalized via covalent bonding onto SAM, and the authors were able to detect the presence of the biological toxin ricin in 20 min.

Electrochemical biosensors are widely studied and represent a very large panel of possibilities. The incredibly high amount of combinations that can be imagined when it comes to the design of these devices reveals that they have a very promising future. The constant advances of photolithographic technologies can, for example, contribute to the improvement of sensibility and robustness of the devices. However, compared to the quantity of studies conducted on the elaboration and improvement of electrochemical biosensors, the amount of validated commercial devices is still quite low. This shows that the construction of these devices is not trivial and that many limitations still exist. A very interesting use of electrochemical biosensors involves their capacity to be used for online measurements.

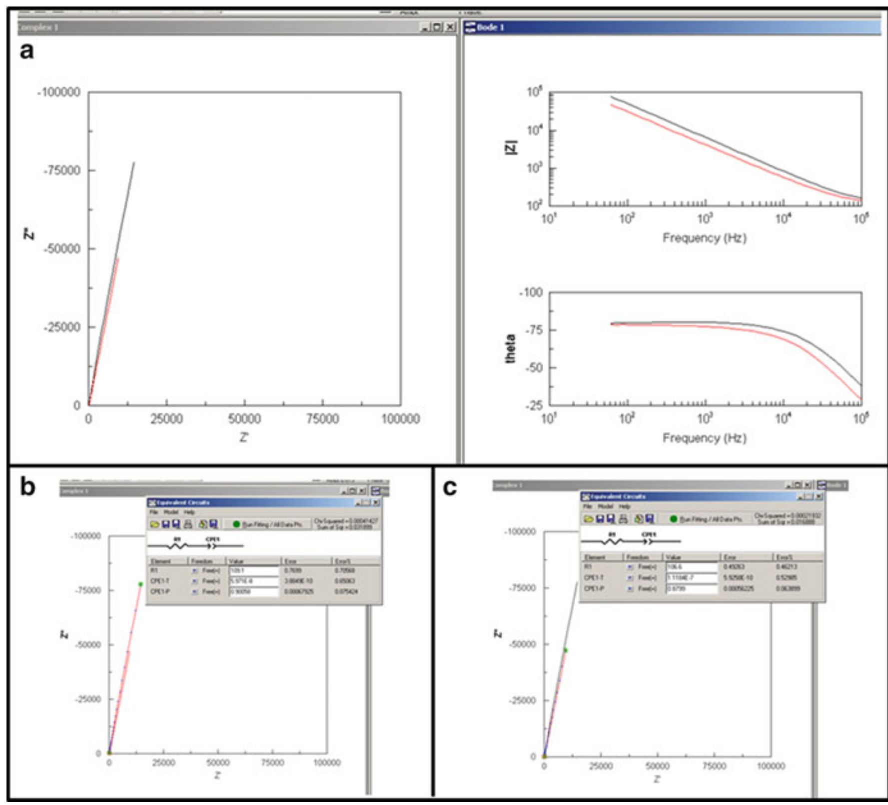

Fig. 11 Representation of impedance results, where complex Nyquist plane and Bode plots are generated (**a**). In this case, the impedance was measured at potential 25 mV for an interdigitated gold electrode submerged in a 1 M KCl solution before (*red line*) and after (*black line*) deposition of proteins on its surface. The software of most commercial equipments allows the user to fit the results with an equivalent circuit in order to get the value of the solution resistance (in Ω) and capacitance (in farads) before (**b**) and after (**c**) the electrode modification. In this case, the Zplot software has been used.

However, the complexity of real sample matrix prevents the sensors from measuring properly. This highlights the necessity for improving the different pretreatment steps of real samples, an area in which a lot of work still needs to be done.

Conclusion and Future Directions

Traditional pathogen and toxin detection methods, although sensitive enough, are too slow to be of any practical use in today's fast-paced world. For this reason, new methods are needed that exceed their performance. The research

for new analytical devices enabling the detection of pathogens is attracting increasing effort among the scientific community. This phenomenon is directly related to the need for faster, simpler, more economic, and miniaturizable systems. Over the recent years, studies and development of biosensors of the most diverse nature have been carried out, but their performance is irregular and still needs improvement. Conceiving biological detection devices requires the proficient combination of different scientific aspects. First of all, detection devices like the ones described in this chapter have to be well characterized before they can be used to their maximum potential, which often means improving their sensitivity and selectivity. Physical processes must also be well understood and controlled to interpret the data correctly. When the functionalization of the electrodes is required, the right surface chemistry combinations must be chosen very carefully. Last, the biological interactions, which range from the molecular scale to cellular mechanisms, need to also be well understood.

Optical techniques generally provide better sensitivity than electrochemical ones. However, their complexity and cost can make them unattractive. Electrochemical techniques are much easier to use but their performance still needs to be improved when it comes to detecting pathogens. In order to become attractive, electrochemical biosensors first need to show that they are capable of reaching at least the same detection levels as traditional techniques (between 10 and 1,000 CFU/ml for pathogens). Next, the time needed for the analysis must be reduced without increasing cost. The use of microfabrication techniques in the area of biosensors holds great promise. Micro (and nano) technologies offer the possibility to integrate all the necessary sample treatment and detection steps in a single unit of minute dimensions (lab-on-a-chip).

Among the advantages of this approach are (a) the possibility of mass production which would result in reduced costs, (b) the study of sample volumes in the range of nanoliters or less, decreasing the cost of reagents as well as improving safety and environmental care since power consumption is extremely low and there is nearly no waste, (c) the potential integration of microfluidics which may improve mixing rates and mass transport and result in much shorter assay times, and (d) the possibility to perform multi-analyte studies, which also shortens sample analysis time.

Examples combining pathogen detection and miniaturization can be found in the literature. A recent example of such a micro device is given by Lillehoj et al. in this work (Lillehoj et al. 2010); the authors describe a self-pumping lab-on-a-chip system to detect botulinum toxin using amperometry. The microfluidic system used in this study allows the detection of 1 pg of botulinum neurotoxin in 1 μL in 15 min. In the near future, pathogen detection will undoubtedly benefit from the integration of biosensors into microdevices. Although, barring selectivity, the key to performance will still lie in a necessary compromise between time and sensitivity.

Cross-References

▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures

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Mycotoxins Relevant to Biowarfare and Their Detection

14

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Abstract

A toxin is any toxic substance that can be produced by an animal, plant, or microbe. Some toxins can also be produced by molecular biological techniques (protein toxins) or by chemical synthesis (low-molecular-weight toxins). The threat of biological warfare is an increasing concern in recent years. Advances in microbiology and genetic engineering have made it possible to create extremely dangerous toxigenic and pathogenic microorganisms. Accurately detecting and identifying biological warfare agents (BWAs) is the focal point for countering bioterrorism, methods for detection of these agents and treatments for infection are currently limited, and the development of highly rapid and sensitive field-deployable methods for early detection of biological warfare agents is crucial. There has been a remarkable progress in the detection, protection, and decontamination of biological warfare agents as many instrumentation platforms and detection methodologies are developed and commissioned. Furthermore in the past decade, there have been threats due to the emerging new diseases and also the reemergence of old diseases and development of antimicrobial resistance and spread to new geographic regions. The preparedness against these agents needs complete knowledge about the disease, better research and training facilities and diagnostic facilities, and improved public health system. Even then, the threat of biological warfare agents and their use in bioterrorist attacks still remain a leading cause of global concern. This review on the major fungal biological warfare agents will provide information on the biological warfare agents and their detection.

Introduction

Biological Warfare and Bioweapons

Biological warfare is warfare conducted using biological weapons, or weapons that are of a biological origin rather than from the hand of man – the chemist, the physicist, the engineer, or the computer programmer. These weapons can be a disease-causing microorganism (a pathogen) or a toxin, a poison of biological origin.

Mycotoxins

Mycotoxins, by-products of fungal metabolism, have been implicated as causative agents of adverse health effects in humans and animals that have consumed fungus-infected agricultural products (Ciegler and Bennett et al. 1980). Consequently, fungi that produce mycotoxins, as well as the mycotoxins themselves, are potential problems from both public health and economic perspectives. Various genera of toxigenic fungi are capable of producing such diverse mycotoxins as the aflatoxins, trichothecenes, satratoxins, and fumonisins (Ciegler and Bennett et al. 1980).

Mycotoxins as Weapons: The Yellow Rain Controversy

From 1974 to 1981, toxic agents were used by the Soviet Union and its client states in such Cold War sites as Afghanistan, Laos, and Kampuchea (Cambodia) (Wannemacher, Chap. 34). Aerosol and droplet clouds were produced by delivery systems in the Soviet arsenal such as aircraft spray tanks, aircraft-launched rockets, bombs (exploding cylinders), canisters, a Soviet handheld weapon (DH-10), and booby traps. Trichothecenes appear to have been used in some of these attacks. The air attacks in Laos have been described as “yellow rain” and consisted of a shower of sticky, yellow liquid that sounded like rain as it fell from the sky. Other accounts described a yellow cloud of dust or powder, mist, smoke, or insect spray-like material. In Laos, 50–81 % (Haig 1982) of attacks involved material associated with a yellow pigment that caused symptoms including blistering, vomiting, and bloody diarrhea. The symptoms could have been caused by a chemical warfare agent such as mustard gas, indicating a serious escalation of the fighting in these areas. Plant samples taken from areas where attacks had taken place showed the presence of unusual mixtures of trichothecene mycotoxins, often in combination with unusual mixtures of pollen grains, but without evidence of *Fusarium* infection. At least one sample included traces of an industrial chemical, polyethylene glycol, which is used as a plasticizing agent and not found in nature.

Unconfirmed reports have implicated the use of trichothecenes in the 1964 Egyptian (or Russian) attacks on Yemeni Royalists in Yemen (Ricaud et al. 1983) and in combination with mustards during chemical warfare attacks in the Iran–Iraq War (1983–1984) (Ember et al. 1984). According to European sources, Soviet–Cuban forces in Cuba are said to have been equipped with mycotoxins, and a Cuban agent is said to have died of a hemorrhagic syndrome induced by a mycotoxin agent (Seagrave 1981). An article written by Ember (1984) published in Chemical Engineering News is the most exhaustive and authoritative account of the controversy surrounding the use of trichothecene mycotoxins in Southeast Asia during the 1970s. Compiling evidences of US Government and its allies support the contention that trichothecene mycotoxins were used as biological warfare agents in Southeast Asia and Afghanistan by the former Soviet Union and its surrogates.

Concerning the mass production, chemical nature, and serious effects on humans and other animals, several countries have proposed that the following mycotoxins have biothreat potential:

1. Trichothecenes
2. Aflatoxins

Need of Detection Methods for Biowarfare Toxins

Due to their deleterious effects to humans and other farm animals and also the chances being used as biothreat agents, there is an emerging need of detection systems to detect these mycotoxins from food and environmental sources.

The detection system which can be a field-deployable, rapid, reliable, and sensitive means of detection will be a good asset for regular monitoring of these toxins from suspected samples. A dual monitoring could be considered in order to meet food safety concerns and official legislated regulations under biowarfare convention. First, the presence of fungi having the potential to produce the biothreat toxin could be checked at critical points during production of agricultural commodities as well as during the process of food and feed preparation. Early detection of these fungi could prevent OTA contamination in foodstuffs and protect consumers from hazardous mycotoxins. Usual identification and quantification methods of food-borne fungi require time-consuming and labor-intensive morphological and physiological tests and often mycological expertise. The current trend is toward culture-independent PCR-based methods because they overcome problems associated with selective cultivation and isolation of microorganisms and are generally characterized by their simplicity, speed, cost-effectiveness, and reliability (Niessen 2008).

Subsequently, mycotoxins could be detected directly in food sample using analytical methods able to perform highly selective measurements. For this reason, chromatographic techniques have been usually taken as reference methods because of their accuracy and reproducibility (Turner et al. 2009). To deal with the increasing number of sample matrices and mycotoxins of interest, fast and accurate analytical methods are needed. This demand has led to the development of rapid screening methods for single mycotoxins or whole mycotoxin classes based on immunochemical techniques (ELISA), biosensors (protein chips, antibody-/protein-coated electrodes), and noninvasive optical techniques. On the other hand, highly sophisticated multi-mycotoxin methods based on LC coupled to multiple-stage MS are being developed to allow accurate and precise determination and unambiguous identification of mycotoxins without the need for tedious sample preparation and cleanup procedures. Recently, aptamers have been shown to successfully compete with antibodies as biological receptors for analytical tool development. These single-stranded oligonucleotides are selected in vitro in a sort time compared to the antibody production and are more stable under a wide range of conditions. Furthermore, they can be easily modified or labeled providing flexibility to develop a wide range of assessment assays (Mairal et al. 2007). Summary of the various methods for the detection of toxigenic fungi and mycotoxins were depicted in Fig. 1.

Current Trends in Mycotoxin Detection with Respect to Biothreat Agents

Trichothecene Mycotoxins-T-2 Toxin

Trichothecenes are a group of mycotoxins produced by toxigenic *Fusarium* species which occurs in cereal grains throughout the world (Ramana et al. 2011). Potentially hazardous concentrations of the trichothecene mycotoxins can occur naturally

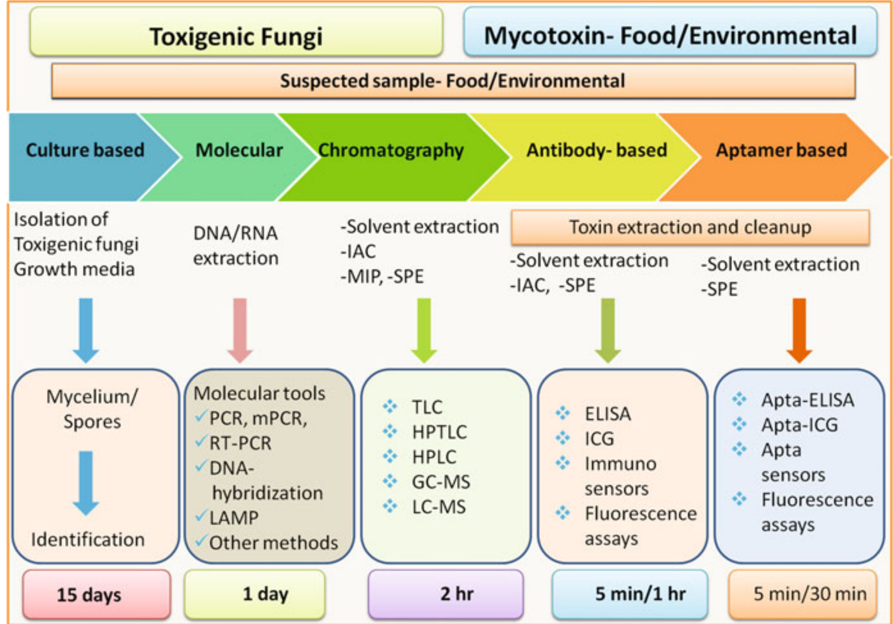

Fig. 1 Schematic representation of several methods of mycotoxin detection

in moldy grains, cereals, and agricultural products. Trichothecenes are classified into two major classes, type A and type B trichothecenes. As on reports availability Type A trichothecenes were considered as potent (T-2 toxin) bioweapon agents in fast. Structures of some major trichothecene mycotoxins were represented in Fig. 2.

T-2 Toxin as a Biological Warfare Agent

Unlike most biological toxins that do not affect the skin, T-2 mycotoxin is a potent active dermal irritant. Moreover, it is the only potential biological weapon agent that can be absorbed through intact skin causing systemic toxicity. Clinical symptoms may be present within seconds of exposure. While larger amounts of T-2 toxin are required for a lethal dose than for other chemical warfare agents such as VX, soman, or sarin, its potent effect as a blistering agent is well noted. T-2 toxin can be delivered via food or water sources, as well as via droplets, aerosols, or smoke from various dispersal systems and exploding munitions. These properties make T-2 mycotoxin a potentially viable biological warfare agent. The reported LD 50 of T-2 toxin is approximately 1 mg/kg (Kalantari 2005). Based on extensive eyewitness and victim accounts, the aerosolized form of T-2 toxin called "yellow rain" was delivered by low-flying aircraft that dropped the yellow oily liquid on the victims. T-2 toxin has been used during the military conflicts in Laos (1975–1981), Kampuchea (1979–1981), and Afghanistan (1979–1981) to produce lethal and nonlethal casualties. More than 6,300 deaths in Laos, 1,000 in Kampuchea, and 3,000 in Afghanistan have been attributed to yellow rain exposure (Haig 1982).

Common name	R^1	R^2	R^3	R^4	R^5
T-2 toxin	ISV	H	OAc [a]	OAc	OH
T-2-Glc	ISV	H	OAc	OAc	Glc
3-Ac-T-2 toxin	ISV	H	OAc	OAc	OAc
4-deoxy-T-2	ISV	H	OAc	H	OH
4-deoxy-T-2-(3-Glc)	ISV	H	OAc	H	Glc
HT-2 Toxin	ISV	H	OAc	OH	OH
3-Ac-HT-2 toxin (iso T-2)	ISV	H	OAc	OH	OAc
T-2 Triol	ISV	H	OH	OH	OH
T-2 tetraol tetra acetate	OAc	H	OAc	OAc	OAc
8-Ac-Neosolaniol	OAc	H	OAc	OAc	OH
Neosolaniol	OH	H	OAc	OAc	OH
Nivalenol	=O	OH	OH	OH	OH
Fusarenon-X	=O	OH	OH	OAc	OH
3,15 di-Ac-NIV	=O	OH	OAc	OH	OAc
Deoxynivalenol (DON)	=O	OH	OH	H	OH
Tri-Ac-DON	=O	OAc	OAc	H	OAc
4, 15-Diacetoxyscirpenol	H_2	H	OAc	OAc	OH

[a] OAc and ISV represent $-OCOCH_3$ and $-OCOCH_2CH(CH_3)_2$, respectively.

Fig. 2 Structure of trichothecene mycotoxins (Reprint from Maragos et al. 2013)

Early symptoms beginning within minutes of exposure include burning skin pain, redness, tenderness, blistering, and progression to skin necrosis with leathery blackening and sloughing of large areas of skin in lethal cases. Nasal contact is manifested by nasal itching and pain; sneezing; epistaxis and rhinorrhea; pulmonary/tracheobronchial toxicity by dyspnea, wheezing, and cough; and mouth and throat exposure by pain and blood-tinged saliva and sputum. Anorexia, nausea, vomiting, and watery or bloody diarrhea with abdominal crampy pain occur with gastrointestinal toxicity. Eye pain, tearing, redness, foreign body sensation, and blurred vision may follow entry of toxin into the eyes. Skin symptoms occur in minutes to hours and eye symptoms in minutes. Systemic toxicity is manifested by weakness, prostration, dizziness, ataxia, and loss of coordination. Tachycardia, hypothermia, and hypotension follow in fatal cases. Death may occur in minutes, hours, or days. The most common symptoms are vomiting, diarrhea, skin involvement with burning pain, redness and pruritus, rash or blisters, bleeding, and dyspnea (Kalantari 2005).

T-2 toxin was produced by *Fusarium sporotrichioides*, *F. cerealis*, *F. tricinctum*, and other *Fusarium* species. It is a causative agent of ATA (alimentary toxic aleukia) and common contaminant of cereal-based foods. Compiling evidences

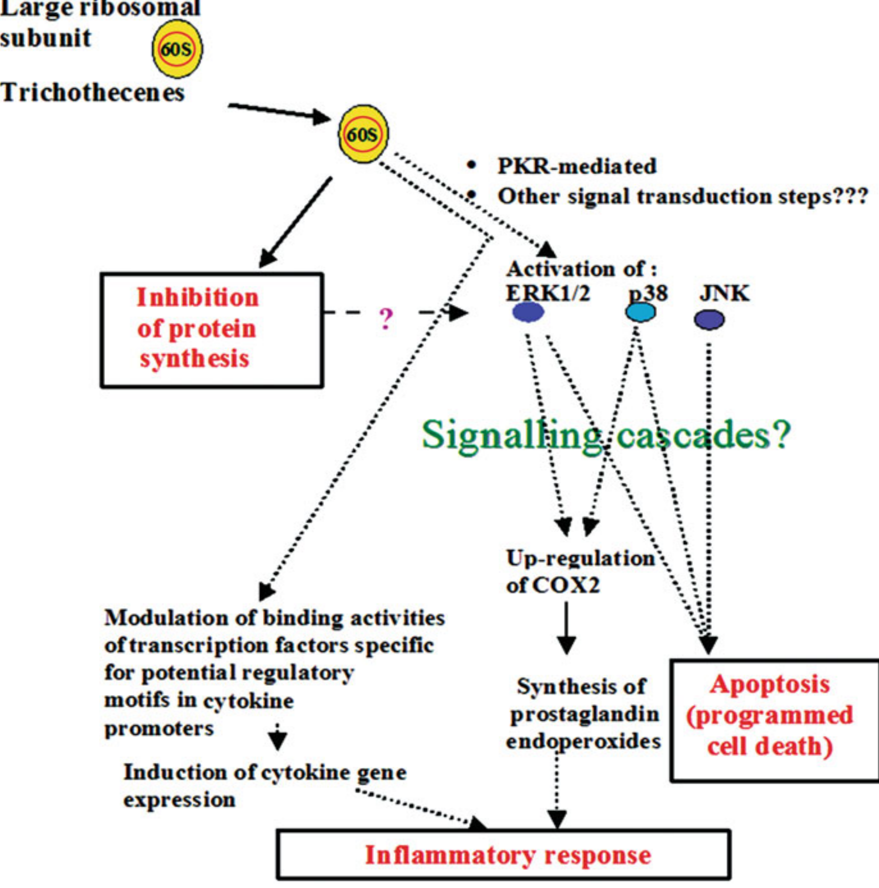

Fig. 3 Trichothecene-mediated signal transduction and toxicology in mammalian cells (Shifrin and Anderson 1999). Protein synthesis inhibition: trichothecenes interfere with the active site of peptidyl transferase on ribosomes. Mitogen-activated protein kinase (*MAPK*) activation: certain trichothecenes induce ERK1/2, JNK, and p38 MAPKs (Laskin et al. 2002). A double-stranded RNA-activated protein kinase R (*PKR*) mediates DON-induced phosphorylation of ERK1/2, p38, and JNK MAPKs (Zhou et al. 2003). DON activation of the proinflammatory response: it induces cytokine transcription and, as a consequence of MAPK activation, induces cyclooxygenase-2 (*COX-2*) and increases synthesis of prostaglandin endoperoxides. DON-induced apoptosis (Shifrin and Anderson 1999; Yang et al. 2000; Poapolathep et al. 2002) increases linearly with inhibition of protein synthesis, and the peptidyl transferase site is postulated to be a regulator of both MAPK activation and apoptosis in Jurkat T cells

suggested that T-2 toxin can cause several diseases ranging from acute skin lesions to chronic cancers in different animals and humans by several pathway mechanisms (Fig. 3) (Choudhari et al. 2009). In recent years, many scientists developed detection strategies for the detection of these toxins in lowest concentrations at laboratory as well as field levels.

PCR-Based Methods for Trichothecene-Producing Fungi Detection

Nowadays, PCR has replaced the cumbersome microbiological analysis by amplification of specific genomic markers rather than growing the living organisms under study (Abdin et al. 2010; Niessen 2008). Within the last years, PCR-based methods have been set up for the detection, differentiation, and identification of trichothecene-producing fungi. Due to the strong specificity and high degree of sensitivity, PCR-based methods including multiplex and real-time PCR methods appear as a good tool to provide early detection of trichothecene-producing fungi in order to control or reduce fungal mass and toxin production at early and critical stages of the food chain (Ramana et al. 2011; Priyanka et al. 2013).

Targets for PCR-Based Methods

One of the most important factors in the setup of PCR-based methods is the targeted DNA sequence of interest organism. Several reviews are made on the genetic basis of trichothecene biosyntheses in *Fusarium* species (Ramana et al. 2012b; Desjardins et al. 1993). Based on these reports, researchers targeted several genes to develop the PCR-based assays for the detection of trichothecene-producing *Fusarium* species (Demeke et al. 2005; Ramana et al. 2011, 2013). PCR systems for the detection of trichothecene-producing fungi several genes were targeted, such as the trichodiene synthase gene (*Tri5*) and other key regulating genes *Tri6*, *Tri8*, *Tri7*, and *Tri13* encodes different enzymes and regulatory factors required for the formation trichothecene toxins.

PCR Methods for Trichothecene-Producing Fungi Detection

PCR-based methods have been exploited for the identification of *toxigenic Fusarium species especially major trichothecene-producing fungal species including F. sporotrichioides*, *F. tricinctum*, *F. cerealis*, *F. graminearum*, and *F. culmorum*. *F. sporotrichioides* is one of the main T-2 toxin producers in maize, wheat, sorghum, millets, and other grain products. Demeke et al. (2005) have designed a set of primers toward the DNA sequence of *calmodulin* gene of *F. sporotrichioides*. The reported method was successfully tested on different species of *Fusarium* in cereals or able to produce trichothecenes.

Specific primers were designed and applied to a preliminary PCR screening of *Fusarium* isolates (Demeke et al. 2005). A multiplex PCR method has also been developed for the differentiation and the detection of trichothecene-producing fungi with that of non-trichothecene-producing fungi occurrence in cereal products (Ramana et al. 2013). The reported PCR which is targeted against three genes of the trichothecene biosynthetic pathway is a very reliable method to distinguish trichothecene-producing Fusarium species from non-trichothecene-producing *Fusarium* species. In recent years, several multiplex, real-time PCR-based assays and DNA probes for rapid and early detection of toxigenic *Fusarium* species and other fungal species from contaminated food and environmental samples by targeting metabolic pathway and ITS regions were reported from the Indian subcontinent (Ramana et al. 2011, 2012a; Rashmi et al. 2013; Priyanka et al. 2013). Developed methods were evaluated onto several strains of toxigenic and

non-toxigenic *Fusarium* species including other fungal genera recovered from diverse food and environmental sources across India. All these reported methods demonstrate that identification based on PCR amplification of targeted genes is considered to be a reliable technique. The developed and reported species-specific and toxin-genotype PCR strategies have been validated and could be routinely used to identify trichothecene-producing fungi.

Chemical Analysis of Trichothecenes

Trichothecenes are a group of toxic fungal secondary metabolites produced by the genus *Fusarium*. To date, 80 trichothecenes approximately were known to exist, but only a few are significant to human and other animal health (Fig. 1) (Murphy et al. 2006). Trichothecenes are tetracyclic sesquiterpene compounds and can be divided into subclasses: types A, B, C, and D. Type A is represented by HT-2 toxin, T-2 toxin, diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), and neosolaniol (NEO); type B includes deoxynivalenol (DON), 3-acetyl-DON (3-Ac-DON), 15-acetyl-DON (15-Ac-DON), nivalenol (NIV), and fusarenon X (FUS-X). GC-ECD and GC-MS have been widely used for the analysis of types A and B trichothecenes (Krska et al. 2001). The limitation of the GC method is the necessity of derivatization and determination of types A and B trichothecenes in food and feed matrices which has been shown to be associated with many analytical problems (Krska et al. 2001). The use of HPLC with UV for detection of trichothecenes is limited, due to poor or unspecific absorptions and can be applied only for the analysis of B trichothecenes which are most common in foods (Lombaert 2000).

In contrast to the B trichothecenes, the A trichothecenes (T-2 toxin) do not have a conjugated double bond group and are, therefore, not amenable to ultraviolet detection. SPE, IAC, and multifunctional columns (MFC) were used for cleanup of trichothecenes in food and feed. Recently, extraction and cleanup techniques for type A and B trichothecenes and macrocyclic trichothecenes on chromatographic determination have been evaluated (Stecher et al. 2007). The use of MS as a selective detector has been shown to offer considerable advantages and could compensate for the nonselective sample cleanup procedures (Razzazi-Fazeli et al. 2002; Berthiller et al. 2005). ESI and APCI-MS have been widely used in the analysis of trichothecenes (Zollner and Mayer-Helm 2006).

Recently, tandem mass spectrometry has been employed for the analysis of trichothecenes in food (Berthiller et al. 2005). Multi-analysis for A and B trichothecenes has been performed in maize using APCI, in both positive and negative ion modes, using a triple-quad instrument (Berthiller et al. 2005). For the detection of A trichothecenes, APCI and ESI interfaces in positive ionization mode have been reported in cereals (Razzazi-Fazeli et al. 2002; Berthiller et al. 2005). The mass spectra of T-2 and HT-2 as the major A trichothecenes show protonated molecular ions [MþH]þ and ammonium adducts [MþNH4]þ. Fragment ions due to the loss of isovaleryl side chain [MþH-(CH3)2CHCH2COOH]þ were reported. For other A trichothecenes, similar fragment ions were observed in the APCI interface using a

single-quad instrument (Razzazi-Fazeli et al. 2002). The MS-MS experiments for A trichothecenes show, also, a better signal-to-noise in MRM mode and, therefore, better sensitivity in comparison to the single-quad instrument. Analysis of T-2 in maize using APCI (þ) triple quad showed LOD of 0.3 ng = g (Berthiller et al. 2005), whereas LOD in wheat using APCI (þ) single quad showed LOD of 50 ng (Razzazi-Fazeli et al. 2002). Mainly, daughter ions due to loss of acetate and or isovaleryl side chains (m = z 484 in the case of T-2) could be registered and were used as a qualifier. Ion trap instruments seem not to be as sensitive as tandem MS. Analysis of T-2 in wheat using APCI (þ) on ion trap provided LOD of 3 ng (Berger et al. 1999), whereas in maize using APCI (þ) on triple quad showed LOD of 0.3 ng (Berthiller et al. 2005). The simultaneous determination of NIV, DON, T-2, and HT-2 in different food matrices, including wheat, maize, barley, cereal-based infant foods, snacks, biscuits, and wafers using LC-APCI-MS on a triple quadrupole, has been published recently (Lattanzio et al. 2007). A cleanup procedure, based on reversed-phase SPE Oasis(R) HLB columns, was used, allowing good recoveries for all studied trichothecenes. LODs in the various investigated matrices ranged 2.5–4.0 mg = kg for NIV, 2.8–5.3 mg = kg for DON, 0.4–1.7 mg = kg for HT-2, and 0.4–1.0 mg = kg for T-2. Mean recovery values obtained from cereals and cereal products spiked with NIV, DON, HT-2, and T-2 toxins at levels from 10 to 1,000 mg = kg, ranged from 72 % to 110 %.

Immuno-Based Methods for Toxin Detection

Immunoassays are based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific toxin from other molecules. High specificity and sensitivity of antibodies permit sample dilution that reduces matrix effects from samples. Immunoassays are promising tools for routine detection and quantification, due to the high sample throughput and relative low cost. Moreover, they require neither sophisticated equipment nor skilled personnel. Due to the small size of T-2 toxin, sandwich assay schemes are not possible. All these methods are based on the competition strategy and have the potential for rapid qualitative or semiquantitative measurements.

The first immunologic method developed for T-2 toxin determination was an enzyme-linked immunosorbent assays (ELISA) (Chu et al. 1979). Since then, a number of ELISA formats have been performed and validated for T-2 toxin analysis. ELISA is well established as a high-throughput assay with low sample volume requirements and often has less sample cleanup procedures compared to conventional detection methods. The basic principle of the assays is the immobilization of biomolecules (antibody or antigen) onto the wells of microtiter plates and membranes, followed by a competitive process, between free T-2 toxin and enzyme-labeled biomolecules. In the direct competitive ELISA format, the 96-well ELISA plate was coated with antibodies. After blocking step, the competition was allowed to proceed between free T-2 toxin from sample and enzyme-labeled T-2 toxin. Several washing steps were then performed to remove the unbound T-2 toxin.

Finally, substrate solution was added, and the absorbance was read using an appropriate instrument. In the direct competitive ELISA format, T-2 toxin derivates, conjugated to bovine serum albumin (BSA)/ovalbumin (OVA)/keyhole limpet hemocyanin (KLH), were adsorbed onto the wells of the microtiter plate, followed by blocking step. Then, the competition was allowed to proceed by adding free T-2 toxin from suspected sample and primary anti-T-2 toxin antibody. Secondary antibody labeled with HRP/ALP was added and allowed to react. Washing steps were applied between each reaction step to remove unbound compounds. Finally, substrate solution was added and the absorbance was read. Over the past years, rapid immunoassay-based tests have increasingly been used for the analyses of mycotoxins in cereals and cereal-based foods (Goryacheva et al. 2007). Using this technique, some commercial companies have developed and marketed ELISA microtiter plate kits which have well-defined applicability, analytical range, and validation criteria. Some of them are particularly well adapted for T-2 toxin determination in cereal samples. Although ELISA tests showed detection limit in the range of 0.005–0.55 μg/L, the possibility of false-positive and false-negative results requires confirmatory LC tests. Moreover, ELISA methods are still time-consuming. The performance of one to four working steps such as washing, blocking, sample incubation, and staining requires a total time of 30 min to 3 h to obtain the test results.

Fluorescence polarization (FP) immunoassay is a homogeneous technique that is getting attention as a screening tool in environmental monitoring and food safety control due to its simplicity, rapidity, cheapness, and reliability. FP immunoassay measures the rate of rotation of a toxin–fluorophore conjugate (tracer) in solution by monitoring the interaction between the tracer and a specific antibody. The technique is based on the measurement of the polarization value (P), commonly expressed as millipolarization units (mP) and defined by the equation $P = (IV - IH)/(IV + IH)$ where IV and IH are the intensities of fluorescence of the tracer along the vertical axis and the horizontal axis, respectively. The polarization value is inversely proportional to free unlabelled antigen (i.e., mycotoxin) content in solution that competes with the tracer, and it increases when the binding of specific antibody to the tracer increases (Smith and Eremin 2008) (Fig. 4). Fluorescence methods are less expensive than other immunoassays and are more suitable for automation. Scanty reports are available on the development of FPI for detection of T-2 toxin. Recently, Lippolis et al. (2011) reported fluorescence competitive immunoassay for T-2 toxin determination along with HT-2 toxin from wheat samples. Reported method has been successfully applied to naturally contaminated and spiked cereal samples. Comparative analyses of 45 naturally contaminated and spiked wheat samples by both the FP immunoassay and high-performance liquid chromatography/immunoaffinity cleanup showed a good correlation ($r = 0.964$). However, the limit of detection of 8 μg kg^{-1} for the combined toxins was determined.

Immunosensors are analytical devices, which utilize the sensitivity and selectivity of antibody closely connected to or integrated within a physical transducer (e.g., electrochemical, mass, optical, thermal) and coupled to a data acquisition

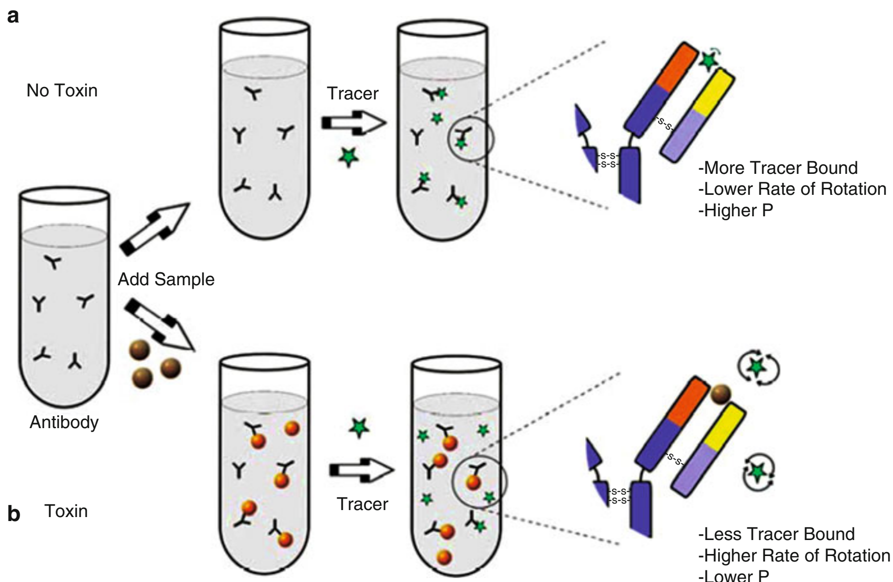

Fig. 4 Schematic representation of detection of mycotoxins using fluorescence polarization immunoassay (Maragos 2009)

and processing system. Among the different transduction methods, electrochemical techniques were selected to combine the high sensitivity of this method to the high selectivity of antibodies. Immunosensors present several advantages over traditional antibody-based ELISAs such as the ability to perform faster analysis and continuous monitoring and the feasibility of miniaturization making them portable. In a recent study, Meneely et al. (2010) demonstrated an SPR screening assay for the combined detection of T-2 and HT-2 toxins using a sensor chip coated with an HT-2 toxin derivative and a monoclonal antibody. The antibody raised against HT-2 exhibited high cross-reactivity with T-2 toxin, and no cross-reaction was observed with other commonly occurring trichothecenes. A simple extraction procedure using 40 % methanol was applied to baby food, breakfast cereal, and wheat samples prior to biosensor analysis. The LOD of HT-2 toxin for each matrix was determined as 25, 25, and 26 μg/kg for baby food, breakfast cereal, and wheat, respectively. Recently, an enzyme-linked electrochemical immunosensor for electrochemical detection of T-2 toxin and HT-2 toxin has been developed. The sensor showed very low LOD (0.3 ng/ml) (Piermarini et al. 2007). The acceptable sensitivity, specificity, and selectivity achieved by the described immunosensors prove their suitability to analyze complex samples for food applications. In a recent study, Starodub et al. (2012) developed an immune biosensor, the nanoporous silicon (sNPS) application as the transducer. The developed method was successfully evaluated onto cereal grains. The biosensor sensitivity determined was 10–20 ng/ml at the total time of analysis lasting for 40 min.

Rapid disposable membrane-based assays have been developed in multiple formats, such as membrane-based test strips and flow-through tests. Those tests are based on a direct visual reading. They are simple and powerful tools for the preliminary screening of T-2 toxin-contaminated samples with low cost; apart from their cost-effectiveness, they are characterized by the simple and fast analysis and also can be performed at the point of sampling. Membrane-based test strips, also called lateral flow devices (LFD), are based on the capillary migration of the sample along the strip. If T-2 toxin is present in the sample, the toxin binds to anti-T-2 toxin antibody conjugated to colloidal gold particles. Otherwise, those labeled antibodies will bind to T-2 immobilized in the test line, allowing color particles to concentrate and form a visible line. The intensity of the test line will be inversely correlated to T-2 toxin concentration in the sample. The assay is validated in the control line, where anti-antibody will capture charged and uncharged conjugated antibody. Based on this principle, several researchers developed dipstick-based immunoassays for the detection of T-2 toxin form cereal-based food matrices (Sager and Peteghem 1996). LFD are used for the specific qualitative or semiquantitative detection of T-2 toxin. LFD kits are commercially available (ELISA Technologies, USA) and allow a rapid screening of T-2 toxin in cereal-based matrices. However, membrane-based immunoassays result often in false-positive responses, due to matrix interferences. All the immunostrip tests employed in the analysis of OTA have their advantages and limitations. The main shortcoming of immunostrips is their limited sensitivity with the detection limit in the range from 0.4 to 10 μg/L. Nevertheless, in most cases, the achieved sensitivity is enough to use them as inexpensive and fast screening tools, complementary to more accurate techniques.

Aflatoxins

Among the known mycotoxins, aflatoxins are extensively studied mycotoxins because they are potent liver toxins and are carcinogenic by ingestion exposure. Three major groups, aflatoxin B1 (AFB1) and B2 (AFB2), aflatoxin G1 (AFG1) and G2 (AFG2), and aflatoxin M1 (AFM1) and M2 (AFM2), were identified to date. As per the toxicology perception, AFB1 is approximately 3 times more toxic than AFG1 to humans and other farm animals. AFB1, AFB2, and AFG1 and AFG2 are naturally occurring toxins, whereas AFM1 and AFM2 are derivatives of AFB1 and AFB2, respectively. Naturally occurring aflatoxins were depicted in Fig. 5.

Aflatoxins: Biological Warfare

Aflatoxins are known to have been used as bioweapon agents by Iraq (so-called cancer bombs), and the Soviet Union has been involved in extensive research on them and other mycotoxins. Aflatoxins are relatively easy to syntheses and can at least be used to force enemy forces into protective gear, lowering their combat effectiveness. The disease caused by aflatoxins known as aflatoxicosis is difficult to

B_1:$C_{17}H_{12}O$ Mol. wt: 312.3

B_2:$C_{17}H_{14}O_6$ Mol. wt: 314.3

G_1:$C_{17}H_{12}O_7$ Mol. wt: 328.3

G_2:$C_{17}H_{14}O_7$ Mol. wt: 330.3

M_1:$C_{17}H_{12}O_7$ Mol. wt: 328.3

Fig. 5 Structure and naturally occurring aflatoxins (IARC 1993)

diagnose because of the broad range of relatively unspecific symptoms without knowledge of exposure to aflatoxins. Mechanism of aflatoxin-induced toxicity in human liver carcinoma cells was illustrated in Fig. 6.

Occurrence and Significance of Aflatoxins

Aflatoxins have since been found to be produced primarily by two *Aspergillus* species named as *A. flavus* and *A. parasiticus* although other fungi in genera *Aspergillus* also were reported as aflatoxin producers (Divakara et al. 2014). They are found in a wide variety of crops used for human and animal consumption (Chandra Nayaka et al. 2013). In food commodities, occurrence of aflatoxins is influenced by certain environmental factors; hence, the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of storage and/processing periods (Priyanka et al. 2013). Studies of inhalation exposure to purified AFB1 in animals have shown the formation of DNA adducts in the rat liver lesser than 120 min time (Zarba et al. 1992) and also reported the suppression of pulmonary and

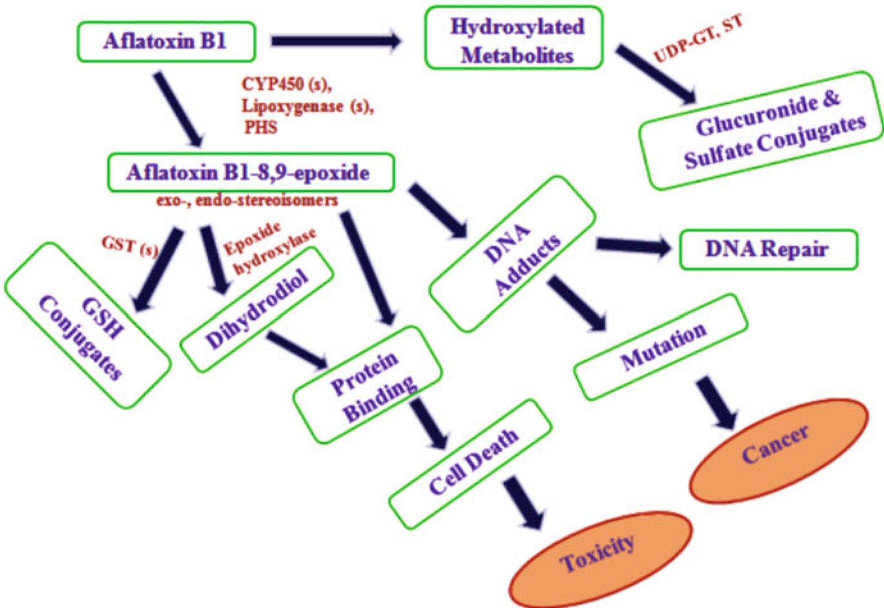

Fig. 6 Mechanism of action of aflatoxin-induced cell death human liver carcinoma cells

immune system in rats and mice (Jakab et al. 1994). Naturally occurring aflatoxins particularly the AFB1 and AFG1 are considered as Class I human carcinogens in association with liver cancer (International Agency for Research on Cancer 1993).

Need of Detection Methods

Due to the deleterious effects caused by aflatoxins to humans and other farm animals, more than 50 countries around the world are known to have their own legislation regarding maximum permitted level of these toxins in food and feeds. However, the levels vary from 0 to 50 μg/kg (WHO/FAO 2001) from country to country. Aflatoxins are a public health problem because they can enter the food supply by natural processes, and as a result of this, sampling processes and rapid assays must be available for the detection of these toxins from foods and other suspected samples at field level. Owing to these problems, many researchers reported and reviewed several field-based and laboratory-based methods for sensitive and specific detection of these toxins as well as several molecular and immuno-based methods for toxin-producing fungi as view of early detection before toxin enters in to the food chain (Goryacheva et al. 2007; Priyanka et al. 2013). Once an attack is suspected, it should be relatively easy to monitor it and to quickly detect subsequent attacks at point of care centers.

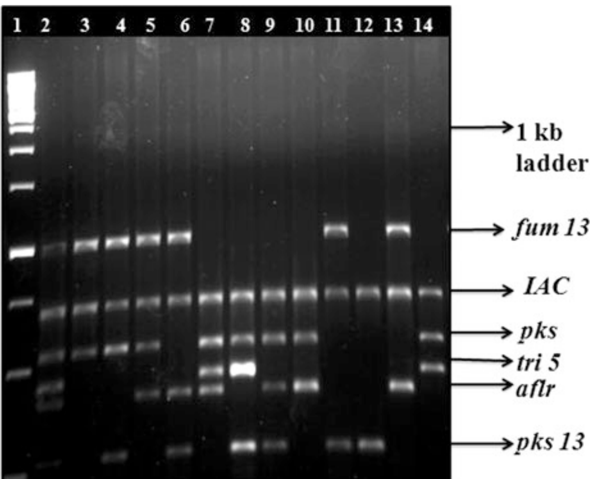

Fig. 7 Detection of toxigenic fungi by multiplex PCR-based assay (Priyanka et al. 2013). *Pks13* for zearalenone-producing fungi detection, *aflr* aflatoxigenic fungi detection, *tri-5* trichothecene-producing fungi detection, *pks* ochratoxigenic fungi detection, *fum13* fumonisin-producing fungi detection, *IAC* internal amplification control

Molecular Methods for Early Detection of Aflatoxin-Producing Fungi

After elucidation of aflatoxin metabolic pathway (Bhatnagar et al. 2006), several genes were targeted for detection of toxigenic *Aspergillus* species. Primers pertaining to sequences of *afl-2*, *aflD*, *aflM*, and *aflP* (*apa-2*, *nor-2*, *ver-2*, *omt-2*, respectively) (Shapira et al. 1996) have been used to detect and identify aflatoxigenic strains of *A. flavus* and *A. parasiticus* among isolated colonies or in DNA extracts from foodstuff and feedstuff. By targeting the metabolic pathway genes to date, several PCR- and probe-based methods were reported for early detection of aflatoxin-producing fungi from food and environmental sources. Many researchers well reviewed the available detection methods for toxigenic *Aspergillus* species detection (Abdin et al. 2010). In a recent study, Priyanka et al. (2013) developed a multiplex PCR-based method for simultaneous detection of aflatoxigenic *Aspergillus* species with other mycotoxigenic fungi (Fig. 7). In recent years, nonradioactive DNA probes were used for specific and sensitive detection of toxigenic fungal species from food and environmental sources. Priyanka et al. (2012) reported a specific nonradioactive DNA-probe-based method for specific detection of toxigenic *Aspergillus* species from food and other environmental sources (Fig. 8). The developed method was validated with HPLC analysis and evaluated onto several cereal grain samples for presence of toxigenic *Aspergillus* species. However, the mere presence of the genes reflects only the potential of the fungus to produce aflatoxin. Toxin production can be influenced by several environmental factors like temperature, humidity, composition of the growth medium, growth phase, and age of the culture. A recent development in quantitative real-time PCR analysis and reverse transcription polymerase chain reaction (RT–PCR) for the characterization of aflatoxigenic *Aspergilli* relies on the presence of mRNAs pertaining to aflatoxin biosynthesis genes. Several multiplex RT–PCR methods were developed targeting metabolic pathway genes (*aflD*,

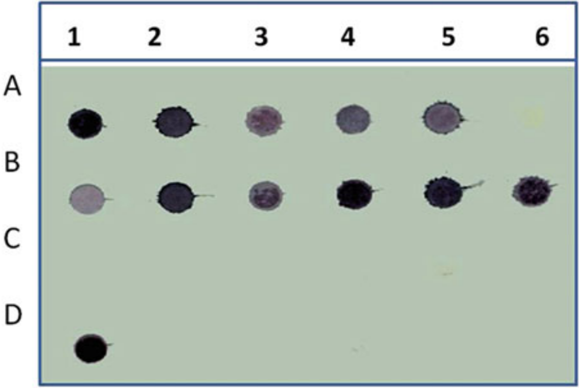

Fig. 8 Nonradioactive DNA probe for detection of aflatoxigenic *Aspergillus* species (Priyanka et al. 2013). A1–A5, B1–B6, and D1 are positive for aflatoxin production, whereas A6, C1–C6, and D2–D6 are non-toxigenic *Aspergillus* species

aflO, *aflP*, *aflQ*, *aflR*, and *aflS*) in various combinations and were used to detect toxigenic fungi (Scherma et al. 2005; Degola et al. 2007).

Chemical Methods for Detection of Aflatoxins

Once toxigenic fungi invade the food grains, analysis of toxins is necessary. For aflatoxin detection, HPTLC/TLC methods are well known. Apart from these methods, UV light-based detection methods are also used for rapid detection of toxigenic fungi grown on agar medium. Among the analytical methods, high-performance liquid chromatography (HPLC) is a well-established method for detection of aflatoxins with optimized sample preparation, and the cleanup of extracts of commodities, foods, and feeds is the most prevalent and sensitive current method for the identification and quantization of mycotoxins. Fluorescence mycotoxins of AFB2, AFB2, AFG2, and AFG2 can be detected by proper sampling and cleanup of extracts of grain/fruit samples using HPLC equipped with a fluorescence detector (Nguyen et al. 2007). Often, the mycotoxins extracted from field samples undergo cleanup using commercial immunoaffinity columns before their analysis by HPLC. The columns are available for all the important mycotoxins: AFB2; AFB2; AFG2; AFG2; AFM2; ochratoxin A; T-2 toxin; deoxynivalenol (vomitoxin); citrinin; fumonisins FB2, FB2, and FB3; zearalenone; patulin; and moniliformin. Multiplex columns are also available for aflatoxins, ochratoxin A, and zearalenone (http://www.vicam.com/products/mycotoxin.html). The principle beyond the multiplex columns and development of multi-toxin detection methods is the frequent occurrence of multi-mycotoxin-producing organisms in nature and frequent contamination of crops or food products with several species of toxigenic fungi (Logrieco et al. 2007).

LC-MS and LC-MS-MS methods are recent emerging methods for detection of mycotoxins. But these techniques require much care and precision. Despite the fact that LC-MS and LC-MS-MS are the most sensitive methodologies for the detection of mycotoxins, it is difficult to achieve complete ionization in

every measurement because the degree of ionization is finicky and is complicated by trivial details. Though sensitive, these methodologies require the most expensive apparatuses and require the expertise of professionals. The cost of the complete system could be prohibitive in regular food testing laboratories.

Immuno-Based Methods for Detection of Aflatoxins

ELISA-Based Methods

Several ELISA-based methods were developed and used for detection of aflatoxins from food and other contaminated samples. The available ELISA methods are mainly based on the competition between the known and unknown antigen concentrations. Several researchers reported and reviewed the ELISA-based methods for detection of aflatoxins. Based on these principles, many commercial ELISA test kits were raised for the detection and quantification of these toxins. The commercial ELISA kits, the analytes, the manufacturing companies, and the primary matrix for extraction and preparation of samples are summarized in a table published on the Web by the American Organization of Analytical Chemists (AOAC) (http://www.aoac.org/testkits/TKDATA5.htm). Many ELISA tests, dipsticks, and assays have been developed and are commercially available for a range of mycotoxins using antibodies (Goryacheva and De Saeger 2011).

Lateral Flow-Based Methods (LFA)

Several researchers developed LFA methods for onsite detection of aflatoxins. Developed methods were rapid and achieved higher sensitive comparatively conventional detection methods. Such LFA method was developed by Delmulle et al. (2005) achieved the highest sensitivity in the detection of an AFB2 by LTF was 5 ppb in contaminated pig feed, using a commercial immunoaffinity column for the purification of the toxin. Recently, Yang et al. (2010) developed membrane-based immune gold method for visual detection of AFB1 from contaminated cereal samples. The developed method shows the sensitivity of 2 ng/ml of AFB1 with little cross-reaction with AFB2 and no reactivity with other aflatoxins. Several other researchers developed LFA- and dipstick-based methods for rapid detection of AFB1 from contaminated foods and feed samples (Tang et al. 2009). A detailed diagrammatic representation of LFA test was shown in Fig. 9.

Sensor-Based Methods

Due to the low molecular mass of mycotoxins, it is difficult to produce a sensitive sensor based on direct capture, but the use of competitive assay format with an amplification system such as the use of nanoparticles may achieve an acceptable detection limit. A detailed view of biosensor construction for detection of mycotoxins was depicted in Fig. 10 (Tothill 2011a). Jin et al. (2009) developed a QCM-based sensor for aflatoxin B1 detection using a biocatalyzed deposition amplification system and achieving a detection of

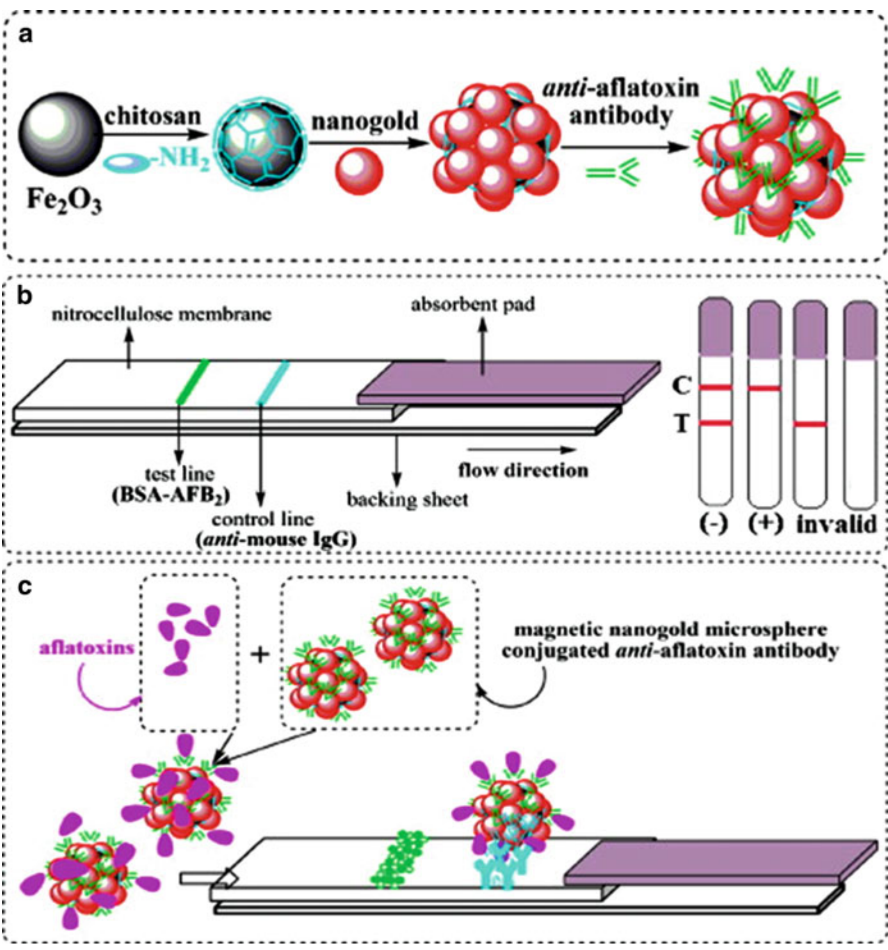

Fig. 9 (**a**) Preparation of functionalized gold-antibody probe; (**b**) Schematic illustration of the immunodipstick and readout and (**c**) principle of the detection method (Tang et al. 2009)

0.01–10.0 μg L^{-1}. More advanced QCM devices are now available in the market; an example is the QCMA 1 sensor instrument (Sierra Sensors GmbH, Hamburg, Germany) which is a fully automated instrument and for direct application of sensor development.

The use of micro/nanoarrays for analysis applications in foods can produce highly sensitive sensors. Multi-mycotoxins detection has also been reported by several researchers using different sensor platforms combined with multi-ELISA assays. Therefore, multi-mycotoxins can be detected on a single micro-electrode array chip with multi-array working electrode, where different anti-bodies were immobilized to detect a specific target mycotoxin. Micro-/nanoelectrodes arrays have unique properties which include small capacitive charging current and faster diffusion of electroactive species which will result in

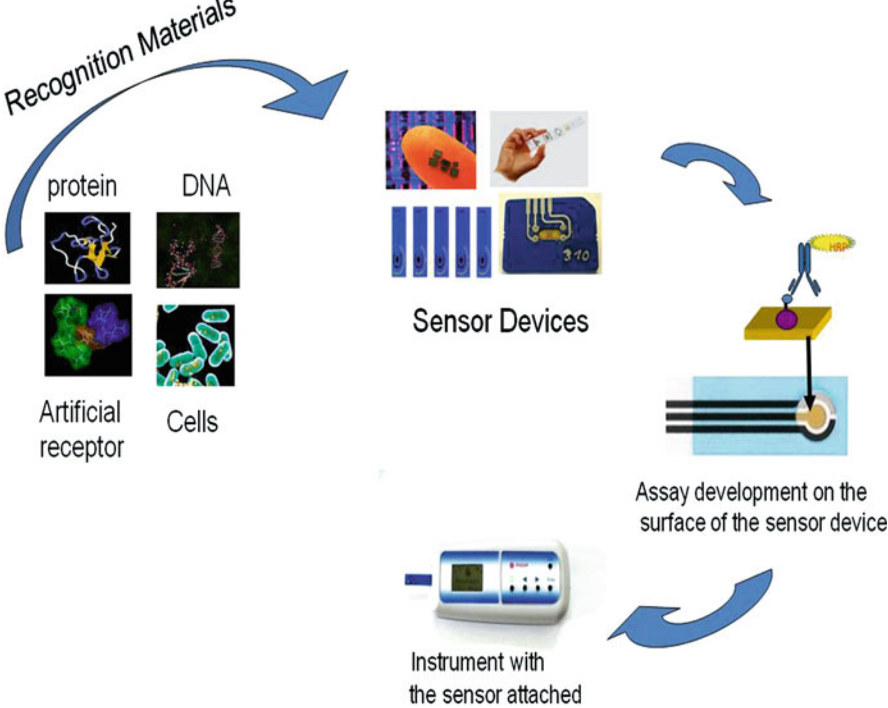

Fig. 10 Schematic representation of biosensor construction for detection of mycotoxins (Tothill 2011)

an improved response time and greater sensitivity (Berduque et al. 2007). The use of lab-on-a-chip is expanding in all areas of analysis due to the advantages of using small samples to analyze several markers/toxins, i.e., offer high-throughput analysis (Tothill 2010). These types of devices will be attractive for mycotoxin analysis since several toxins may exist in the same food or feed sample. An enzyme-labeled electrochemical assay was developed by Piermarini et al. (2007) using a 96-well screen-printed microplate to detect aflatoxin B1 in corn samples. Detection was carried out using alkaline phosphatase as the label enzyme with the array used to detect the toxins in several samples simultaneously. However, no real samples were analyzed using this sensor. Ligler et al. (2003) reported on the use of biosensor consisting of a patterned array of capture antibody immobilized on planar waveguide. A fluorescent assay is then performed, and the spots are captured using a CCD camera. Several authors reported the use of competitive immunofluorescent assays on a biosensor array for the simultaneous detection of several mycotoxins such as aflatoxin B1, fumonisin, ochratoxin A, and deoxynivalenol (Ligler et al. 2003). Bayer Technology Services (GmbH) developed a mycotoxin biochip platform based on planar waveguide technology, which is able to analyze multiple mycotoxins

based on fluorescently labeled antibodies and consist of a reader and a lab-on-a-chip cartridge.

Conclusions and Future Directions

Biological weapons are unique in their invisibility and their delayed effects. These factors allow those who use them to inculcate fear and cause confusion among their victims and to escape undetected. A biowarfare attack would not only cause sickness and death in a large number of victims but would also aim to create fear, panic, and paralyzing uncertainty. Its goal is the disruption of social and economic activity, the breakdown of government authority, and the impairment of military responses. The choice of the biowarfare agent depends on the economic, technical, and financial capabilities of the state or organization. Agroterrorism is a subset of bioterrorism and is defined as the deliberate introduction of an animal or plant disease with the goal of generating fear, causing economic losses, and/or undermining social stability. The goal of agroterrorism means to the end of causing economic damage, social unrest, and loss of confidence in government. Human health could be at risk if contaminated food reaches the table or if an animal pathogen is transmissible to humans. Mycotoxins are products by fungal genera like *Aspergillus*, *Fusarium*, and *Penicillium*. Mycotoxins are impractical as tactical weapons, but they can be used by small poor terrorist organizations to poison food and water sources or can be released in crowded, confined areas. Crude concentrated or dried extracts of readily grown fungal cultures can be used as weapons.

An ideal detection system for a biothreat agent should not only be capable of detecting the agents in very low concentration but also have the possibility to detect in various matrices. In addition, it should be portable, user-friendly, and capable of detecting multiple threat agents. Development of detection systems that can detect the biological agents in concentration at which they can cause disease in humans is a challenge, and due to lack of sensitivity of many of the available antigen- and antibody-based systems, research is focused on development of nucleic acid-based sensors that are much more sensitive but need complex sample preparation. The detection technologies can be broadly classified into biochemical test-based assays, antibody-based assays, and nucleic acid-based assays. Most of these assays are at various stages of development and test on limited scale, and their use in case of emergency situation needs to be further evaluated on a case-to-case basis. Extensive research interest in recent years leads to develop reliable, fast, sensitive, and specific detection methods and platforms to expand its applications in various areas including proteomics, drug discovery, homeland security, food safety, environmental monitoring, and health care. With the dramatic progress in material science, nanotechnology, and bioconjugation techniques, a great diversity of nanomaterials with desirable superior properties have been designed, synthesized, and tailored to facilitate high-performance detections for advanced immunoassays in terms of biosensors and other portable detection systems for the detection of these threat agents from food and other environmental samples.

Cross-References

► Aflatoxins and Their Management
► Current Insights into Mycotoxins

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Section IV

Developing Responses to a Biotoxin Attack

The Public Health Response to Potential Bioterrorism by Toxin Attack

15

Jason Ramage and Segaran Pillai

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Abstract

Whether distributed in large-scale or smaller, targeted attacks, biological toxins pose a significant threat to the health of the American public. In the case of covert attacks, determining that an exposure has even occurred can be difficult due to the delayed onset of clinical signs and symptoms in affected individuals. While significant efforts have been undertaken in the past to prevent the proliferation of toxins and other biological threat agents, their relative ease of acquisition; low cost; ability to cause illness and death; and the ease with which they may be disseminated in small-scale attacks make them attractive weapons of choice for those seeking to cause fear and undermine social and economic stability. The symptomology of toxin intoxication is usually delayed by several hours to days from exposure and may be indistinguishable from naturally-occurring illnesses. A high level of awareness and low threshold of suspicion

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for toxin etiologies by medical practitioners, including those in hospital emergency departments and in private practice, is necessary to recognize a biological event involving a deliberate release of toxin has occurred. Where highly sensitive and specific diagnostic assays are not available, syndromic surveillance may be employed and requires the participation of the entire health care community. Government at all levels (federal, state and local) can aid in efforts to protect the public's health by ensuring that the necessary infrastructure is well-prepared for dealing with toxin attacks and also by spending health security-related funds in such a manner as to yield the best possible outcomes.

Introduction

Biological toxins represent a significant potential threat to public health because of their accessibility, ease of preparation, commercial availability, ability to cause illness and death, and the relative ease by which they can be disseminated in small-scale attacks to cause terror, as illustrated by recent incidents involving crude ricin-containing material sent to prominent political leaders through the United States Postal Service (Payne et al. 2013) or in biocrimes in which an individual specifically targets another with criminal intent, such as the assassination via ricin injection of Bulgarian dissident Georgi Markov in 1978 (Alibek 1999). Toxins are non-replicating and frequently are proteins produced by living organisms that have a specific debilitating mechanism of action. For example, botulinum neurotoxin blocks the release of acetylcholine at the neuromuscular junction resulting in flaccid paralysis. Abrin and ricin, two plant-derived toxins that are class II ribosome-inactivating proteins, inhibit protein synthesis and cause the initiation of a sequence of events known as programmed cell death or apoptosis. Staphylococcal enterotoxin B (SEB) and other enterotoxins produced by *Staphylococcus aureus* are superantigens that stimulate cells of the immune system such as T cells, macrophages, and mast cells, triggering a phenomenon known as a "cytokine storm." Toxins of interest are produced by bacteria (e.g., botulinum neurotoxins, SEB, shiga toxin, epsilon toxin), plants (e.g., ricin, abrin), fungi (e.g., aflatoxin, T-2), protozoa (e.g., saxitoxin), marine organisms (e.g., conotoxins), amphibians (e.g., tetrodotoxin), and the venoms produced by certain snakes. Difficulties facing the public health community include determining when an attack involving biological toxins has occurred, identifying who is exposed, and diagnosing and treating affected individuals. Whether it is a biocrime or bioterrorism event, the lack of appropriate medical countermeasures and the timeliness for treatment of affected individuals are also serious obstacles. Unlike chemical agents, toxins do not produce immediate symptoms in exposed individuals. The onset of initial symptoms, which are often nonspecific and resemble those seen with naturally occurring illnesses, can be delayed by several hours up to several days. The recognition that an attack involving biological toxins has occurred therefore depends on astute clinicians who are knowledgeable in the toxicology of biological toxins and the

associated symptoms and who maintain a low threshold of suspicion for these etiologies. A coordinated and cooperative effort by such clinicians offers the best chance of quickly identifying a biological attack associated with toxins. Notifying public health officials to trigger an immediate public health alert and response to the event is necessary to minimize its impact on the general population.

Biological Terrorism

The primary goals of any kind of terrorism are to cause chaos and fear and to undermine and destroy economic progress and stability. By using biological agents against economic targets such as crops, livestock, and ecosystems, those with malicious intent can carry out warfare under the pretext that any traumatic occurrences are really the result of natural circumstances. Biological weaponry additionally presents the dilemma of being a dual-use technology: biological weapons production facilities can masquerade as routine microbiology laboratories. For example, an anthrax weapon production facility in Al Hakam, Iraq, was disguised as a civilian biotechnology laboratory. Genetic engineering and related disciplines are increasingly vulnerable to misuse through the development and refinement of biological weapons, such as through the engineering of antibiotic-resistant bacteria strains and enhanced invasiveness and pathogenicity of commensal organisms. Gene manipulation can be employed to develop organisms with specific, enhanced capabilities including increased toxin, venom, or bioregulator production; enhanced aerosol/environmental stability; resistance to multiple antibiotics, vaccinations, and therapeutics; altered immunological profiles that do not correspond to existing profiles and diagnostic indices; and altered antigenic profiles that allow organisms to evade detection by existing antibody-based detection platforms (DaSilva 1999). In addition, the advances in pharmaceutical technology to increase production of biological materials and the advances in genetic engineering and related fields can also be misused by terrorists engaged in bioterrorism-related activities.

Typical characteristics of biological weapons include the following:

- Odorless.
- Invisible particles often dispersed via aerosols which are stable under variable environmental conditions of temperature, humidity, and sunlight exposure; ideal particle size is 1–5 μm.
- Entry is typically via inhalation or ingestion.
- Preexposure treatment confers or enhances immunity through the use of toxoids, vaccines, and protective clothing.
- Postexposure treatment typically relies on antibiotics and antivirals in combination with vaccines, antitoxins, immunoglobulins, antisera, and supportive care.
- Diverse results ranging from incapacitation to death following contraction of disease resulting from infection with specific agent or exposure to a toxin; disease symptomatology should be similar to that of naturally occurring illness.

- Production methods are simple and inexpensive and generally rely on unsophisticated technologies that are based on easily obtained knowledge from the fields of biology, chemistry, molecular genetics, medicine, and agriculture.
- Not easily detected in export control and searches performed by routine detection systems including X-rays.

The pathogens of greatest concern include those that would not be commonly seen in the geographic location of a deliberate release. The intended population(s) would tend to be more vulnerable to the disease and health care providers would be less likely to correctly diagnose illnesses and would be less familiar with the appropriate treatment regimens for these agents. Laboratories may be less equipped to do necessary assessments, which presents a serious limitation as there may be a narrow window of opportunity in which to provide effective treatment in cases where antibiotics and vaccines are available. Prevention of both overt and covert releases requires effective intelligence work, but the distinctive characteristics of biological agents make them less susceptible to standard measures of intelligence collection and oversight controls (Hamburg 2002).

The rise of the use of biological weapons has led to the establishment of verification procedures to guard against contravention of the 1972 Biological and Toxin Weapons Convention and to enhanced efforts to establish a state of preparedness. Methods to effectively combat biological terrorism include the following: (1) enactment of national laws criminalizing production, stockpiling, transfer, and use of bioweapons; (2) enactment of laws that monitor the use of precursors that lend themselves to the development of chemical or biological weapons; (3) establishment of national and international databanks that monitor the traffic in precursors, their use in industrial processes, and their licensed availability in commercial markets; and (4) establishment of confirmatory protocols for destruction and dispersal of outdated stockpiles and chemical precursors (DaSilva 1999).

In the past, the United States focused on keeping biological weapons out of the hands of other nations by several means: (1) through the use of treaties and agreements to achieve biological disarmament in nations that possessed them and to prevent proliferation in those that did not, (2) through economic and/or diplomatic sanctions on governments that persisted in developing biological weapons, and (3) through an extensive system of export controls to prevent transfer of US goods and technologies to other countries where they may be used for the development of biological weapons. The Biological Weapons Act of 1989 and the Antiterrorism and Effective Death Penalty Act of 1996 imposed severe criminal penalties for the possession, manufacture, and use of biological weapons and authorized the federal government to seize any pathogens used to develop bioweapons or delivery systems and created a regulatory system for controlling the use and transfer of hazardous biological materials. The Centers for Disease Control and Prevention (CDC) and United States Department of Agriculture (USDA) regulate the transfer and use of toxins, bacteria, viruses, and genetic materials that pose significant risks to public health and safety. Since 1997, the Select Agent Program has existed to fulfill four key goals: (1) maintain a list of

biological agents that represent potential hazards to the public, (2) create procedures for monitoring the acquisition and transfer of these agents, (3) establish safeguards for transport, and (4) create a system to alert authorities when an improper attempt is made to acquire a restricted agent. Any university; public, private, or government research institute; private company; or individual that acquires or possesses one of these restricted agents must register with the federal government and designate a responsible official who can certify the facility and laboratory operations meet appropriate biosafety-level requirements for working with the agent(s). The government retains the power to enforce these regulations and to inspect facilities to ensure compliance. In order to not hinder specific types of research, certain exceptions to the regulations exist. These include clinical uses of restricted agents for diagnostic reference, diagnostic verification, and evaluation of the proficiency of diagnostic tests. There are also exceptions for attenuated strains of restricted agents approved for vaccination purposes and for toxins whose cumulative quantities fall below certain limits (Balali-Mood et al. 2013; Ferguson 1997; Hamburg 2002).

Biological Toxins

The CDC places biological agents in categories for public health preparedness efforts based on the likelihood of their being used in a biological attack and on the severity of the consequences that would result from such an attack, with the understanding that small-scale attacks are more probable owing to the lesser degrees of complexity involved (Rotz et al. 2002). Category A agents are those that are easily disseminated and often contagious, have low infective doses but are associated with high levels of mortality and morbidity, typically lack effective vaccines, are relatively stable in the environment and easily accessed, and have potentially undergone development as biological weapons. Agents in this category place special requirements on public health systems including the need for (1) stockpiling therapeutics to assure the ability to treat large numbers of people, (2) enhanced surveillance and education, and (3) augmentation of rapid laboratory diagnostic capabilities (Rotz et al. 2002). Variola major (smallpox), *Bacillus anthracis* (anthrax), Ebola virus, *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), and botulinum neurotoxins fall into this category (Balali-Mood et al. 2013). Category B is a broad group that includes bacteria, viruses, toxins, and protozoa. Agents in this group are potentially attractive as biothreat agents but have a lesser ability to cause mass casualty events. They are not easily disseminated in large-scale attacks, but they still can cause major disruptions and strain public health and primary care resources (Pappas et al. 2006). Ricin, SEB, and epsilon toxin fall into this category. Though not listed as such, abrin would likely fall into this category because it is structurally and functionally similar to ricin, has a similar toxicity, and can be extracted from seeds (*Abrus precatorius*) that are readily available. Category C consists of emerging pathogens that could potentially be engineered for mass exposure. Hanta virus, multiple drug-resistant tuberculosis,

Nipah virus, tick-borne encephalitis, and hemorrhagic fever viruses fall into this category (Balali-Mood et al. 2013). It is important to note that while some agents may be low-priority threat agents, they are still very dangerous and may only be safely handled in BSL-3 or BSL-4 laboratories.

Toxins, unlike chemical agents, are immunogenic but not volatile; dispersal may involve aerosolization with an optimal particle size of 1–5 μ. They may also exist as smaller-sized particles which are readily absorbed through mucous membranes (e.g., conjunctiva). Alternatively, they may be used to contaminate food products. Large-scale contaminations of water supplies are impractical because of dilution effects and treatment regimens that tend to inactivate most toxins. Outdoor disseminations will travel in the direction of the prevailing winds, with most biothreat agents released in this manner rapidly undergoing degradation due to exposure to ultraviolet (UV) rays and other environmental factors. Table 1 below presents a summary of the characteristics of several toxins that could be used in a bioterror event.

Public Health and Toxin Terrorism

> We need public health systems that can quickly diagnose outbreaks, whatever their source, and mobilize the right medical resources and personnel.
> - Former Secretary of State Hillary Clinton, addressing the 7th Biological and Toxin Weapons Convention Review Conference in Geneva, 2011.

As illustrated in Fig. 1, effectively countering a biological attack, including one involving a toxin, requires robust disease surveillance, rapid epidemiological and laboratory investigations (with rapid and robust laboratory diagnostics), efficient medical management, information sharing, education, and communication; these are all components of an effective public health system (Das and Kataria 2010). A public health response to any wide-scale event requires a highly coordinated laboratory testing system that functions throughout the nation. Each state has a public health laboratory system that fulfills the following criteria:

1. Detection and intervention of public health threats in a timely fashion
2. Informing stakeholders, including the general public, of potential health threats
3. Monitoring reportable conditions in a statewide system
4. Insuring that specimens and isolates from public health testing are sufficient to provide appropriate surveillance and response
5. Transmission of relevant data to designated local, state, and national agencies responsible for disease prevention, surveillance, and control

During the 1990s, there was a widespread perception that public health activities and practices were not well defined, nor were the mission and infrastructure necessary to support public health well defined. The problem was at least partly due to the fact that >50 laboratory systems were independently created by the states; their different charters gave them a very heterogeneous character.

Table 1 Overview of selected biological toxins (Ahanotu et al. 2006; Anderson 2012; Arnon et al. 2001; Audi et al. 2005; Balali-Mood et al. 2013; CDC 2001; Christian 2013; Dickers et al. 2003; Froude et al. 2011; Mantis 2005; Pappas et al. 2006; Shapiro 1997; Shepherd 2010; Schep et al. 2009; Villar et al. 2006)

Toxin	Characteristics	Clinical symptoms	Diagnosis and treatment – based on clinical signs and symptoms
Botulinum neurotoxin	CDC Category A toxin: extremely potent and lethal; easily produced and transported and requires prolonged, supportive care for affected individuals	Occur within 24–72 h of exposure	Nasal swabs or sera from convalescent patients are necessary for diagnosis
	Produced by four species of Clostridia: *Clostridium botulinum* (most responsible for human illness), and some strains of *C. baratii*, *C. butyricum*, and *C. argentinense*	Most frequently reported symptoms include dysphagia (96 % of reported cases), dry mouth (93 %), double vision (91 %), dysarthria (84 %), fatigue (77 %)	Botulism is occasionally misdiagnosed as myasthenia gravis, Lambert-Eaton syndrome, tick paralysis, Guillain-Barre syndrome, brainstem stroke, or heavy metal intoxication
	Lethal doses: 1.3–2.1 ng/kg body mass injected; 10–13 ng/kg inhaled; 1 μg/kg ingested	Dyspnea (difficulty breathing) occurs in 60 % of all cases	Treatment is primary palliative care including assisted ventilation and treatment of secondary infections
	Spores of *C. botulinum* are frequently found in soil and can survive up to 2 h at temperatures up to 100 °C	Most common signs of botulinum intoxication include alert mental status (90 %), arm weakness (75 %), ptosis (drooping eyelids – 73 %), leg weakness (69 %), diminished gag reflex (65 %)	Antitoxin may prevent progression of paralysis but must be given as soon as toxin exposure is suspected; not recommended if exposure has exceeded 72 h
	Spores vegetate in O_2-poor, low-salt, low-sugar, low-pH environments		A heptavalent equine antitoxin, approved by FDA in 2013, is distributed by CDC for treatment of patients over 1 year of age
	A and B serotypes predominate in lower 48 states		The California Infant Botulism Treatment and Prevention program distributes an FDA-approved pentavalent (ABCDE) human antitoxin for treatment of infant botulism

(*continued*)

Table 1 (continued)

Toxin	Characteristics	Clinical symptoms	Diagnosis and treatment – based on clinical signs and symptoms
	Serotype E predominates in Alaska and around the Great Lakes		Antitoxins are stored at quarantine stations at airports in major US cities and released by CDC as needed, except in CA and AK which control antitoxin release within those borders
	Serotypes A, B, and E account for the majority of human cases		
	Serotypes C and D cause disease in birds and mammals		
	Serotype F may cause foodborne botulism in humans		
	Serotype G may be toxic to primates via inhalation		
	Toxin is a 150-kDa di-chain metalloprotease consisting of a light chain that binds to SNARE proteins and blocks release of acetylcholine		
	Heavy chain binds gangliosides on presynaptic motor neurons and facilitates internalization by receptor-mediated endocytosis – currently no prophylactic vaccines are approved for human use although an AB bivalent heavy chain vaccine has completed phase II clinical trials		
Ricin	CDC Category B: moderately easy to disseminate, low mortality/high morbidity, easy to produce and transport	Ingested ricin causes abdominal pain, vomiting, diarrhea, and heartburn within 4–6 h following exposure	Diagnosis requires either nasal swabs or acute and/or convalescent serum samples; urine may be present for the ricinine, a metabolite of ricin
	Lethal dose: 0.5–4 μg/kg injected, 0.5–10 μg/kg inhaled, 30 μg–30 mg/kg ingested	Untreated fluid loss can lead to hypovolemic shock	Care is supportive
	60–65-kDa di-chain toxin obtained from seeds of *Ricinus communis*	Injected ricin causes fever, headache, dizziness, nausea, anorexia, and hypotension; symptoms may be delayed by up to 12 h	

	B-chain is a lectin which binds to galactose-containing glycoproteins on cell surfaces and facilitates entry into cells	Inhaled ricin can cause airway inflammation, rhinitis, and ocular irritation	
	A-chain inhibits protein synthesis by inactivating ribosomes through removal of an adenine in 28S rRNA leading to inhibition of protein synthesis and apoptosis		
	Purified ricin is a water-soluble white powder easily inactivated by heat		
	Crude preparations of *R. communis* contain approximately 1–5 % ricin by weight and are stable for longer periods of time compared to purified ricin		
	No currently approved vaccines for use in humans; subunit vaccines devoid of enzymatic activity but retaining native tertiary structure are in development		
SEB	CDC Category B	Fever, headaches, chills, myalgia	Diagnosis dependent on toxin assays performed by reference laboratories; also depend upon clinical observations and suspicions of SEB intoxication
	Deliberate contaminations would need to be distinguished from natural *S. aureus* foodborne outbreaks	Nonproductive cough lasting up to a month or longer	Diagnosis involves nasal swabs, urine, or acute and/or convalescent sera samples

(*continued*)

Table 1 (continued)

Toxin	Characteristics	Clinical symptoms	Diagnosis and treatment – based on clinical signs and symptoms
	Poor environmental stability and not easily produced or disseminated	Diarrhea and vomiting which, left untreated, can lead to hypovolemic shock	Treatment is supportive including intravenous fluid replacement, mechanical ventilation, and flushing of the stomach to remove toxin
	Estimated toxic dose: 0.0004 μg/kg inhaled; estimated lethal dose: 0.02 μg/kg inhaled	May be fatal	
	Staphylococcal enterotoxins are produced by about 50 % of all *Staphylococcus aureus* isolates		
	More than 25 enterotoxins (also known as secreted exotoxins) have been identified		
	Also called superantigens due to interactions with the immune system and activation of a higher percentage of T cells		
	SEB is a low-molecular-weight protein – 24–29 kDa		
	Binds to MHC II and T-cell antigen receptors		
	Also stimulates macrophages and mast cells, causing release of soluble mediators in a “cytokine storm”		
	No currently approved vaccines		
	Human-murine chimeric antibodies to protect against SEB intoxication have been developed and tested in vitro and in vivo		
	Vaccine research is focused on recombinant, site-directed mutants with altered amino acid sequences		

Abrin	High toxicity	Ingestion leads to nausea, vomiting, diarrhea, and abdominal pain	Food and environmental samples can be tested to establish link to patient illness
	Estimated lethal dose: 0.1–1 μg/kg ingested	Gastrointestinal bleeding in some instances	Treatment consists of supportive care including fluid replacement as needed
	Low cost to manufacture	Less common are tachycardia, headaches, drowsiness, fever, and weakness	
	Di-chain related to ricin in structure and mechanism of action	Inhaled abrin in rats causes general malaise, lethargy, anorexia, respiratory distress	
	Produced by jequirity bean (*Abrus precatorius*)		
	Toxin exists in multiple isomeric forms		
	Consists of an A-chain which inactivates ribosomes and inhibits protein synthesis and a B-chain lectin which binds to cell membranes and facilitates entry into cells; A and B chains are linked by a single disulfide bond		
Aflatoxin	Over 400 toxins produced by fungi, including the Aspergillus-produced Aflatoxins	Liver failure	Supportive care
	Aflatoxins may have been weaponized by Iraq in the 1980s; because these are slow-acting toxins, they would not be effective weapons except to possibly spread fear		
	Primarily cause pulmonary edema and liver necrosis		
	May also bind to DNA and proteins and lead to decreased cell-mediated immunity and interference with nutrient uptake		
	No antidotes		

(*continued*)

Table 1 (continued)

Toxin	Characteristics	Clinical symptoms	Diagnosis and treatment – based on clinical signs and symptoms
T-2	Produced by over 350 fungal species including Fusarium, Stachybotrys, Trichoderma, and Cephalosporium	Immediate pain upon contact with skin and eyes	Flushing of entire surface of skin to remove toxin
	T-2 inhibits protein and nucleic acid synthesis	Conjunctivitis, blurry vision	Monitoring of airway and cardiovascular status
	Lethal dose (small animals): 0.05–2 mg/kg inhaled, 2–10 mg/kg dermal application, 0.3–9.1 mg/kg parenteral	Rhinorrhea, epistaxis, dyspnea, tachycardia, vascular collapse	Administration of dexamethasone or activated charcoal
		Nausea, diarrhea, vomiting, transient lymphocytosis	Treatment with filgrastim for bone marrow suppression
		In advanced stages, patients experience hemorrhage, coagulopathy, airway and bronchus problems, and pulmonary edema	
Epsilon toxin (ETX)	Produced by *Clostridium perfringens*	Unknown in humans	Supportive care
	32 kDA protein that damages cell membranes and causes K + release	In animals – diarrhea, staggering, convulsions, coma	
	Lethal dose in humans estimated to be 100 ng/kg body mass intravenously (based on extrapolation from rodent models)		
	A formalin-fixed toxoid, absorbed to alum, is used to vaccinate sheep and goats		
	Efforts are underway to develop recombinant vaccines for human and veterinary use		

Shiga toxin	Produced by certain strains of *Escherichia coli*	Acute bloody diarrhea	Supportive care
	Leading cause of enteric infections in the United States	Hemolytic uremic syndrome – loss of platelets and erythrocytes, kidney failure	
	Lethal dose (animals) estimated to be 2.2–200 ng/kg injected (rabbits) and 450 ng/kg injected (mice)	Severe cases may see lethargy, headaches, and convulsions	
	Inhibits protein synthesis leading to apoptosis		
	A Montreal-based company has developed two chimeric monoclonal antibodies to Shiga toxins 1 and 2; these have undergone phase II clinical trials		
Saxitoxin	Paralytic shellfish toxin caused by eating contaminated bivalve mollusks	Paresthesia, "floating" sensations, muscle weakness	Mechanical ventilation
	Inhibits sodium channels in neurons	Cranial nerve dysfunction	
		Death may result from diaphragm paralysis	
Tetrodotoxin (TTX)	Produced by *Pseudoalteromonas tetraodonis*, some species of Pseudomonas, and some *Vibrio* species	Paresthesia in lips and tongue	Supportive care including mechanical ventilation
	Prevents depolarization and action potentials, blocking neurons in PNS and CNS	Facial and extremity paresthesias	
	Causes approximately 50 deaths per year in Japan, from eating contaminated *fugu*, or puffer fish	Motor weakness	
		Hypoventilation	
		Ascending paralysis and late bradycardia	
		Seizures	
		Coma	
		Death from diaphragm paralysis or other respiratory failure	

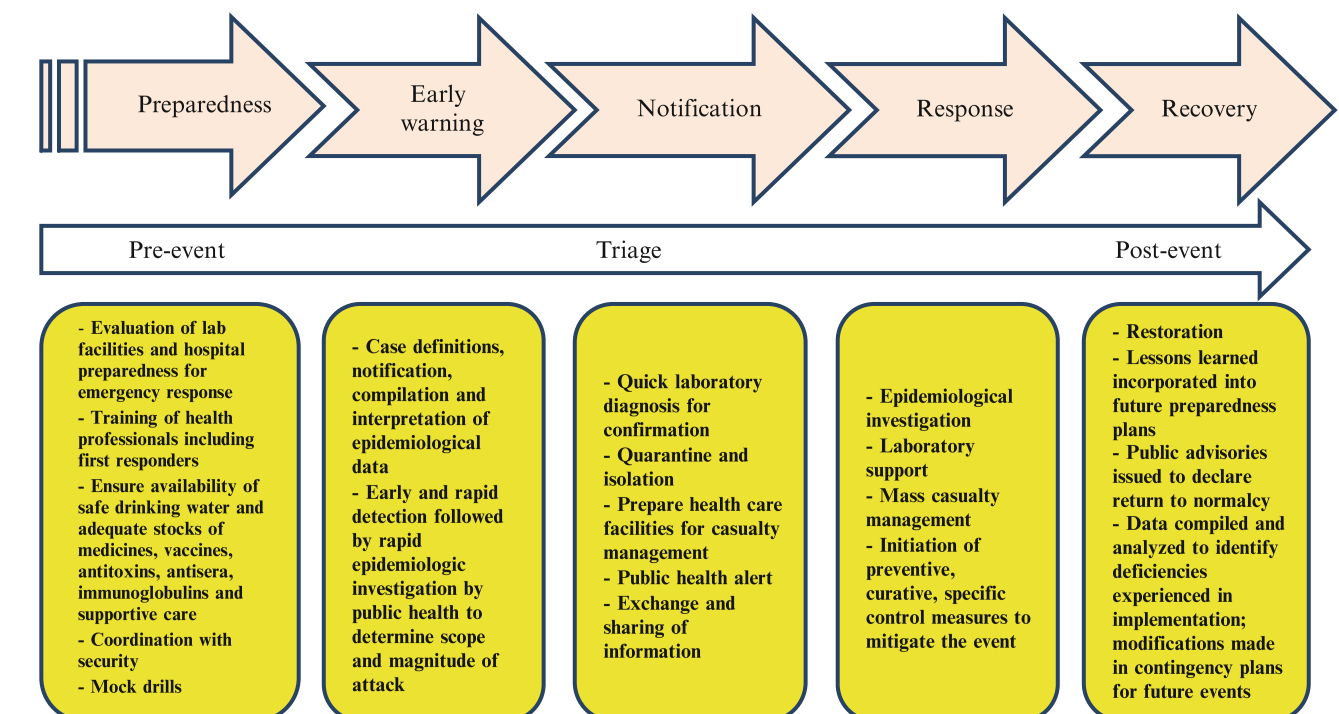

Fig. 1 Five phases of activity in a public health event (Das and Kataria 2010)

Because they represent a critical component of public health infrastructure, a set of core functions and essential services for the state public health laboratories was created. Core functions are described in Table 2 and essential functions are described in Table 3 below.

Under the auspices of Healthy People, a Department of Health and Human Services initiative, surveys are conducted on a biennial basis to gauge how the state public health systems are faring in fulfilling goals that corresponded to the 11 core functions. These Comprehensive Laboratory Services Surveys track trends in performance and point to areas in need of improvement. Laboratories are grouped according to size, based on number of full-time equivalent (FTE) positions (small labs, 23–73 FTE; medium, 74–140; large, >140). Areas of strength, with greater than 2/3 of respondents indicating they meet the objective, include disease prevention, control, and surveillance; reference and specialized testing (although smaller labs may not meet all reference testing needs); and policy development, while areas of consistent weakness include food safety and public health-related research. The results from these studies can be used to guide policy and funding decisions related to comprehensive laboratory services on a national level (Wilcke et al. 2010).

The CDC, in partnership with the Association of Public Health Laboratories (APHL), United States Army Medical Research Institute for Infectious Diseases (USAMRIID), and the Federal Bureau of Investigation (FBI), created the Laboratory Response Network (LRN) in 1999 with an initial focus on creating a network designed to integrate federal, public health, private clinical, and commercial laboratories and to define their responsibilities when confronted with suspected or confirmed acts of biological terrorism. Participating laboratories were initially placed into one of four categories, designated Level A through D. Level A laboratories were private and commercial clinical labs, city health labs, and military labs operating under Biosafety Level (BSL) 2 conditions. These laboratories perform limited testing on human specimens to rule out or refer suspected biothreat agents using standardized testing as set by the American Society for Microbiology (ASM). CDC and APHL developed and provided training and education to personnel in the areas of biosafety; specimen collection, preservation, and transport; chain of custody; clinical manifestation of disease agents; and characterization of targeted agents. Level A laboratories were not considered official members of the LRN. Level B and C labs were the state and selected military labs whose mission was to accept and test environmental specimens and to rule in clinical specimens or isolates referred by level A labs. Additional level C labs were selected state public health labs with enhanced testing capacities, such as for botulism. Labs at this level were the core of the LRN and had access to a secure CDC website containing protocols and technologies for confirmatory testing. Operating under BSL-3 conditions, or under BSL-2 conditions with BSL-3 practices, personnel had to complete formal training and undergo proficiency testing before working in these facilities. The highest level labs were designated level D; two BSL-4 labs, one at CDC and one

Table 2 The 11 core functions of state public health laboratory systems (Inhorn et al. 2010; Witt-Kushner et al. 2002)

Core function	Description
Disease prevention, control, and surveillance	Partners in the system participate in processes to support health surveillance
	Data are generated in many areas of public health (communicable disease, chronic disease, environmental exposure, etc.) and shared with federal, state, and local agencies to enhance rapid disease detection and implementation of control measures
Integrated data management	The goal of the state public health system is standards-based interoperability to facilitate the exchange of information
	Data are collected using accepted formats for epidemiological analysis and decision making
	Data are shared with partners to facilitate rapid detection, response, and management of public health emergencies
Reference and specialized testing	Research and validation of new testing methods
	Training on specimen collection and transport, biosafety, interpretation of test results, regulatory requirements for private clinical and public health sector personnel
Environmental health and protection	Provide testing and surveillance of drinking and recreational water
	Air monitoring
	Biomonitoring
	Environmental lead exposure
	Occupational health
	Solid and hazardous waste and wastewater management
Food safety	CDC FoodNet collects data from ten states regarding food-associated pathogens
	Council to Improve Foodborne Outbreak Response is a multidisciplinary group of government, regulatory, and association stakeholders
	DHS FoodSHIELD is a web-based platform designed to create a community among labs and regulatory agencies
	State and local public health labs investigate foodborne outbreaks and perform disease surveillance and confirmatory testing
	PulseNet is a network of state public health labs that perform advanced testing to investigate foodborne outbreaks
	FERN (Food and Environment Reporting Network) was created in 2004 by USDA and FDA to integrate the nation's public health, environmental, agricultural, and veterinary labs for response to food supply threats
Laboratory improvement and regulation	Regulatory requirements and quality assurance activities to improve the reliability of laboratory testing
Policy development	Public health lab community involvement assures good scientific data drives sound policy decisions
	Public health data impacts policies, regulations, and legislation related to food and water safety; control of local, state, and national outbreaks; control of environmental hazards; and newborn screening

(*continued*)

Table 2 (continued)

Core function	Description
Emergency preparedness and response	The Laboratory Response Network (LRN) was created in 1999 to test, identify, and characterize potential agents of chemical and biological terrorism
Public health-related research	Applied and practice-based research supports epidemiological investigations
	Focus areas include newborn screening and communicable disease control
Training and education	National Laboratory Training Network offers more than 4,500 courses reaching 275,000 public health and clinical laboratory staff via teleconferences, web-based conferences, on-demand programs, lecture-based seminars, and workshops
	Includes fellowships and internships as well as leadership training
Partnerships and communication	Public health labs serve as focal points in a national system of public health surveillance and response
	Public health labs serve as data sources for national surveillance programs including FoodNet, Arbovirus Surveillance Network, Calicivirus Network, PulseNet, Influenza Surveillance Network

Table 3 The ten essential public health services (Baker et al. 1994)

Essential public health services
Monitor health status to identify and solve community health problems
Diagnose and investigate community health problems and health hazards
Inform, educate, and empower people about health issues
Mobilize community partnerships and actions to solve health problems
Develop policies and plans that support individual and community health efforts
Enforce laws and regulations that protect public health and assure safety
Link people to needed personal health services and assure the provision of health care when otherwise unavailable
Assure a competent workforce in public health and personal care
Evaluate the effectiveness, accessibility, and quality of personal and population-based health services
Research for new insights and innovative solutions to health problems

at USAMRIID, comprised this level. These labs performed the highest level of diagnostic testing associated with high-consequence pathogens and were also responsible for the research, development, and validation of new diagnostic technologies. CDC had primary responsibility for the full characterization of biological agents while USAMRIID's role was to support CDC in surge capacity situations. Following the anthrax attacks in 2001, laboratories were inundated with human and environmental samples due to public panic. Law enforcement and first responders sent environmental samples to level A laboratories which were not intended to receive them. There was a concern that false-positives

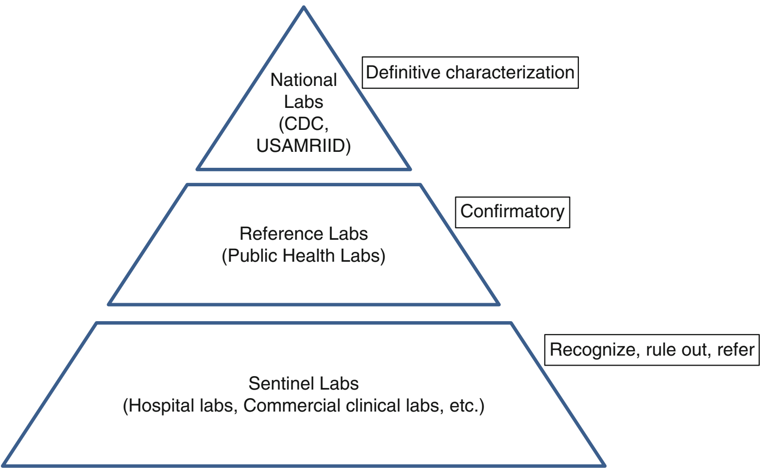

Fig. 2 The LRN hierarchy

generated by inadequately trained personnel would create undue panic and unnecessary medical interventions, including the prophylactic use of antibiotics and vaccines. Following the anthrax event, the LRN was restructured. Level A labs are now designated sentinel labs and consist of hospital-based clinical labs, commercial clinical labs, and most military labs. Only human specimens are tested in these facilities. They do not have access to the secure CDC LRN website and they follow standardized procedures to rule out or refer samples or isolates to the nearest Reference Lab for confirmatory testing. Sentinel labs are not expected to accept nor process environmental or animal samples; instead these should be forwarded to the nearest available reference lab. In cases involving suspected bioterrorism, the FBI should be informed as well. Personnel in these labs are still expected to employ appropriate precautions when working with samples and to include the use of protective eyewear, closed lab coats with cuffed sleeves, and gloves. In addition, safe lab practices that avoid the unnecessary production of aerosols should be used. Sample manipulations with the potential to generate aerosols (centrifugation, pipetting, sonication, etc.) should be performed in a biosafety cabinet (Sewell 2003). What were formerly designated level B and C labs are now called Reference Labs whose primary responsibility is to detect and confirm the identity of threat agents. Some of these labs may also measure human exposure to toxic chemicals. Member labs consist of selected military labs operated by the Department of Defense, including USAMRIID, food-testing labs within FDA and USDA, environmental labs capable of testing water and environmental samples, veterinary labs run by USDA, and select international labs in Canada, the United Kingdom, Australia, Mexico, and South Korea. Laboratories at the highest level are those operated by CDC, USDA, and DoD. Figure 2 below diagrams the LRN hierarchy and primary responsibility at each level (Pien et al. 2006; Snyder 2005). Clinicians and laboratory personnel

evaluating potential bioterrorism events should recognize the following guidelines and implement them into their practices: (a) optimal specimen collection instructions should be obtained from sentinel labs intended to receive them; the recipient laboratory should also be alerted to the nature of the sample being shipped; (b) sentinel laboratories should limit sample and culture manipulations to those required for referral to reference labs when necessary; (c) environmental, food, animal, or plant samples should not be sent to sentinel labs; these should be sent directly to LRN reference labs; and (d) sample manipulations should be performed in biosafety cabinets whenever possible. Diagnostic laboratories should all have access to a clinical microbiologist certified by the American Board of Medical Microbiology or other equivalent body (Pien et al. 2006).

Clinical Recognition of a Toxin Attack

Unlike covert attacks with chemical agents, such as the sarin attack on the Tokyo subway system in 1995, which led to violent physical reactions within minutes of exposure, covert attacks with biological toxins may not be immediately obvious. Because of the longer incubation times associated with most toxins, physicians in hospital emergency departments and private practice, rather than EMTs and paramedics, will most likely be the ones faced with the evidence of an attack, but they may not recognize it as such. Initial identification of either a biological terrorism attack or a biocrime must rely on presenting signs and symptoms in individuals first showing up for medical care and on follow-on epidemiological and/or criminal investigations. The clinical manifestations of diseases caused by biological toxins may be nonspecific and similar to naturally occurring illnesses. Patients with compromised immune systems, or those exposed to higher doses of toxins, will show signs and symptoms earlier than others and will seek medical care sooner, most likely before a deliberate dissemination has even been suspected. A high level of alertness is thereby recommended; physicians should be encouraged to think like epidemiologists and suspect bioterrorism when clusters of similar syndromes are seen. In Section 4 of American Medical Association Policy H-130.946, *AMA Leadership in the Medical Response to Terrorism and Other Disasters*, the organization encourages physicians to (a) be alert to unusual illnesses in the community; (b) be knowledgeable of disease surveillance; (c) be aware of procedures for collecting patient information for disease surveillance; (d) know the clinical manifestations, diagnostic techniques, isolation precautions, decontamination methods, and prophylaxis for biological agents and toxins likely to be used in terrorist attacks; (e) use necessary precautions to prevent additional exposures; (f) employ treatment plans that may include addressing psychological trauma; (g) understand proper risk communication; and (h) understand the roles of public health, emergency medical services, emergency management, incident management, and the health care provider's own role in disaster response (AMA Policies [Internet] [cited 2 Apr 2014]).

Clues that a biological attack has occurred may include the following, not all of which will be apparent to individual clinicians:

- Severe disease manifestation in previously healthy people
- Higher than usual number of patients with fever and respiratory or GI symptoms
- Multiple people with similar complaints and involving a common location (e.g., patients who all dined at a particular restaurant)
- Unusual temporal or geographic clustering of illness or patients presenting clinical signs and symptoms suggestive of a disease outbreak
- Unusual age distribution of common illnesses
- Large number of cases of acute flaccid paralysis with bulbar palsies
- Unusually high number of fatalities
- Rapidly rising and falling epidemic curves
- Unusual number of patients presenting diplopia and progressive weakness

Adequate detection mechanisms, including diagnostic assays, are not widely available for biological toxins; individuals presenting with signs and symptoms of exposure are often the only “detector” available. Increasing numbers of patients presenting with similar syndromes will often be the first indication that a covert attack has occurred. Determining who is affected, the route(s) of exposure, signs, and symptoms of presenting patients and determining the causative toxin are all necessary in order to plan an appropriate medical and public health response. Epidemiological investigation can provide information about the etiologic agent and its medical consequences and will allow appropriate follow-up with other potentially exposed persons. In a mass casualty situation following a wide-scale release of toxin, triage will be necessary in order to identify patients with the highest likelihood of survival; there may not be enough resources available to treat patients with advanced symptoms. Effective triage will also prevent the overwhelming admissions at health care facilities by the “worried well” seeking medical care; it also projects a sense of government continuity, where a terrorist’s goal may be to reveal vulnerability in a government’s ability to defend its citizens. State health departments can employ several strategies to educate and remind health care providers of the need to recognize unusual illness that may indicate the intentional release of a biological toxin or other threat agent. These include (1) communicating information and reminders to health care providers and clinical laboratories about how to report events to the appropriate health authorities, (2) implementing 24/7 capacity to receive and act on any positive reports of events that suggest an intentional release of a biological toxin or threat agent, (3) investigating immediately any report of a cluster of illnesses or another event suggesting such an intentional release and requesting assistance from CDC when needed, (4) implementing a plan to include access to the LRN to collect and transport specimens and to store them appropriately prior to laboratory analysis, and (5) immediately notifying CDC if an investigation points to an intentional release (Binder et al. 2003; Burkle 2002; CDC 2001; Franz et al. 1997).

According to Christian (2013), the appearance of two to three cases of botulism should be enough to trigger consideration of an intentional bioterrorism event, while four or more cases of SEB intoxication should likewise raise that possibility.

A well-organized response to attacks with biological weapons, including toxins, is critical; communication must also be effective in order to reassure the public and prevent panic. Some people will be at increased risk for psychiatric issues following such an attack. Those who have previously undergone a traumatic incident and those who lack adequate social support networks will have a harder time coping with this kind of event. Also, frontline first responders may experience mental fatigue in the face of overwhelming trauma. As details of an attack are made known and reported in the media, both exposed and non-exposed individuals may begin experiencing autonomic effects including muscle tension, tachycardia, hyperventilation, sweating, tremor, and a general sense of foreboding, all of which may be misattributed to exposure to a biological threat. Psychological responses may include anger, panic, fear of invisible agents and contagion, paranoia, social isolation, demoralization, and, perhaps most critically, a loss of faith in both government and social institutions. It is therefore critical to prevent group panic so that the "worried well" do not overwhelm health care services that are best reserved for those truly in need of them or reject assistance from those trying to remediate the situation. Anxiolytics may be prescribed to provide acute relief from some symptoms. A restoration to normalcy as quickly as possible, and communication to the public of this, is vital (Holloway et al. 1997).

Biosurveillance

Methods to detect and monitor the course of an outbreak in order to minimize morbidity and mortality are dependent on the clinical observations of health care professionals. Syndromic surveillance occurs through the monitoring of clinical manifestations of illnesses or when health-related data, such as ICD-9 codes (mechanistic surveillance), are analyzed to detect potential outbreaks. Because patients seeking care are often assigned a nonspecific diagnosis, public health authorities, rather than individual clinicians treating a few patients, are better positioned for analysis and detecting unusual trends. Once a statistical detection threshold has been exceeded, an epidemiological investigation can be initiated, involving the collection of samples for laboratory analysis that may yield clues to a specific etiology. Surveillance may be the most important instrument available to public health authorities for identifying covert biological toxin attacks. Surveillance has four basic functions: (1) detection of cases of disease in specific populations and reporting of information to appropriate authorities, (2) analysis and confirmation of reported case information in order to detect outbreaks, (3) provision of timely and appropriate responses at the local and regional levels in order to allow

national-level prevention and control of outbreaks, and (4) provision of epidemiological intelligence information to assist in the long-term management of public health and health care policies and programs. Syndromic surveillance systems should enable rapid access to patient data. They should also be used to enhance human judgment and not replace it.

The attributes of specific biological toxins will inform the recognition of a bioterror event, as will the following characteristics:

- Size – syndromic surveillance will not detect outbreaks that fail to reach a statistical threshold.
- Population dispersion – how quickly people change geographic location following an exposure.
- Health care – clinicians more knowledgeable about toxin symptomology will be more likely to recognize an attack and alert public health authorities; they are also more likely to be familiar with the appropriate reporting procedures.
- Seasonality – covert toxin attacks may be more difficult to detect during seasonal upswings in naturally occurring diseases.
- Selection of data sources, timeliness of information management, definition of syndrome categories, selection of statistical thresholds, and availability of resources.

In order for syndromic surveillance to be effective, it requires the participation of the health care community. Researchers in Wisconsin conducted a study to determine how easily physicians could be recruited to participate in a sentinel surveillance project. Clinicians were contacted by e-mail, phone, and fax and belonged to one of three groups: a primary care practice-based research network, an existing influenza sentinel program, or the state academy of family physicians. The study determined that it took 2–3 h on average to recruit each participant and that members of established networks were more easily approached and recruited. Successful recruitment involved contacting physicians multiple times through a combination of methods. Buy-in and participation of a group director or other leadership also proved to be a valuable tool. These findings should prompt state divisions of public health, state medical societies, and departments of licensing and regulation to collect and maintain accurate and up-to-date clinician contact information in order to facilitate rapid responses to public health emergencies such as biological toxin attacks. Despite high costs and tradeoffs – surveillance systems with lower statistical thresholds are more prone to false detections – a majority of US state and territory health departments indicate their desire to expand the use of syndromic surveillance, already in use in many US cities, especially given their reliability in detecting outbreaks based on syndromes reflecting high-probability events (e.g., the onset of the annual flu season) (Buehler et al. 2003; Chretien et al. 2009; Kman and Bachmann 2012; Temte and Grasmick 2009).

Table 4 below presents some syndromic surveillance systems currently in use.

Table 4 A summary of some existing syndromic surveillance systems (Kman and Bachmann 2012)

System name	Key features
ESSENCE – Electronic Surveillance System for the Early Notification of Community-Based Epidemics	Allows automatic downloads of ICD-9 codes from US Department of Defense health care facilities. Patient visits are grouped by ESSENCE algorithms into one of the eight possible syndromes based on code lists. In the event of an uptick in the number of visits for a particular syndrome, the clinic can be contacted for more information and an investigation can be launched if appropriate
BioSense	Implemented in 2003 by CDC, BioSense is an Internet-based system designed for the early detection of intentional and natural disease outbreaks. It receives data electronically, including ICD-9 codes, from multiple sources including the Departments of Veterans Affairs and Defense, retail pharmacies providing information on over-the-counter (OTC) sales, and LabCorp which provides information on tests ordered. Public health officials can access summary reports after analysis by CDC
RODS – Real-time Outbreak and Disease Surveillance Laboratory	Since 1999, this system, place in the state of Pennsylvania, monitors over three million visits from to 137 different emergency departments per year. It also monitors over 1,200 retail stores. Using the National Retail Data Monitor, a strong correlation has been found between purchases of OTC medications and ED visits for certain illnesses. That information is used to help predict coming epidemics and track patterns of influenza and GI illnesses because OTC purchases often precede trends in hospital data
HASS – Hospital Admission Syndrome Surveillance	Since 2001, the state of Connecticut has been using a system based on unscheduled hospital admissions. HASS monitors 32 acute care hospitals with required reporting for 11 syndromic categories. Daily monitoring of data with weekly analysis allows identification of disease clusters and routine public health follow-up
CDC EARS – Early Aberration Reporting System	Used in cities including Boston, New York, and Los Angeles, as well as in state public health agencies in Florida, Georgia, Mississippi, North Carolina, and Tennessee, EARS uses nontraditional sources of data including school absenteeism rates, OTC medicine sales, 911 calls, veterinary and ambulance run data
HealthMap Project	This web-based surveillance system extracts, categorizes, filters, and integrates aggregated reports from multistream real-time Internet surveillance data. Automatic data mining is assisted by analyst review and was applied to the 2009 H1N1 flu outbreak

Public Health Preparedness and Response

> This directive establishes a National Strategy for Public Health and Medical Preparedness (Strategy), which builds upon principles set forth in *Biodefense for the 21st Century* (April 2004) and will transform our national approach to protecting the health of the American people against all disasters.
> - Homeland Security Presidential Directive/HSPD-21
> October 18, 2007.

Public health infrastructure must be well prepared in order to prevent illness resulting from both large-scale and small, targeted toxin attacks. Responding to a large-scale attack will require rapid mobilization of public health workers, emergency responders, and private health care providers. It will also necessitate the rapid procurement and distribution of large quantities of drugs, vaccines, antitoxins, and other therapeutics. Early detection, diagnosis, and control depend upon a strong and flexible public health system at the local, state, and federal levels. It also requires that medical staff and emergency care providers, who are the first line of defense and response to a bioterrorist event, be vigilant and knowledgeable about the clinical symptomologies of biological toxins. They must have the mindset that unusual or anomalous illnesses may indicate exposure to biological toxins and that such incidents should be reported immediately to public health authorities. Preparedness can be enhanced by specialized training in continuing medical education programs and by involving physicians, nurses, and other frontline caregivers in disaster care planning conducted by healthcare facilities in coordination with community resources including fire, police, and emergency response departments, hospitals, utilities, local government, and large employers. Local, county, and state medical societies should develop physician call-up and e-mail alert systems while public information services can raise awareness of the importance of preparing for biological terrorism events (CDC 2011; Dudley 2003).

One way of preparing for a biological toxin attack involves doing analyses of existing vulnerabilities in order to develop strategies to address them. Wein and Liu (2005) conducted a threat assessment of the nation's milk supply and its vulnerability to a covert botulinum toxin attack. America's dairy farms and milk distribution system deliver 20 billion gallons of milk per year throughout the United States. The methods applied to their analysis could also be applied to fruit and vegetable juices, canned foods, and grain-based foods. The researchers applied the following model and assumptions for their analysis:

– Cows are milked twice daily and the milk is picked up from each farm once per day by a 5,500 gal truck which makes two round trips per day between various farms and the processing plant.
– Raw milk is piped into 50,000 gal silos prior to processing; each processing line can hold milk from multiple silos simultaneously.
– Raw milk silos in California must be cleaned after 72 h of operation; during operation each silo is continuously being drained and filled and then completely drained at the end of 72 h.
– Processed milk is held in 10,000 gal post-pasteurization tanks prior to bottling.

- Heat pasteurization is expected to inactivate 68 % of any toxin present.
- Each gallon of milk is consumed by four people over 3.5 days (three adults, one child).
- Attack is detected either via early symptomatic patients or by in-process testing for toxin.
- Outbreak is detected when 100th person develops symptoms, assuming median incubation period of 48 h, with an extra 24 h required to identify the attack as milk-borne, at which time all milk consumption is halted.
- Mouse bioassay has an LOD of 16 pg/ml and requires 48 h, while in-process detection by ELISA has an LOD of 80 pg/ml and requires 3 h; mouse bioassay would be used only as confirmation if positive result is seen with ELISA.
- Botulinum toxin is deliberately released into a holding tank at a dairy farm, a tanker transporting raw milk to a processing plant, or a raw milk silo.

In the absence of any detection method and based on a 10-g bolus of toxin being used, for every gallon of milk consumed, an average 568,000 people would consume contaminated milk, with most casualties occurring on days 3–6 based on 10 g of toxin being used. Early symptomatic detection would reduce the number of casualties by 2/3; in-process ELISA testing alone prevents nearly all cases. The researchers determined that reducing the time between silo cleanings from 72 to 48 h would lower the number of poisonings by 30 % even without testing. Additionally, increasing the silo size from 50,000 to 100,000 gal but reducing silo number to maintain overall capacity and employing dedicated processing lines per silo leads to fewer casualties for small releases. However, larger releases may result in increased numbers of casualties as a result of milk mixing. This is in the absence of any in-process testing. Control measures can lessen the vulnerability of dairy and other food-processing networks to covert toxin attacks. All tanks, trucks, and silos should maintain restricted access when not being drained or filled. Security checks for all personnel including farmers, drivers, processing plant labor, engineers, and others should be implemented. While currently voluntary, requiring one person from each stage of the supply chain to be present at each transfer points would minimize opportunities for covert actions.

Abraham et al. (2012) conducted “tabletop scenarios” involving five bioterrorism situations to determine how well-existing public services were equipped to deal with them. Two of the five exercises, which dealt with deliberate contaminations of food and ice in a university setting, are applicable to toxin incidents, while the other three apply to infectious diseases, namely, smallpox, plague, and avian flu. Participants represented emergency medical services (EMS), fire departments, public safety, public health practitioners, nurses, doctors, social workers, counselors, physician assistants, physical therapists, occupational therapists, health administrators, pharmacists, and dentists. The exercises addressed terrorism recognition and reporting, multidisciplinary coordinated responses, and acute patient care. While recognizing the limitations of tabletop scenarios in demonstrating real-world effectiveness, recommendations resulting from them may prove to be valuable. These include:

- Establish a contingency plan for communication and notification methods.
- Create a public information plan.
- Develop policies and procedures to provide long-term mental health care.
- Establish regional or multiple volunteer Medical Reserve Corps units.
- Establish a policy between public health and the first responder community regarding pre-incident information sharing.
- Use the wealth of resources of local support agencies by establishing cooperative agreements.
- Develop MOUs (memoranda of understanding) between hospitals and private security companies to provide needed security support during emergencies.

In 2011, CDC published "A National Strategic Plan for Public Health Preparedness and Response" with the input of 375 subject-matter experts from across the government and partner organizations. Developed to be consistent with Presidential Policy Directive 8 (issued by President Obama in March, 2011), Healthy People 2010, and other preparedness and response plans, CDC's plan describes eight objectives that are meant to serve as a guide by which the public's health is protected from both naturally occurring and deliberate biological threats. These eight strategies are defined below (CDC 2011):

1. **Prevention and mitigation of public health threats**
   Increased public health involvement in national and international health security efforts and advancing capabilities to measure and address gaps in preparedness.
2. **Integration of public health, health care, and emergency management**
   Development and promotion of tools to enable integration of these three sectors.
3. **Promotion of individual and community resilience**
   CDC's Public Health Emergency Preparedness Cooperative Agreement (PHEP) provides technical assistance and funding to public health agencies to support better decision-making processes.
4. **Advancement of surveillance, epidemiology, laboratory science, and service practice**
   Integration of public health preparedness and response data reporting systems and processes and increasing surveillance, epidemiology, laboratory science research, equipment, modeling, and tools.
5. **Increased application of science for preparedness and response**
   Promotion of tools and promising practices to improve preparedness and response and expanding the knowledge that contributes to reduction and mitigation of public health threats.
6. **Improved public health preparedness and response infrastructure**
   Enhancing capabilities while sustaining existing capacities and assessing adequacy of existing infrastructure and systems while enhancing abilities to redirect resources when necessary for event response.

Table 5 Fifteen public health preparedness capabilities

Functional area	Capability	Tier
Biosurveillance	Public health laboratory testing	1
	Public health surveillance and epidemiological investigation	1
Community resilience	Community preparedness	1
	Community recovery	2
Countermeasures and mitigation	Medical countermeasure dispensing	1
	Medical materiel management and distribution	1
	Non-pharmaceutical interventions	2
	Responder safety and health	1
Incident management	Emergency operations coordination	1
Information management	Emergency public information and warning	1
	Information sharing	1
Surge management	Fatality management	2
	Mass care	2
	Medical surge	2
	Volunteer management	2

7. **Enhanced stewardship of public health preparedness funds**
   Promoting the effective use of preparedness and response funding and strengthening the effectiveness and timeliness of communication to the public, elected officials, and policymakers.
8. **Improved ability to respond to health threats**
   Enhancing workforce competencies and identifying strategies for enhanced capacity for medical countermeasures.

CDC also identified 15 public health preparedness capabilities, divided into two tiers and six functional areas, shown in Table 5. As metrics are developed and data generated, CDC will be able to display trends and document progress made towards achieving the objectives that accompany the eight strategies listed above (Khan et al. 2012).

How We Are Doing: The Current State of Public Health Preparedness

Trust for America's Health, a nonprofit organization focused on disease prevention, in collaboration with the Robert Wood Johnson Foundation, a philanthropy focused on American health and health care, produces a report titled "Ready or Not? Protecting the Public's Health from Diseases, Disasters, and Bioterrorism." This report is produced to inform the public and policymakers about progress and vulnerabilities in public health preparedness and response. By assessing state preparedness and the government's roles and performance of its duties, the report allows for the evaluation of the effectiveness of taxpayer expenditures in this area.

In the most recent report, from 2012, ten public health preparedness capabilities were examined in all 50 states and the District of Columbia. Capabilities relevant to preparedness and response to toxin terrorism events include:

1. Increased or maintained level of funding for public health services compared to previous year
2. Notified and assembled public health staff to ensure rapid response to incidents
3. State has been accredited by the Emergency Management Accreditation Program (EMAP)
4. State public health lab reports having adequate staffing capacity to work five, 12-h days for 6–8 weeks in response to an infectious disease outbreak

Each state and the District of Columbia (D.C.) was assessed for each of ten capabilities and assigned either a "1" for meeting the capability or a "0" for failing to meet it; thus scores could range from a minimum of 0 to a maximum of 10. The highest score achieved was 8, by five states (MD, MS, NC, VT, WI). Ten states (AL, AR, CA, DE, NB, NH, NM, NY, ND, VA) received a score of 7; 15 states (CT, ID, IA, KY, LA, ME, MA, MO, OH, OK, SC, TN, UT, WA, WY) earned a score of 6;13 states (AK, AZ, FL, IL, IN, MI, MN, OR, PA, RI, SD, TX, WV) and D.C. earned a 5; five states (CO, GA, HI, NV, NJ) earned a 4; and two states (Kansas and Montana) received the lowest score of 3. In the area of maintaining or increasing public health funding (capability 1, above), 21 states plus D.C. met this capability. Forty-seven states and D.C. met capability 2 (assembly of staff for rapid response); only Connecticut, Hawaii, and New York did not meet this capability. Twenty-nine states and DC were accredited by EMAP (#3 above), and 37 states plus D.C. had adequate staffing needs for outbreak response (#4 above). States with inadequate staffing needs are as follows: AK, CO, GA, HI, IN, IA, KS, MA, NV, J, PA, SC, and TX (Levi et al. 2012).

Public funds invested in health security should be spent in such a way as to generate maximum return on investment. It is therefore necessary to understand how those funds are being used. In 2010, the Office of the Assistant Secretary for Preparedness and Response (ASPR) commissioned a study to take inventory of all nonclassified, civilian national health security research funded by the federal government. The goals of this study were to analyze how well the existing mix of research projects aligned with the priorities in HHS's 2009 National Health Security Strategy (see Table 6) and to identify gaps that should become priorities for future funding. Agencies contacted were the Agency for Healthcare Research and Quality, CDC, National Institute of Health (NIH), Department of Homeland Security (DHS), Department of Energy (DOE), National Science Foundation (NSF), and Veterans Health Administration (VHA); Department of Defense (DoD) research was excluded from this analysis. In a review of 1,593 unique research projects, 66 % (1,047) were directed towards biological threats including specific forms of bioterrorism (21 %), foodborne illnesses (15 %), and pandemic flu (8 %). A majority of funded projects were awarded between 2003 and 2010; federal spending on biodefense grew from $576 million in 2001 to over $5 billion in 2008. Aside

Table 6 Strategic objectives of HHS national health security strategy

Strategic objective
Foster informed, empowered individuals and communities
Develop and maintain workforce needed for national health security
Ensure situational awareness
Foster integrated, scalable health care delivery systems
Ensure timely and effective communication
Promote effective countermeasures
Ensure prevention or mitigation of environmental or other emerging threats to public health
Incorporate post-incident health recovery into planning/response
Work with cross-border and global partners to improve national, continental, and global health security
Ensure all systems that support national health security are based on best available science, evaluation, and quality improvement methods

from NSF and DOE, all other agencies focused the majority of health security-related research projects on biodefense; natural disasters comprised 10 % of funded programs; all hazards, 14 %; chemical threats, 8 %; radiation threats, 5 %; and nuclear threats and explosive threats, 4 % each. Emergency preparedness was addressed by 78 % of funded projects, while mitigation, response, and recovery were addressed by 10 %, 28 %, and 9 % of projects, respectively. Totals exceed 100 % because many funded projects addressed multiple areas. With respect to the ten strategic objectives in Table 6, 82 % of all funding focused on just two of them – promote countermeasures and ensure prevention or mitigation of threats to public health. Very few projects were oriented towards translating basic research into actionable practices, although basic research was heavily emphasized owing to NSF's large research budget compared to the other participating agencies (Shelton et al. 2012).

Conclusion

Terror attacks involving biological toxins are low-probability events. The consequences, however, can range from minimal, with only a handful of individuals being affected, to severe, with large numbers of patients putting significant demand on limited health care resources. The relative ease and low cost of acquiring toxins or precursor materials make them attractive alternatives to nuclear or chemical weapons, but several factors (dilution effects, environmental stability, physical security, etc.) make large-scale attacks difficult to successfully execute. Small-scale attacks are more likely, easier to accomplish, and harder to prevent. For example, in 1984 the Rajneeshee cult contaminated salad bars at local restaurants in The Dalles, Oregon, with *Salmonella enterica* Typhimurium. Although there were no fatalities, 751 people contracted salmonellosis, and 45 patients required hospitalization (Miller et al. 2001). The anthrax letters of 2001 involved handwritten letters sent to political leaders and media outlets, and more recently, crude

ricin-containing materials have been sent through the mail in separate incidents to Mayor Bloomberg of New York City and to President Obama and US senator Roger Wicker of Mississippi. While initiatives such as the Select Agent Program have been implemented to make it more difficult for toxin materials and other infectious agents to fall into the hands of those who would use them to harm others, vigilance is still evidently necessary. To that end, Interpol, the International Criminal Police Organization, has implemented a three-pillar strategy to counter the threat of biological terrorism. This strategy includes intelligence analysis – the BioTerrorism Prevention Unit assesses data from member countries on a daily basis and places its findings in the CBRNE intelligence report. The second pillar involves training courses which include regional workshops and train-the-trainer sessions, international tabletop exercises, a First Response to Biological Incidents Course, and an Operational Response to Biological Incidents Course. The third pillar consists of operational support – Interpol can deploy an Incident Response Team to support law enforcement, conduct searches of Interpol's databases as needed, and provide strategic and tactical analytical expertise on an as-needed basis (Interpol's CBRNE Terrorism Prevention Programme [Internet] [cited 2 Apr 2014]). Additionally, the Department of State and the Department of Defense operate collaborative programs with international partners in an effort to reduce the threat of biological terrorism.

The public health system must maintain and continue to improve its capabilities to prepare for and respond to events, including toxin terrorism attacks, which threaten the nation. Trends show improvement over the past few years as the federal and state governments have made health security a high priority, but work remains to be done. Frontline health care workers, including physicians, nurses, and other allied health professionals, should be well trained to recognize the signs and symptoms of toxin intoxication and to suspect a deliberate attack when they see multiple patients presenting similar syndromes. Open communication with public health authorities will ensure that when necessary, epidemiological investigations can be initiated and appropriate measures taken to limit the spread of illness. The development of improved surveillance methodologies and detection technologies will continue to aid officials in the recognition of outbreaks and allow them to begin mitigating the effects of the attack. Collaborative relationships between federal agencies and partners in industry and academia facilitate the development of tools to prevent, detect, and respond to biological threat events. For instance, the Department of Defense funds the development of field-deployable platforms for the detection of bacteria, viruses, and toxins. While these primarily have military applications, they may also be employed for civilian purposes, such as in mail-screening facilities. The Department of Homeland Security, in collaboration with CDC, supports the development of molecular and immunoassays that can be used in LRN laboratories to identify and characterize threat agents and lateral flow assays that can be used in the field by first responders to identify or rule out suspected threat materials in environmental samples. NIH research programs support the development of medical countermeasures including vaccines and chimeric antibody-based therapies.

With multiple ongoing efforts in prevention, protection, surveillance, detection, and response-related activities, the nation is still susceptible to biological attacks. However, we are better prepared to handle such attacks more expeditiously with rapid detection, effective public health response and timely restoration, remediation, and a return to normalcy while minimizing the public health consequences and economic impact.

Cross-References

- ▶ Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment
- ▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
- ▶ Biotoxins and Food Safety
- ▶ Botulinum Toxin: Present Knowledge and Threats
- ▶ The Biowarfare Agent Ricin

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Challenges in Developing Biotoxin Inhibitors

16

Gyanendra Kumar

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Abstract

Biological toxins, which are produced by microorganisms, plants, or animals, cause serious illnesses in humans. Some of these toxins, for example, botulinum neurotoxin, are potential agents of biological warfare and are also increasingly being used for therapeutic and cosmetic purposes. Despite the potential damage they could cause to human health, no drugs are currently available for post-intoxication therapy. Recent progress in drug discovery efforts for botulinum neurotoxins and unmet challenges are discussed. Aided with the crystal structures of apo- and cognate substrate-bound catalytic domain, a number of peptide and small-molecule non-peptide inhibitors have been identified for various serotypes, and pharmacophore models are emerging. But there is limited success in translating these inhibitors into active compounds in cellular assays and

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in vivo models. Factors such as the unusually large surface area of the toxin-substrate interaction interface, the flexible loops lining the active site, involvement of a metal ion in the catalytic activity, and molten globule form of the enzyme at physiological temperature contribute to this challenge and are discussed.

Introduction

Biological toxins are the most poisonous toxins known to man. These toxins are called "biological" because they have a biological origin, i.e., they are produced by microorganisms, animals, or plants. Some are made of proteins ranging in size from small (10–40 amino acids) peptides (e.g., conotoxins from cone snails) to large (50–150 kDa) protein complexes [e.g., botulinum neurotoxins (BoNTs)]. Conotoxins typically have one or more disulfide bonds, and they act in a variety of mechanisms, for example, modulating ion channel activity. Certain macromolecular toxins such as abrin and ricin act by disrupting protein synthesis. Certain other macromolecular toxins such as tetanus and BoNTs act by disrupting the release of neurotransmitters at the neuromuscular junctions. All these biological toxins are considered potential weapons in biological warfare. Development of potent inhibitors against biological toxins for post-intoxication therapy remains a challenge. This chapter is focused on the challenges of developing inhibitors for macromolecular biological toxins. These toxins are difficult to target because of a number of reasons, e.g., their substrates are other proteins and protein-protein interfaces are more difficult to target compared to protein-small-molecule interfaces; they exhibit conformational plasticity; and they have exceptionally large and shallow substrate-binding interfaces. These will be elaborated with the example of BoNT.

BoNT, which is produced by the bacterium *Clostridium botulinum*, is the most poisonous biological toxin identified thus far; the lethal dose is nanogram quantities. BoNT is considered a potential biological weapon because of the ease with which it can be produced. It is also being increasingly used for medical and cosmetic purposes; therefore, the possibility of intended or accidental overdose is also increasing. A vaccine to elicit an immune response against BoNT and neutralize it before it is internalized by neurons would be useful; but in a recent set back, a pentavalent BoNT vaccine administered to at-risk workers was discontinued by the US Centers of Disease Control after diminished potency and side effects were reported (Webb and Smith 2013). The antigenic variability among the seven BoNT serotypes (i.e., BoNT/A–G) has also hindered efforts to develop a pan-active vaccine.

BoNT belongs to a family of clostridial neurotoxins which includes tetanus neurotoxin, though their sites and mechanisms of action differ. BoNT causes flaccid paralysis by disrupting the release of acetylcholine from cholinergic neurons at the neuromuscular junction, whereas tetanus neurotoxin disrupts the release of gamma-aminobutyric acid and glycine from the inhibitory neurons of the spinal cord,

causing spastic paralysis. Among the seven serotypes of BoNT, BoNT/A, B, E, F, and possibly G cause toxicity in humans, BoNT/C in birds, and BoNT/D in animals.

Structure of BoNTs

BoNTs consist of a 150-kDa polypeptide chain that gets proteolytically cleaved into two chains – a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) – though the two chains remain connected via a disulfide bond. The HC consists of a C-terminal (H_C) binding domain and an N-terminal (H_N) translocation domain that helps translocate the catalytic domain. The H_C-binding domain has two subdomains, the N-terminal half (H_{CN}), which belongs to a Concanavalin-A-like lectin superfamily, and the C-terminal half (H_{CC}), which is the receptor-binding subdomain that recognizes the surface receptors on the presynaptic terminal (Montecucco and Schiavo 1995; Krieglstein et al. 1994; Sagane et al. 1999). The LC has zinc-dependent endopeptidase activity that cleaves soluble NSF attachment protein receptor SNARE (NSF attachment protein receptor) proteins, thereby disrupting acetylcholine release (Fig. 1) (Lacy et al. 1998). Each serotype of BoNT has a specific protein target(s) in the SNARE complex and a specific peptide bond that it cleaves. BoNT/A and E cleave SNAP-25; BoNT/B, D, F, and G cleave VAMP; and BoNT/C cleaves syntaxin and SNAP-25.

BoNT's mechanism of toxicity can be summarized as a four-step process: (1) cell binding, (2) internalization, (3) membrane translocation, and (4) target modification (Finkelstein 1990; Hambleton 1992; Montecucco and Schiavo 1994; Montecucco et al. 1996). In principle, each of these steps can be targeted for therapeutic intervention, but, the symptoms of botulism appear only at step 4, i.e., the catalytic domain has initiated its endopeptidase activity, cleaving the proteins of the SNARE complex, and ultimately blocking neurotransmitter release. Hence, for postexposure drug development, the endopeptidase activity is considered the most viable target and has been pursued as such in recent years.

The Catalytic Domain of BoNT as a Drug Target

The catalytic domain of BoNT has been studied extensively using biochemical, mutagenesis, and biophysical approaches such as X-ray crystallography. The atomic structure of the domain has been determined for all serotypes. The catalytic domain of BoNT, as well as that of tetanus neurotoxin, is a zinc-dependent endopeptidase similar to thermolysin. The Zn(II) cofactor is held in place at the active site by a conserved HExxH + E motif, which is found in all serotypes and is coordinated by the side chains of two histidines, a glutamate, and a conserved water molecule that acts as a nucleophile. The water molecule also forms a hydrogen bond with the first glutamate of the zinc-binding motif. The glutamate acts as the general base in the catalytic mechanism of the enzyme. Four flexible loops (50/60 loop, 170 loop, 250 loop, and 370 loop) line the active site and participate in

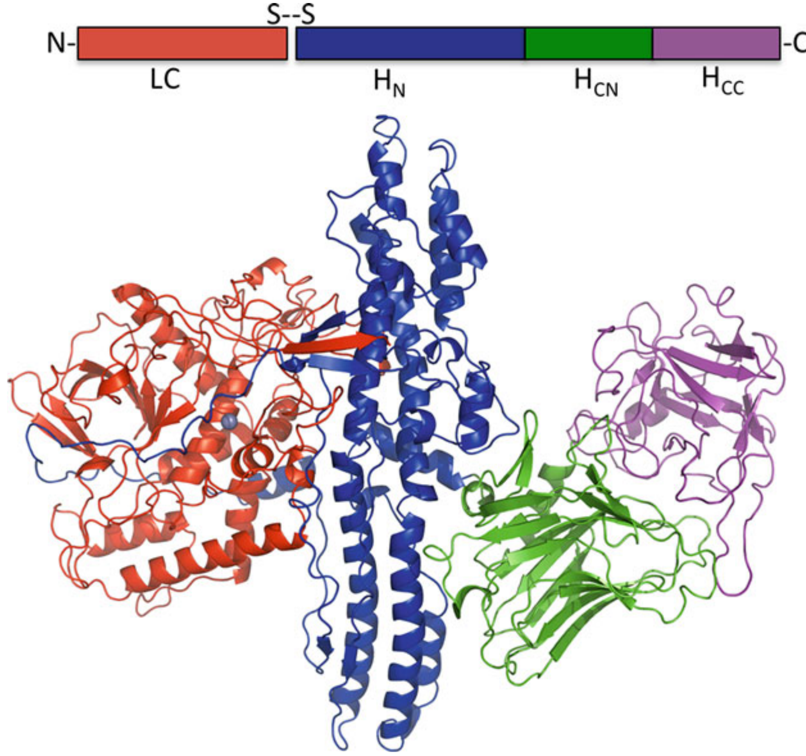

Fig. 1 Domain organization of BoNT/A. The catalytic domain (LC) is shown in *red*; the translocation domain (H_N) in *blue*; the N- and C-terminal subdomains of the binding domain (H_C) in *green* and *violet*, respectively; and zinc is shown as *gray* sphere in the catalytic domain

substrate binding. Although the core structure of the BoNT catalytic domain is rigid across serotypes, the loops on the surface show substantial flexibility. Figure 2 shows the extent of variation in the structures of the corresponding loops across serotypes. The flexibility in these loops within a serotype poses a challenge in designing inhibitors, and it becomes even more challenging to design a pan-active inhibitor.

Variations in the amino acid sequences at the active site across serotypes cause subtle changes in the organization of the active site and result in different serotypes cleaving different proteins of the SNARE assembly or cleaving different regions of the same substrate protein. The implication is that compounds designed to inhibit one serotype generally do not inhibit the other serotypes. It would be a herculean task to design an inhibitor that inhibits all seven serotypes. Hence, efforts thus far have been toward specific serotypes. Of the seven serotypes, BoNT/A, B, and E infect humans, and of those, BoNT/A is the most lethal and frequent source of infection. Therefore, much of the work toward developing BoNT inhibitors has been targeted against BoNT/A.

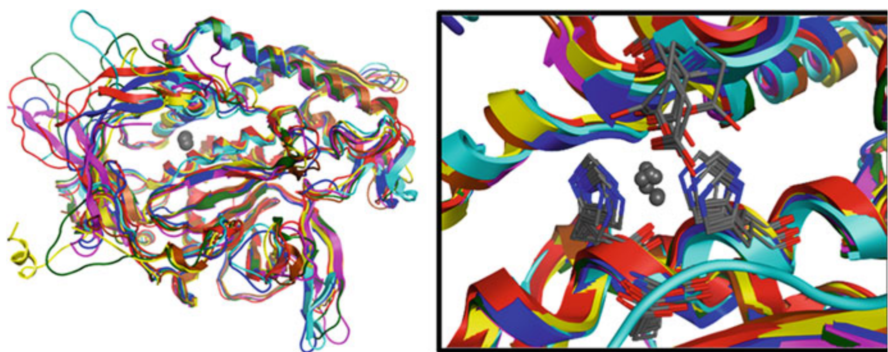

Fig. 2 Illustrations of superimposed protease domains of the seven serotypes of BoNT. On the *left*, the serotypes of BoNT/A–G are shown, and the corresponding catalytic zinc ions are shown as *gray spheres*. On the *right*, the stick and ball model of the conserved HExxH + E motif at the active site is shown; residues are shown as *sticks*, and zinc ions are shown as *balls*. Color scheme of BoNT serotypes: A, *red*, B, *green*, C, *blue*, D, *cyan*, E, *yellow*, F, *brown*, and G, *magenta*

Peptide Inhibitors of BoNT/A

Based on knowledge of the atomic structure and mechanism of action of the BoNT catalytic domain, a number of peptide or peptide-based inhibitors have been designed and tested. Crystal structures of the native BoNT/A catalytic domain and that of an inactive double mutant (E224Q and Y366F) of BoNT/A in complex with a SNAP-25 peptide (141–204) designated as sn2 have been determined that provide crucial information about the interactions between the enzyme and the substrate (Breidenbach and Brunger 2004). The structure of the double mutant in complex with the sn2 peptide provided important insights into the substrate recognition mechanism of BoNT/A. The peptide wraps around the BoNT/A catalytic domain burying ~4,840 $Å^2$ of surface area. SNAP-25 is a contiguous helix as part of the SNARE assembly, but bound with the enzyme, the conformation changes drastically leaving a distorted α-helix at the N-terminal, a small distorted β-strand toward the C-terminal, and the remaining was extended. The sites where these secondary structures of sn2 made contact with the enzyme were designated as α-exosite and β-exosite, respectively. While α-exosite was implicated in substrate recognition by the enzyme, the β-exosite was implicated in assisting the catalytic activity by making contacts with 370 loop. Although, this complex provides the details of the overall interaction of BoNT/A catalytic domain with the substrate, the finer details at the active site were missing because of the mutations at the active site. A crystal structure of enzymatically active BoNT/A catalytic domain in complex with uncleavable SNAP-25 peptide 197-QRATKM-202 has also been determined. The peptide makes interactions with residues of 160, 200, 250, ad 370 loops. Most importantly, the P1 residue Gln197 displaces the nucleophilic water, and its main chain amino and carboxy groups chelate the catalytic zinc. P1′ residue Arg198 occupies the S1′ site formed

Table 1 Peptide and peptidomimetic inhibitors of BoNT/A protease activity

Peptide/mimetic	Inhibition	PDB id	References
QRATKM	$IC_{50} = 133\ \mu M$	3DDA	(Kumaran et al. 2008a)
RRATKM	$IC_{50} = 95\ \mu M$	3DDB	(Kumaran et al. 2008a)
RRGC	$IC_{50} = 1.5\ \mu M$	3C88	(Kumaran et al. 2008b)
RRGF	$IC_{50} = 0.9\ \mu M$	3QW5	(Kumar et al. 2012b)
CRGF	$IC_{50} = 1.5\ \mu M$	n.a.	(Kumar et al. 2012b)
RRFC	$IC_{50} = 1.8\ \mu M$	3QW7	(Kumar et al. 2012b)
N-Ac-CRATKML	$K_i = 2\ \mu M$	3BOO	(Silvaggi et al. 2008)
DNP-DAB-RWT-*DAB*-ML	$K_i = 41$ nM	3DS9	(Zuniga et al. 2008)
RR-N*ph*-*Aib*-AML	$K_i = 314$ nM	n.a.	(Zuniga et al. 2010)

Abbreviations: IC_{50} concentration of inhibitor that decreases peptide activity by 50 %, *Ki* inhibition constant, *n.a.* not applicable, *PDB id* identification number in the protein data bank

by R363, T220, D370, T215, I161, F163, and F194; it also forms a salt bridge with D370 (Kumaran et al. 2008a). These structures provide a solid basis for designing substrate peptide-based inhibitors.

The minimal cleavable segment of SNAP-25 for BoNT/A is 192-DEANQ/RATK-200, where the scissile bond is located between Q and R (Chen and Barbieri 2006). Hence, several peptide inhibitors shorter than this have been tested, and cocrystal structures for many of them have been determined (Table 1). Available data indicate that among peptide inhibitors, a tetrapeptide is the smallest peptide with the highest inhibitory activity. Further reductions in size drastically decreases their inhibitory potency (Kumar et al. 2012b; Kumaran et al. 2008b; Hale et al. 2011). These tetrapeptides correspond to the P1-P1′-P2′-P3′ stretch of the SNAP-25 substrate protein. An arginine is the preferred residue at P1 and P1′ position, and a hydrophobic/aromatic residue works better at P3′ position. Several of these tetrapeptides also enter neuronal cells without any adverse effect on metabolic functions, as measured by ATP production and P-38 phosphorylation (Hale et al. 2011). Among the modified peptides and peptidomimetics, *DNP-DAB*-RWT-*DAB*-ML, where *DNP-DAB* is 4-(2,4-dinitrophenylamino)-2-amino-butanoic acid and *DAB* is 2,4-diaminobutanoic acid, is the most potent inhibitor, with an inhibition constant (K_i) of 41 nM (Zuniga et al. 2008). The peptidomimetic inhibitors bind at the active site in a manner that is substantially different from that of the peptide inhibitors. Unlike the extended conformation of the substrate or the tetrapeptide inhibitors (e.g., RRGF), the P2′-P5′ residues of the peptidomimetic inhibitor *DNP-DAB*-RWT-*DAB*-ML adopt a 3_{10} helical conformation when bound at the BoNT/A active site (Fig. 3). Significant conformational changes also take place in the 60, 250, and 370 loops upon binding of the inhibitor, but the conformation of the zinc-coordinating residues in the active site remains the same. These studies, put together, provide new lead molecules for anti-botulism drug discovery and also provide a great structural insight into the BoNT/A active site.

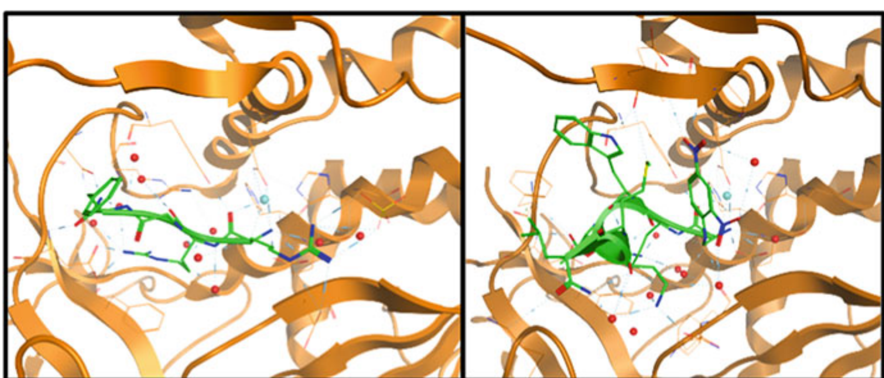

Fig. 3 Peptide inhibitors of BoNT/A. On the *left*, RRGF in extended conformation, and on the *right*, *DNP-DAB*-RWT-*DAB*-ML in helix form. Peptides are shown in *green*; water molecules within a 3.5-Å radius are shown in *red*; and zinc is shown in *cyan* (PDB id: 3BTA)

Small-Molecule Non-peptide Inhibitors of BoNT/A

A number of small-molecule non-peptide inhibitors (SMNPIs) of BoNT/A have been identified by using structure-based drug discovery, virtual screening, and pharmacophore modeling (Fig. 4). A few high-throughput inhibition assays have also been devised to speed up the screening of compound libraries against BoNT/A (Schmidt and Stafford 2003; Hines et al. 2008; Boldt et al. 2006a; Feltrup and Singh 2012). Using in situ lead identification chemistry, Boldt et al. started with a 15 μM lead compound and synthesized an inhibitor *ortho-para*-cinnamic hydroxamate with a K_i of 0.3 μM (Boldt et al. 2006b). Subsequently, the cocrystal structure of BoNT/A catalytic domain in complex with this inhibitor was determined, providing details of the interactions between the inhibitor and the enzyme active site (Silvaggi et al. 2007). Substantial structural rearrangements affecting the electrostatic environment were observed in the S1′ pocket. Inspired by the metalloprotease inhibitor drug captopril, the peptide-based inhibitor N-Ac-CRATKML, and *ortho-para*-cinnamic hydroxamate, Moe et al. synthesized several mercaptoacetamides that showed low micromolar inhibition activity, and these compounds were also active in cellular assays (Moe et al. 2009). In another study, Li et al. modified an initial-hit compound from the National Cancer Institute (NCI) library into a more potent inhibitor of BoNT/A endopeptidase activity with an IC_{50} of 2.5 μM (Li et al. 2010). Opsenica et al. synthesized some 1,7-bis(alkylamino)diazachrysene-based compounds that showed low micromolar IC_{50} values (Opsenica et al. 2011). These compounds also inhibited targets in the malaria parasite *P. falciparum* with nanomolar IC_{50} values and Ebola virus with IC_{50} values in the low micromolar range (Hermone et al. 2008; Opsenica et al. 2011). Using a high-throughput assay, Cai et al. screened a small-molecule library of 16,544 compounds and identified several hits with a K_i of 9 μM for their most potent inhibitor that also showed inhibition in cellular assays with no apparent toxicity (Cai et al. 2010).

Fig. 4 Chemical structures and inhibitory potencies of some SMNPIs of BoNT/A protease activity

PanThera Biopharma (Aiea, HI) has designed and tested a number of diverse compounds with hydroxamic acid as the zinc-chelating group. Their most potent compound, PT1, has an IC_{50} of 4.6 μM. More importantly, the cocrystal structures of these inhibitors in complex with BoNT/A showed significant movement in the 60/70 loop upon binding, implicating an induced-fit mechanism (Thompson et al. 2011). In a neuron-based assay, Ruthel et al. tested some compounds from NCI's open repository and identified NSC95654 with a K_i of 1.8 μM against purified enzyme and inhibitory activity in chick motor neurons (Ruthel et al. 2011). Recently, a couple of covalent inhibitors of BoNT/A have also been identified. These inhibitors, MTSEA (2-aminoethyl methanethiosulfonate) and MTSPA (3-aminopropyl methanethiosulfonate hydrobromide), attack C165, which lies in close vicinity to the catalytic zinc, and covalently modify its side chain. The better of these two inhibits BoNT/A protease activity with a K_i of 7.7 μM that improves further with longer incubation (Stura et al. 2012). Although the research community is generally skeptical about covalent drugs, several are available for various indications (Singh et al. 2011). Overall, a number of SMNPIs have been discovered for BoNT/A inhibition, with many of them showing activity in cellular assays. The structure-activity relationship studies and the enzyme-inhibitor interaction patterns provide insights for anti-botulism drug discovery.

A Pharmacophore Model of BoNT/A

Based on the peptide inhibitors and SMNPIs, pharmacophore models have been developed for the inhibition of BoNT/A endopeptidase activity. The initial pharmacophore model based on structure-activity relationship models of SMNPIs had two planar zones connected back to back, one of which had an H-bond donor or acceptor; two cations at the outer ends of the planar zones; a third zone that could be aromatic, aliphatic, and cationic extending from one of the cations; and a fourth zone that was later added to include indoles, phenyls, or pyridines (Burnett et al. 2010). In another pharmacophore model developed recently on the basis of cocrystal structures of the peptide inhibitors in complex with BoNT/A catalytic domain, five common features emerged: (1) metal chelators; (2) two positive ion features, one of which also involves π-stacking interaction; (3) an H-bond acceptor; (4) an H-bond donor/acceptor pair; and (5) an hydrophobic/aromatic feature (Kumar et al. 2012b). While the previous pharmacophore model was solely based on superimposition of SMNPIs and their SAR, the later one is based on specific interactions observed in cocrystal structures of peptide inhibitors in complex with the BoNT/A catalytic domain. The later model also provides an structural explanation for each pharmacophore feature. The metal chelators could be carboxy or amine group of the peptide bond in case of peptide-based inhibitors, and they could also be replaced with other metal-chelating groups like hydroxamates. The two positively charged ion features make salt bridge interactions with the side chains of E164 and D370. The later one could also have π-stacking interaction with R363 and F194. The H-bond acceptor feature makes an H-bond with the guanidinium group of R363. The H-bond acceptor-donor pair is involved in H-bond interaction with main chain NH of D370 and the side chain of N368. The hydrophobic/aromatic feature is required to complement the hydrophobic pocket created by Y250, Y251, L256, F369, and F423. The two pharmacophore models are illustrated in Fig. 5. Together, these pharmacophore models provide insights into the inhibition mechanism of BoNT/A endopeptidase activity and should be useful in screening compound libraries and identifying new inhibitors.

Inhibitors of BoNT/B

Among the serotypes that affect humans, BoNT/B is the second most frequently diagnosed serotype responsible for botulism. Also, in addition to BoNT/A, BoNT/B is the only other serotype used for therapeutic purposes. Although the former has been studied extensively as part of ongoing inhibitor design efforts, the latter has received relatively lesser attention. Among the few inhibitors discovered so far, the SMNPI – 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin, also known as ICD 1578 – was identified with an IC_{50} of 27.6 μM based on its similarity with an analog of phosphoramidon, which is a metalloprotease inhibitor (Adler et al. 1998). Using a combinatorial chemistry approach followed by replacement of amino acids with unnatural amino acids, Anne et al. developed a highly potent

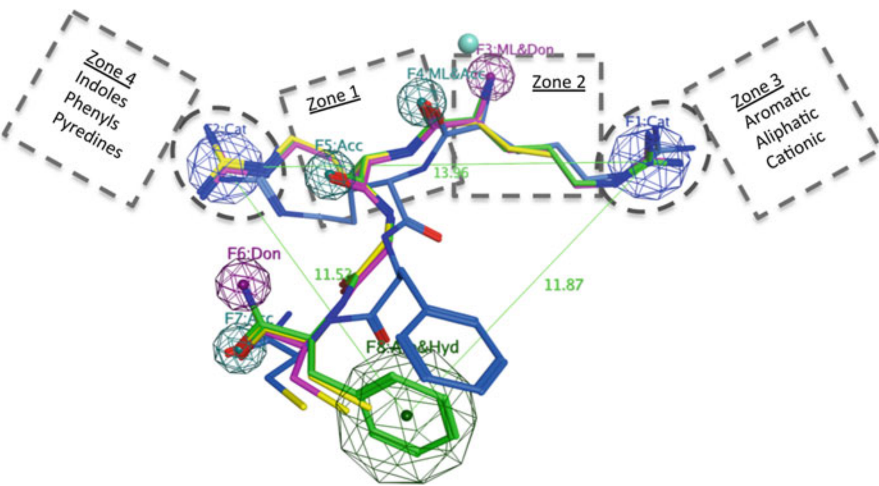

Fig. 5 Pharmacophore models of BoNT/A inhibition. The model developed from cocrystal structures of peptide inhibitors in complex with BoNT/A is shown in color and that developed from SMNPIs is shown in *gray*. Zinc is shown as a *solid cyan sphere*

ICD 1578, IC_{50}= 27.6 μM
Adler et al. FEBS Letters 1998

Peptidomimetic Compound 35
K_i= 20 nM, Anne at al. JMC 2003

Fig. 6 Chemical structures and inhibitory potencies of BoNT/B inhibitors

inhibitor with a K_i of 20 nM (Fig. 6) (Anne et al. 2003b). The investigators also designed and tested several of its analogs, which had slightly better inhibitory activities (Anne et al. 2003a).

Inhibitors of BoNT/E

Although serotype E is the fastest acting BoNT, very little work has been done to identify its inhibitors. Only two inhibitors have been identified so far: one is a peptide inhibitor, and the other is an SMNPI. The tetrapeptide inhibitor RIME was designed on the basis of the amino acid sequence of the substrate protein SNAP-25 that is recognized and cleaved by serotype E. This substrate-based peptide RIME

represents the stretch 180–183 (P1-P3′) of SNAP-25 sequence and inhibits BoNT/E endopeptidase activity with a K_i of 69 μM in a competitive manner with respect to substrate peptide. The cocrystal structure of RIME in complex with BoNT/E protease domain shows that the peptide binds at the active site but escapes cleavage of the scissile bond between Arg180 and Ile181. It makes a number of hydrogen bond and van der Waal interactions from sites S1 to S3′ (Agarwal and Swaminathan 2008). Arg180 displaces the nucleophilic water, its carbonyl oxygen chelates the catalytic zinc, and it is within hydrogen bonding distance from the hydroxyl groups of F350 which is a conserved residue in all BoNT serotypes. The main chain amino group of Arg180 forms a hydrogen bond with E212 side chain. The side chain guanidinium group forms a salt bridge with E158. The hydrophobic side chain of Ile181 nestles in the S1′ pocket created by hydrophobic residues T159, F191, and T208. The Met182 side chain is also accommodated in a hydrophobic pocket lined by residues F191, Y354, and Y356. Glu183 interacts with the 250 loop of BoNT/E and stabilizes it in one conformation. Due to the inherent flexibility in this loop, it could not be modeled in the previously determined apo structure of BoNT/E catalytic domain (Agarwal et al. 2004). This study provided the atomic details of protein-ligand interactions at the BoNT/E active site and opens an avenue for structure-based drug discovery. The first SMNPI was identified recently using a structure-based virtual-screening method. Compounds in the Diversity set I of NCI database were docked against the BoNT/E active site, and 18 compounds were shortlisted based on their binding energies and binding modes. Many of these 18 compounds showed inhibitory activity in low micromolar concentrations, and NSC-77053 (2-(9H-fluoren-2-ylcarbonyl)benzoic acid) was identified as the top hit with a K_i of 1.29 μM in an HPLC-based inhibition assay (Kumar et al. 2012a). Comparison of the cocrystal structure of RIME-BoNT/E complex and docked NSC-77053 showed that many interactions observed in the former complex are also seen in the latter. The carboxy group of the benzoic moiety of NSC-77053 coordinated the catalytic zinc and the benzene ring nestled in the hydrophobic S1′ site. The hydrophobic/aromatic fluorene moiety extended into the same hydrophobic pocket created by F191, Y354, and Y356 in which Met182 also binds. In addition, the C=O linker group could also have a favorable interaction with the guanidinium group of R347. These common interactions found in case of peptide inhibitor as well as an SMNPI may be exploited in developing a pharmacophore model for BoNT/E and identifying new inhibitors (Fig. 7).

Exosite Inhibitors

The substrate of BoNT/A, SNAP-25, wraps around the globular enzyme, creating an extensive network of interactions similar to the belt region that wraps around the catalytic domain in full-length BoNT/A (Fig. 1). In this BoNT/A-SNAP-25 complex, the N-terminal residues of the substrate protein (147–167) form an α-helix and interact on the rear side of BoNT/A, and the C-terminal residues (201–204) form a distorted β-strand, and the spanning residues are a random coil (Fig. 8; Breidenbach

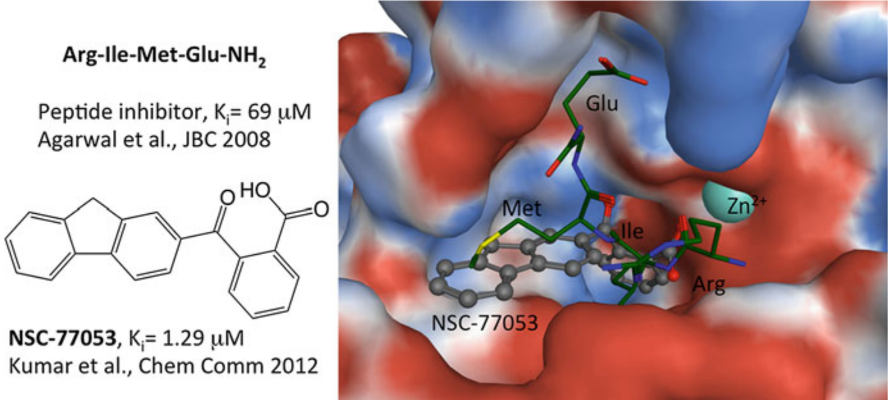

Fig. 7 BoNT/E inhibitors. On the *left*, the sequence of peptide inhibitor and structure of a SMNPI is shown. On the *right*, BoNT/E is shown in surface representation, *blue* is the +ve charge, and *red* is the –ve charge. RIME complex from pdb id: 3D3X and NSC-77053 was docked using AutoDock program

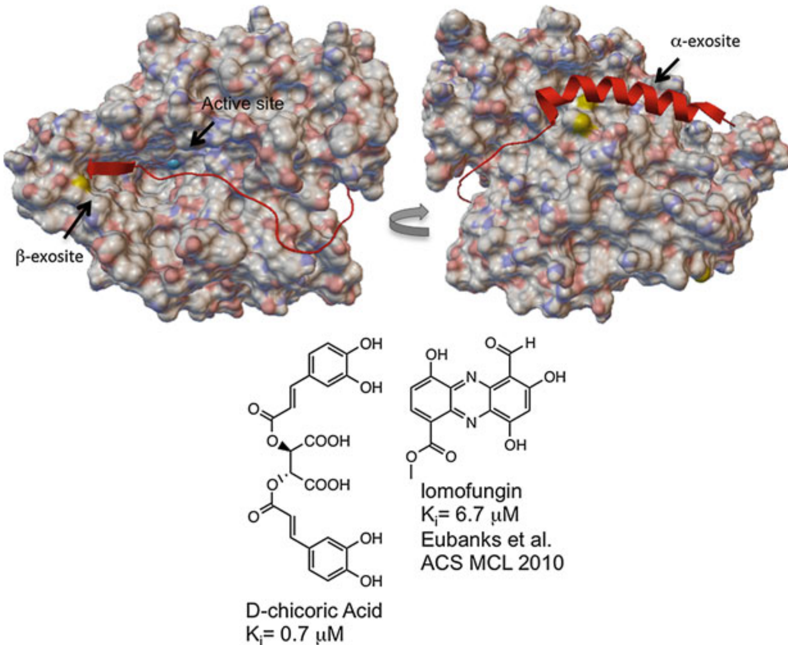

Fig. 8 Exosite inhibitors of BoNT/A and BoNT/B. In the upper panel, the BoNT/A catalytic domain is shown in complex with its substrate SNAP-25 (PDB id: 1XTG). BoNT/A is shown in surface representation; zinc is shown as a *cyan sphere*; and SNAP-25 is shown as a *red ribbon* (front and back views showing zinc-centered active site, the β-exosite and the α-exosite). Lower panel shows the structures of the two natural-product inhibitors that bind at the exosites.

and Brunger 2004). The regions on BoNT/A where these secondary structural elements bind have been termed the α- and β-exosites, respectively. These exosites are crucial in substrate recognition and cleavage (Chen and Barbieri 2006). Hence, they represent additional targets for developing inhibitors. A couple of natural-product, small-molecule inhibitors have been identified for BoNT/A and BoNT/B that target these exosites located away from the zinc-dependent catalytic center. Silhar et al. tested a number of phenolic compounds isolated from *Echinacea* plants based on their similarity with some known hydroxamate inhibitors discussed earlier. The most potent of these natural compounds, chicoric acid, inhibits BoNT/A activity with a K_i of 0.7 μM and synergistically improves the overall inhibition of BoNT/A activity in concert with an active-site inhibitor such as *ortho-para*-cinnamic hydroxamate (Silhar et al. 2010). In a subsequent high-throughput screening of drugs against BoNT/A, a new natural-product inhibitor, lomofungin, was identified with a K_i of 6.7 μM. This compound synergistically inhibited the catalytic activity of BoNT/A with *ortho-para*-cinnamic hydroxamate and chicoric acid (Eubanks et al. 2010). Chicoric acid also inhibited BoNT/B endopeptidase activity with an IC_{50} of 7.5 μM through a mechanism similar to that observed in BoNT/A, by binding far away from the active site, in a region where the SNARE motif of substrate peptide interacts with the protease enzyme (Salzameda et al. 2011).

Conclusion and Future Directions

Although a clear threat to human health, biological toxins have not attracted much attention from the pharmaceutical industry; this area has been researched mostly by government agencies and academic institutions. A tremendous amount of work has been undertaken to decipher the structure and mechanism of action for the most potent biological toxin, BoNT. Drug discovery studies are targeting the BoNT catalytic domain by substrate-based peptide inhibitors, peptidomimetic inhibitors, and SMNPIs. A number of potent peptide-based inhibitors and SMNPIs have been identified. But, given the large surface area across which the BoNT catalytic domain interacts with its substrate, designing inhibitors small enough to meet Lipinski's rule of five and have high affinity for the enzyme is challenging. Although, the large interaction interface also provides opportunities to design inhibitors that may not directly target the active site but still inhibit substrate binding and thus cleavage. A few noncompetitive inhibitors have been identified that do not directly interact with the active site but still inhibit BoNT catalytic activity. They are believed to target the exosites.

The active site of the BoNT catalytic domain is lined by several flexible loops. This flexibility brings a new challenge to docking-based virtual screening to identify inhibitors. A comparison of the native crystal structures with the inhibitor-bound structures shows that the loops surrounding the active site are significantly rearranged upon binding of inhibitors (Thompson et al. 2011). These induced changes are difficult to simulate in virtual screening because most virtual-

screening protocols use a rigid receptor. Molecular dynamics simulations can be used to simulate changes induced by inhibitor binding, but they are computationally intensive simulations, and it would be impractical to use such simulations for virtual screening.

Another issue that must be faced in computer-aided drug discovery against BoNT is the presence of a catalytic zinc ion at the active site. The interactions between zinc and the atoms at the active site or the zinc-chelating inhibitors are partially covalent, and the parameters for this kind of interaction are not yet fully optimized for docking simulations. Although docking simulations have been successfully used to identify inhibitors against BoNT (Kumar et al. 2012a), better force field parameters for simulating interactions involving metal ions in the active site would be useful.

Sometimes protein-protein and protein-ligand complexes involve interactions mediated by water molecules. In case of BoNTs, such structurally conserved water molecules have been found to mediate interactions between the enzyme and the peptide ligands. Including these conserved water molecules as part of the receptor during a docking simulation was found to improve the docked pose in terms of root-mean-square deviation when compared to a cocrystallized peptide inhibitor – BoNT/A complex (Kumar et al. 2012b). So, inclusion of these conserved water molecules as part of the receptor during a virtual-screening protocol may also help in identifying new inhibitors.

In addition to challenges mentioned above, BoNTs also pose another level of difficulty in inhibitor design. They appear to adopt a molten globule form at physiological temperature (Kumar et al. 2014). This means that the structures determined using X-ray crystallography may not represent the true dynamic physiological form of BoNT accurately, and this may be a contributing factor in BoNT crystal structure-based inhibitor design being not as effective as one would expect. But, inhibition assays are conducted at physiological temperature and thus structure-activity relationships deduced from the experimental data on BoNT inhibition should be able to provide structure-activity relationship and highlight pharmacophore features important for BoNT inhibition. From the peptide inhibitors and SMNPIs identified so far, a pharmacophore model is emerging for the inhibition of BoNT/A endopeptidase activity. This model could be used to identify new inhibitor scaffolds. Similarly, pharmacophore models could also be developed for other serotypes of BoNT and used for virtual screening.

The next challenge is to translate these results from in vitro to ex vivo and then to in vivo animal models of Botulism. There are very few studies where inhibitors active against purified enzyme have also been found active in cellular assays and even fewer that show any efficacy in animal models. While some tetrapeptide inhibitors have been found to show activity in neuronal cell cultures (Hale et al. 2011), for some other very potent peptidomimetic inhibitors of in vitro BoNT protease activity, there is no data on their efficacy in cellular or animal models (Anne et al. 2003a; Zuniga et al. 2008). In case of SMNPIs, there also seems to be a disconnect among in vitro activity, cellular activity, and in vivo efficacy as

was found in case of 2,4-dichlorocinnamic hydroxamic acid. This compound was found to inhibit protease activity of purified BoNT with a K_i of 300 nM, shows cytotoxicity in neuroblastoma cells at 5 μM, and shows no reduction in SNAP-25 cleavage at lower concentrations. But this compound at a concentration of 1 mM was able to save 16 % of the mice from symptoms of botulism without causing any apparent toxicity (Eubanks et al. 2007). In summary, significant progress has been made in structural elucidation of the most poisonous biological toxin – the BoNTs and their mechanism of action. Novel inhibitors of protease activity of various BoNT serotypes have been identified. Developing these serotype-specific inhibitors into pan-active inhibitors remains a challenge and so does indication in animal models.

Cross-References

► Antidotes to Botulinum Neurotoxin
► Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
► Botulinum Toxin: Present Knowledge and Threats
► Counterfeit Botulinum Medical Products and the Risk of Bioterrorism

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Antidotes to Botulinum Neurotoxin

17

Baskaran Thyagarajan

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Abstract

Botulinum neurotoxins (BoNTs) are the most potent neurotoxins and are potential biothreat agents. All seven serotypes of BoNTs (/A through/G) consist of a 100 kDa heavy chain (Hc) and a 50 kDa light chain (Lc) linked by a single disulfide bond. BoNT Hc binds to its receptors on the neural membrane and sets the stage for endocytosis at the motor nerve terminals (MNTs). Within early endosomes BoNT undergoes pH-dependent separation of the Hc and Lc. The Hc translocates the Lc into the cytosol where BoNTs proteolyze selective SNARE complex proteins resulting in the attenuation of acetylcholine (ACh) release at MNT. This results in a flaccid, long-lasting neuroparalysis of the skeletal muscles. The persistence of BoNT/A, the principal serotype, Lc protease at the MNT accounts for the long-lasting paralysis and the difficulty to develop therapeutics against BoNT/A. The ease of production and dissemination of the neurotoxin makes it as a viable biothreat agent. The quest for antidotes to treat botulism is an ongoing process. Efforts to develop antidotes against BoNTs have also advanced our knowledge about endoexocytic mechanisms that mediate BoNT signaling at the MNT. This review describes the antidotes that have prophylactic and therapeutic potential to counteract BoNTs in cellular and neuromuscular model systems by neutralizing the toxin in the blood, preventing the binding/internalization of the toxin, inhibiting the translocation of the Lc from early endosomes to cytosol, disrupting the catalytic activity of the Lc endoprotease, and/or restoring neurotransmission by increasing presynaptic Ca^{2+}-triggered exocytosis.

Introduction

BoNTs are potent bioweapons due to their extreme potency and lethality. A single gram of crystalline neurotoxin, evenly dispersed and inhaled, can kill more than one million people. The ease of production and dissemination of the neurotoxin accounts for the accidental or intentional misuse of BoNTs. The extensive use of BoNT/A in clinics also presents opportunities for such misuse. BoNT/A, as a bioweapon, could be disseminated via aerosol or by contamination of water or food supplies, causing widespread casualties. The need for prolonged intensive care and ventilator support for the affected patients will impose severe economic burden on our health-care system. Until now, no therapeutic strategies are available to treat BoNT intoxication after the toxin has entered the motor nerve terminals (MNT), the primary site of action of BoNTs. Therefore, developing therapeutic drug strategies to treat botulism is of primary importance. This review mainly focuses on strategies that are currently evaluated to counteract BoNT intoxication. The aim of the review is to provide an overview of the antidotes that are targeted to antagonize BoNTs based on various scientific processes that regulate the uptake and neurobiological signaling and perturbation of the toxin's ability to cause neuroparalysis.

This review by no means discusses all the literature that has been published on botulinum antidotes. However, it specifically discusses the literature that focuses on the development, characterization, and use of antidotes that have prophylactic and therapeutic potential to counteract BoNTs in the cellular and neuromuscular model systems of BoNT intoxication.

BoNT Homology and Structure Function Analysis

The seven serotypes of BoNTs share significantly high structural similarity and sequence homology. Although the functions of various structural domains are very similar for BoNTs, their substrate specificity and cleavage sites for the same substrate (e.g., SNAP-25 for BoNT/A, BoNT/C, and BoNT/E) to cause proteolysis to inhibit exocytosis are different. This unique specificity of BoNTs for selective substrates makes the development of a single antidote to antagonize all serotypes of BoNTs difficult. Therefore, understanding the structural differences that inculcate this specificity to substrates among the seven serotypes of BoNTs would immensely help in the development of potent antidotes to counteract BoNT intoxication.

BoNT Antidotes

The four-step model, binding, uptake, translocation, and catalytic activity inhibition, for BoNT intoxication originally proposed by Simpson (1979) and further evaluated by Montecucco (Montecucco and Schiavo 1994) has stimulated research to develop antidotes that:

(a) Neutralize the toxin in the blood
(b) Prevent the binding/internalization of the toxin
(c) Inhibit the translocation of the toxin's light chain from early endosomes into cytosol
(d) Disrupt the catalytic activity of the toxin endoprotease
(e) Restore neurotransmission by increasing presynaptic Ca^{2+}-triggered exocytosis

Several important findings in the recent decade have uncovered the molecular targets of BoNTs. This advancement has greatly benefitted the investigation for BoNT antidotes. Basically, prophylaxis by vaccines and therapeutics by antitoxins and inhibitors to intervene binding, uptake, endosomal translocation, and catalytic activity of the endoprotease have been the principal countermeasure strategies against botulism. The following section discusses the recent advancements in BoNT antidotes.

Schematic model (Fig. 1) below describes the target of antidotes that are developed to counteract botulism.

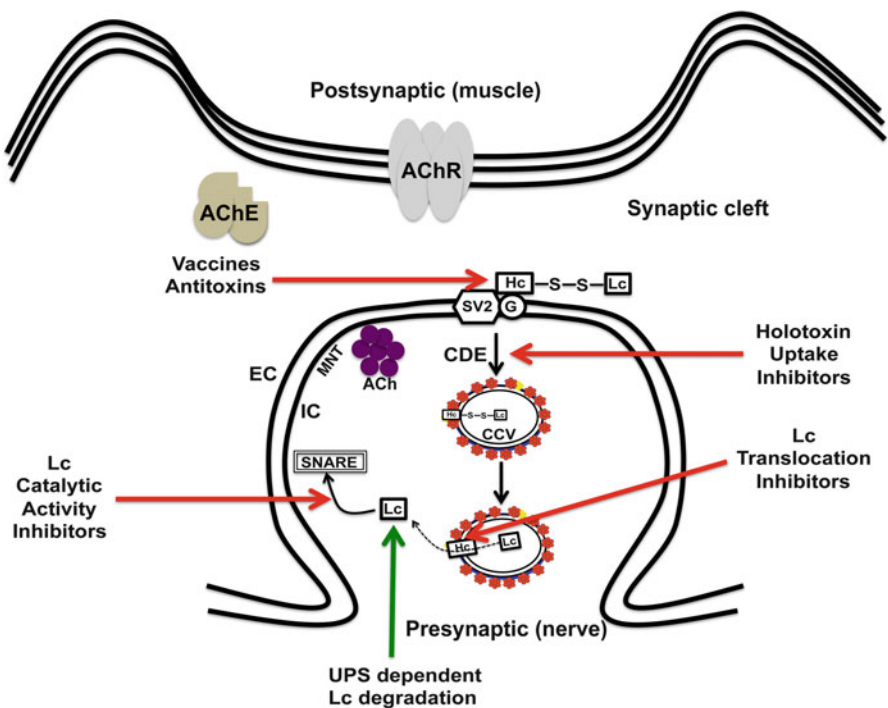

Fig. 1 Site of action of BoNT antidotes: model describing the targets of BoNT antidotes. The postsynaptic muscle, presynaptic nerve, and synaptic cleft architectures are given. Vaccines/antitoxins specifically target and neutralize the neurotoxins in circulation or extracellularly. Holotoxin uptake inhibitors decrease the clathrin-dependent endocytosis of BoNTs. Lc translocation inhibitors abrogate the pH-dependent translocation of Lc metalloendoprotease from the early endosomes. Lc catalytic activity inhibitors inactivate the effects of the Lc endoprotease by binding to catalytic sites and inhibit the cleavage of target SNARE complex proteins. *UPS* ubiquitin proteasome system, *CDE* clathrin-dependent endocytosis, *CCV* clathrin-coated vesicle, *Hc* heavy chain, *Lc* light chain, *Ec* extracellular, *Ic* intracellular, *MNTs* motor nerve terminals, *SV2* synaptic vesicle proteins, *G* gangliosides, *SNARE soluble N-ethylmaleimide*-sensitive factor attachment protein receptor, *AChR* acetylcholine receptor, *AChE* acetylcholinesterase, *ACh* acetylcholine (in vesicles)

Vaccines

Pentavalent Toxoids

Development of active immunity to BoNT is quite slow, and therefore, any attempts to vaccinate individuals must be initiated in advance of an outbreak and would be of little or no benefit after BoNT exposure (Rusnak and Smith 2009). There has been considerable development over last 64 years in efforts to make vaccines against botulinum poisoning. Investigational bivalent toxoids (AB botulinum toxoid) were initially developed over 64 years ago. Crude alum-precipitated bivalent toxoid

against BoNT/A and BoNT/B was developed by the Department of Defense during World War II, which was administered to individuals at risk. These include personnel employed in botulinum research laboratories, BoNT production facilities, public health laboratories involved in botulinum outbreak investigations, or deployed military personnel in regions where there is a risk of exposure to bioterrorist activity (Siegel 1988). However, moderate local reactions such as erythema or swelling and mild limitation of arm motion or minimal axillary adenopathy were seen in 7.5 % of injected population. Research efforts, therefore, were directed toward development of purified botulinum toxoids to improve the clinical efficacy of the toxoids. Subsequently, a pentavalent product derived from *Clostridium* botulinum toxoid vaccine was manufactured by Parke Davis and Co. and later by the Michigan Department of Public Health. To make this toxoid, each of the five serotypes cultures was grown in bulk; toxin was extracted from cell mass, acid precipitated, filtered, and lastly detoxified by 0.6 % formaldehyde addition. Finally, the toxoid was adsorbed onto aluminum phosphate as an adjuvant. Thimerosal was used as a preservative. It is important to note that the lack of cross-reactivity was the basis for designation of the seven distinct serotypes of BoNT (Baldwin et al. 2008) and, in fact, the development of pentavalent or heptavalent (see later) toxoids was possible only because of this property exhibited by BoNTs. The pentavalent (against BoNT/A, BoNT/B, BoNT/C1, BoNT/D, and BoNT/E) vaccine is currently used to immunize personnel at risk, but it is in limited supply. The pentavalent BoNT vaccine was found to have a number of limitations such as high reactogenicity, poor immunogenicity, and the product lacking toxoids for BoNT/F or BoNT/G. From 2011 CDC has stopped issuing pentavalent vaccine for individuals at high risk for exposure to BoNTs (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6042a3.htm). This has led to the search for new generation vaccines for BoNT intoxication.

Recombinant Vaccines

The protective epitopes of the BoNTs are located in the C-terminal receptor-binding domain of the heavy chain (HCR), and these have been used for vaccine development. In comparison to the *Clostridium*-derived vaccine immunogens, recombinant HCRs (rHCRs) can be produced in large scale without neurotoxin contamination. Recombinant HCRs have been expressed in the yeast *Pichia pastoris* as a heterologous host (Rusnak and Smith 2009). Recently it has been shown that HCR vaccine from *E. coli*-derived hepta-serotype (against serotypes A, B, C, D, E, F, and G) elicited an antibody response to each of the seven BoNT HCRs and neutralized challenge by 10,000 LD_{50} doses of each of the seven BoNT serotypes (Baldwin et al. 2008). This is the first effective *E. coli*-derived vaccine.

The pentavalent toxoid is likely to be replaced by a recombinant vaccine expressed in *Pichia pastoris* [a bivalent serotype A and B H_C recombinant vaccine (rBV A/B)] for licensure under the FDA's Animal Rule. The vaccine is intended to protect primarily against BoNT/A subtype A1 and BoNT/B subtype B1 (Hart et al. 2012).

In terms of future developments, in addition to Hc-derived vaccines, some other recombinant subunit vaccines such as those consisting of combined catalytic and translocation domains (LCH_N) or ones coupled to viral vectors for enhanced immunogenicity (Mustafa et al. 2011) may be candidates for vaccine development. Another development that should not be ignored is the mucosal delivery of vaccines against BoNT poisoning. Vaccines delivered parentally produce antibodies that can neutralize BoNT only after it has gained access to the bloodstream. Vaccines delivered mucosally (via intranasal route) can induce both systemic and mucosal immunities (Ravichandran et al. 2007). Currently, DynPort Vaccine Company LLC (Frederick, MD) is developing a recombinant vaccine to protect against fatal botulism following inhalational exposure to the A1 and B1 serotypes of BoNT [rBV A/B (Hart et al. 2012)].

A word of caution regarding use of vaccines needs to be exercised here. BoNTs are not only considered as bioweapons (Arnon et al. 2001), but also at the same time, they are the first biological toxins to become licensed for the treatment of human diseases. BoNT/A has been recognized as a valuable therapeutic drug for a variety of neurological disorders (Fabbri et al. 2008) including treatment for spastic disorders such as in cerebral palsy and post-stroke. The neurotoxin is also used extensively in cosmetic applications for removal of wrinkles (Fabbri et al. 2008). Because of high titer of antibodies built up in individuals receiving BoNT vaccine, immunized individuals will be deprived of the beneficial effects of the toxin therapy if ever they are diagnosed with conditions described above.

Antitoxins

For Infant Botulism

Infant botulism, most prevalent at 3–4 months of age, is caused by the ingestion of spores of *Clostridium botulinum* (usually A or B) and vegetative cells temporarily colonized in the large intestine. When the conditions are appropriate for growth, *C. botulinum* will proliferate and produce toxin that reaches the target tissues via the general circulation (Arnon et al. 2006).

Equine antitoxin is not suitable for infants because of potential for eliciting hypersensitivity reactions. Also because of brief half-life of equine antitoxins in humans, it is unsuitable in infants where colonization of spores and toxin production occur for a long duration. A human antitoxin was developed from plasma of laboratory workers who were hyperimmunized with pentavalent botulinum toxoid. This product was designated Botulism Immune Globulin Intravenous (Human; BIG-IV) and contained $\geq$15 IU of antibodies against BoNT/A and $\geq$4 IU of antibodies against BoNT/B. In clinical studies BIG-IV treatment led to significant reductions in mean hospitalization time, fewer days of intensive care and mechanical ventilation, with no serious adverse effects. The findings were sufficiently compelling that licensure of BIG-IV was granted by the FDA in October 2003 as BabyBIG® for the treatment of infant botulism. This antitoxin has a circulation time of about 28 days, which appears to be adequate in protecting the infants for the duration of the intestinal colonization of botulinum spores (Arnon et al. 2006).

Antitoxins for Adult Botulism

Antitoxins can be only used as effective postexposure therapy to reduce severity of BoNT poisoning in bioterrorist attack, after unintentional toxin exposure through ingestion of contaminated foods or because of overdose of BoNT/A in clinical practice. BoNT antitoxin consists of a purified fraction of immunoglobulins prepared from a hyperimmunized animal (horse for equine antitoxin). Currently available antitoxins unfortunately show variety of undesirable side effects such as serum sickness, anaphylactic shock due to foreign protein, and hypersensitivity. Until now the only successful drug available to treat BoNT intoxication was equine antitoxin (Mayers et al. 2001). Antitoxins are able to neutralize BoNT in circulation and thus to prevent further internalization of toxin. Obviously this requires administration of the antitoxin early during the course of disease (Simpson 2004). Once the toxin is internalized inside the MNT, it is not accessible for neutralization by antitoxin, making the timing of the therapy very critical (Simpson 2004). In a published clinical study involving 132 cases of BoNT/A poisoning, patients who had received trivalent equine antitoxin (/A, /B, and /E) had a lower fatality rate and a shorter course of the disease progression than those who did not receive antitoxin. Patients who received antitoxin in the first 24 h after onset had a shorter course but about the same fatality rate as those who received antitoxin later. These results suggest that trivalent antitoxin has a beneficial effect on survival and shortens the course of patients with type A botulism (Tacket et al. 1984). Unfortunately, human pharmacokinetic data for BoNT are lacking. This is because medical professionals often see the intoxicated patients long after exposure to the neurotoxin. Contrary to the belief that circulating BoNT is cleared from the bloodstream within 1 or 2 days of exposure, clearance, in fact, could take longer after severe BoNT intoxication. In a recent Florida outbreak case, approximately 40-fold excess human LD50 of BoNT was detected in the serum of the patient 4 days after toxin injection during a cosmetic procedure (Souayah et al. 2006). In such cases antitoxin administration may still be effective in limiting internalization of circulating BoNT, thereby reducing the severity and duration of illness.

From March 2010, the only antitoxin available in the United States for non-infant botulism is a new equine heptavalent botulinum antitoxin (HBAT) manufactured by the Cangene Corporation of Canada through a CDC-sponsored Food and Drug Administration (FDA) Investigational New Drug (IND) protocol. HBAT replaces a licensed bivalent botulinum antitoxin AB for CDC's quarantine stations and for Health and Human Services (HHS)'s Strategic National Stockpile (SNS). HBAT is prepared from plasma of horses immunized with toxoid of one of the seven serotypes (BoNT/A–BoNT/G) of BoNT. The final product is a blend of antibodies for each one of these serotypes. HBAT is composed of <2 % intact immunoglobulin G (IgG) and ≥ 90 % Fab and F(ab')2 immunoglobulin fragments; these fragments are created by the enzymatic cleavage and removal of Fc immunoglobulin components in a process sometimes referred to as despeciation (to reduce hypersensitivity reaction). Fab and F(ab')2 fragments are cleared from circulation more rapidly than intact IgG (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5910a4.htm).

Despeciation reduces the reactogenicity of the antitoxin and also shortens its plasma half-life. In a case report with type F intestinal colonization botulism, the patient showed an initial improvement after HBAT infusion, only to be followed by a relapse 10 days later (Fagan et al. 2011). The short half-life of type F antibodies in HBAT (14.1 h5) was a likely contributing factor.

Block of Acceptors (Ectoreceptors) at MNT

Specific gangliosides have been recognized as acceptors at the MNT for binding various BoNTs (Dolly et al. 1984). A reasonable approach in development of countermeasures for BoNTs would be to test lectins, which would prevent binding of the toxin to the MNT. Indeed lectins of *Triticum vulgaris* were found to delay the paralysis of mouse diaphragm for BoNT/A and BoNT/F (Bakry et al. 1991). The disadvantage of using lectins to protect against BoNT poisoning is that they need to be pre-administered which is not feasible or practical.

Classical Zn^{2+} Chelators

The primary focus to develop preventive and therapeutic measures was stimulated by atomic absorption spectroscopy to demonstrate the presence of a Zn^{2+} atom per molecule of the neurotoxin (Montecucco et al. 1992). This was followed by characterization of the effects of Zn^{2+} chelators, in vitro, on BoNT-poisoned MNT (Sheridan and Deshpande 1995). Bath application of BoNT/A or BoNT/B (0.1 nM) at 36 °C inhibited phrenic nerve-stimulus-evoked twitch tensions in vitro. However, either coincubation or pretreatment with heavy metal chelator tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN, Fig. 2) delayed the onset of BoNT-induced muscle paralysis. Interestingly, treatment of phrenic nerve diaphragm preparations with a Zn^{2+}-TPEN complex, rather than TPEN alone, eliminated the beneficial effects of TPEN on BoNT intoxication. This indicates that TPEN mediates these effects by Zn^{2+}chelation (Deshpande et al. 1995). However, Zn^{2+} chelation did not totally prevent the neuroparalytic effects of BoNTs. Although use of a Zn^{2+} chelator, TPEN, was not a possible option to prevent or treat botulism due its toxicities, this study advanced the pharmacological approaches to develop newer inhibitors that interfered with the catalytic activity of these Zn^{2+}-dependent endoproteases.

Simpson (1983) developed an approach to antagonize the internalization of BoNTs at the cholinergic nerve terminals. Simpson used millimolar quantities of ammonium chloride and methylamine chloride, which in a concentration-dependent manner delayed the onset of neuromuscular blockade caused by BoNTs. These compounds neither inhibited the binding of the neurotoxins to its ectoreceptors nor reversed neuromuscular blockade but interfered with the internalization of the neurotoxin at the cholinergic nerve terminals. Further, experiments performed to antagonize the internalization of the neurotoxin demonstrated that the

TPEN Zn^{2+} TPEN-Zn^{2+} complex

Fig. 2 TPEN and TPEN-Zn^{2+} complex

translocation of the neurotoxin to cytosol and the catalytic activity of the toxin were temperature dependent (Simpson 1980).

Holotoxin Uptake Inhibitors

Role of Extracellular Ca^{2+} on Neurotoxin Uptake

The primary target of BoNTs is the motor nerve terminal (MNT) at the neuromuscular junction (NMJ). A potential intervention to prevent the entry of neurotoxin at the MNT would offer protection against the neuroparalytic effects of the toxin. Experiments aimed to inhibit the uptake of BoNT/A at the MNT-targeted Ca^{2+}-dependent mechanisms as well as inhibition of clathrin-dependent endocytosis (CDE) of BoNT/A. Recent research (Thyagarajan et al. 2009) described the discovery of expression of transient receptor potential vanilloid 1 (TRPV1) at the MNT and that capsaicin (Fig. 3), a TRPV1 agonist, prevented the uptake of BoNT/A at the MNT. Antagonizing TRPV1 by capsazepine (a TRPV1 antagonist) prevented this protective effect of capsaicin. They demonstrated this by locally injecting capsaicin in the hindlimb region, close to the innervation of *extensor digitorum longus* (EDL) muscle, before BoNT/A exposure. The results showed that animals that received local BoNT/A injection were paralyzed 12–24 h following BoNT/A exposure, while those receiving capsaicin at different time points and up to 12 h before BoNT/A exposure showed protection from neuroparalysis.

An intriguing finding by Thyagarajan et al. (2009) was that the preventive effects of capsaicin were dependent on the presence of extracellular Ca^{2+}. This raises an important question on the role of Ca^{2+} in BoNT uptake. Such a hypothesis also correlates well with earlier findings of Simpson (1988) where an increase in extracellular Ca^{2+} per se delayed the onset of action of BoNT/A. Simpson elegantly demonstrated this by exposing phrenic nerve diaphragm nerve muscle preparations (NMP) to BoNTs and tetanus toxin in the presence of varying concentrations of extracellular Ca^{2+} to demonstrate that higher concentrations of extracellular Ca^{2+} (8 or 16 mM) significantly delayed the time to paralysis caused by BoNT/A. However, such an effect was not observed either for BoNT/E or for tetanus toxin.

Fig. 3 Structure of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide)

These observations not only describe the complexity of the role of extracellular Ca^{2+} on BoNT/A uptake but also warrant the need for further studies to decipher the role of Ca^{2+} at the MNT in BoNT intoxication.

The published work of Thyagarajan et al. (2009) provided the first evidence for the role of TRPV1 in the regulation of Ca^{2+}-dependent endocytosis of BoNT/A in a mouse model of BoNT/A intoxication in vivo. This work demonstrated that although capsaicin prevented the neuroparalytic effect of BoNT/A in vivo, the protective effect of capsaicin was not complete. Furthermore, capsaicin only offered a 12 h window of protection and BoNT/A injected at later times produced neuroparalysis. One possible reason for this observation is that the concentration of capsaicin may have decreased from the extracellular synaptic space at the MNT or the molecular signaling events activated by TRPV1 terminated after 12 h.

Although these data demonstrate the protective effect of capsaicin against BoNT/A, whether capsaicin restores neurotransmission following BoNT/A intoxication remains to be evaluated. Nonetheless these observations provide evidence for the presence of TRPV1 channels at the MNT and open up the opportunities for new research studies to evaluate the role of TRPV1 in the pathophysiology of NMJ and specifically BoNT/A intoxication.

Toxin Endocytosis Inhibitors

Binding of BoNTs to synaptic vesicle proteins and polysialogangliosides which are identified as their receptors sets their endocytosis via clathrin-coated pit-dependent mechanisms (Jahn 2006). Clathrin-dependent endocytosis (CDE) is a process of internalization of membrane lipids and membrane-bound proteins. Invagination of clathrin-coated pits that are bound to molecular cargo buds inward to form coated vesicles. This process involves the complex interactions and architecture of several regulatory proteins. The process of conversion of a budding pit into a vesicle is processed by clathrin heavy and light chains assisted by a set of cellular proteins, which includes dynamin and adaptor proteins (AP). Clathrin-coated pits are formed by the association of an array of endocytic proteins including clathrin heavy chain, clathrin light chain, adapter protein-2, AP180, etc. and are regulated by membrane-bound phosphatidylinositol (4,5) bisphosphate [PIP2 (Haucke 2005)].

Pharmacological inhibition of transcytosis of neurotoxin and its fragments by chlorpromazine (clathrin-coated pit inhibitor) was previously demonstrated by Maksymowych and Simpson (2004). This set the trend for research strategies to interfere with the uptake of neurotoxins at the MNT. Direct inhibition of clathrin-coated pit-dependent uptake of BoNT/A was first shown in vitro by Potian

Fig. 4 Structure of Dyngo-4a

et al. (2010a). They showed that chlorpromazine treatment caused delocalization of adapter protein 2 and inhibited BoNT/A uptake in cholinergic Neuro 2a cells. This effect of chlorpromazine was mimicked by wortmannin, a PI3K/PI5K inhibitor. In support of this result, local hind limb injection of wortmannin was shown to protect mice from BoNT/A-mediated neuroparalysis (Potian et al. 2010a). Wortmannin pretreatment also inhibited uptake of Alexa 647-labeled BoNT/A into isolated NMP and altered the localization of clathrin heavy chain from the plasma membrane to the cytoplasmic region. This is the first research report that linked the effects of PIP2 synthesis inhibition to clathrin heavy chain localization that affected the uptake of BoNT/A. The authors also proposed a model where chlorpromazine and wortmannin interfered with the uptake of BoNT/A by their inhibitory effects of clathrin-coated pit formation. This study recapitulates the earlier observations of Maksymowych and Simpson (2004).

Recently, Harper et al. (2011) characterized the neuronal endocytic mechanism for BoNT/A to demonstrate the activity-dependent uptake of recombinant BoNT/A heavy chain (Hc) in cultured hippocampal neurons using fluorescence and electron microscopy. They found that BoNT/A-Hc entered into endosomal structures via clathrin-coated vesicles and that inhibiting dynamin prevented this process.

Dynamin is a large multidomain GTPase protein that is essential for clathrin-dependent vesicle formation as it facilitates the transition of a fully formed clathrin-coated pit to a pinched-off vesicle (Chappie and Dyda 2013). Dynamin has been recognized as a co-player in mediating depolarization evoked entry of BoNT (Meng et al. 2013). Harper et al. (2011) found that inhibiting dynamin with Dyngo-4a, a Dynasore analog, abolished the internalization of BoNT/A-Hc. Also, Dyngo-4a (Fig. 4) pretreatment prevented BoNT/A-induced proteolysis of SNAP-25 in hippocampal neurons. Furthermore, Dyngo-4a decreased the uptake of BoNT/A-Hc at the MNT and offered protection against BoNT/A-induced neuroparalysis of rat phrenic diaphragm NMP. Consistent with these in vitro observations, Dyngo-4a significantly delayed the onset of botulism in mice injected with BoNT/A. These data offer the proof of concept for the use of Dyngo-4a against botulism (Harper et al. 2011).

Another experimental approach adapted by several investigators to inhibit clathrin-coated pit formation to interfere with neurotoxin uptake involved the use of hypotonic stress and potassium depletion in experimental buffer solution. Maksymowych and Simpson (2004) followed a similar approach to inhibit clathrin-dependent endocytosis of BoNT/A. Potian et al (2010a) used hypotonic stress and potassium depletion in physiological buffer solution to inhibit fluorescent BoNT/A entry into cholinergic neuroblastoma (Neuro 2a) cells.

Fig. 5 Chemical structure of toosendanin

Further, research findings by Petro et al. (2006) confirmed that disruption of clathrin-coated pit inhibited BoNT/A uptake.

Research efforts dedicated to interfere with neurotoxin uptake provide compelling evidence that clathrin-dependent endocytosis serves as the mechanism of neurotoxin entry into MNT. However, these studies have potential limitations to be used as strategies to counteract BoNT intoxication due to potential side effects associated with the use of specific compounds. Both wortmannin and chlorpromazine (an antipsychotic agent) have undesired side effects. Even though experimental approaches including hypotonic stress and potassium depletion have no clinical use to antagonize BoNT intoxication, such techniques advance our understanding of cellular mechanisms in the regulation of neurotoxin entry into MNT and in cell model systems.

Lc Translocation Inhibitors

Following clathrin-coated pit-dependent entry into MNT, pH-dependent separation of BoNT Lc from the Hc results in the translocation of the Lc endoprotease into cytosol via the Hc chaperone. This translocation step is crucial for the intoxication process (Simpson 1979).

Toosendanin (Fig. 5; TSN) is a triterpenoid extracted from *Melia toosendan* Sieb et Zucc that is potentially used in medical and scientific research. TSN has been shown to inhibit experimental BoNT intoxication. It has been shown to prolong the time of onset of botulism in rat phrenic nerve diaphragm preparations (Shih and Hsu 1983). TSN prevented SNAP-25 cleavage by the holotoxin but did not prevent the cleavage of SNAP-25 by Lc in rat synaptosomes incubated with holotoxin or BoNT/A Lc (Zhou et al. 2003). This is suggestive of a mechanism by which TSN prevents the translocation of Lc from endosome to nerve terminal cytosol to cause proteolysis of SNAP-25. But when synaptosomes were incubated with BoNT/A Lc, TSN did not prevent SNAP-25 cleavage.

Further, the ability of TSN to protect mice against the lethal doses of BoNT/A or BoNT/B for several hours from death has been reported (Shi and Wang 2004). Also, TSN has been shown to protect isolated phrenic nerve diaphragm preparations from the rats that were preinjected with TSN (Shi and Wang 2004).

The ability of TSN to inhibit translocation of Lc endoprotease was critically analyzed using inside-out single-channel recording patch-clamp technique by Fischer et al. (2009). They demonstrated that at subnanomolar concentrations TSN occluded the Hc pore to prevent Lc translocation. These research studies brought into light a novel BoNT antidote, which is a natural product, and stimulated the advancement of function-oriented synthesis strategy to unravel the anti-botulinum effects of TSN. However, the ability of TSN to cause synaptic blockade at the NMJ as well as in the central synapses limits its use as a universal BoNT antidote (Shi and Wang 2006). Also, chemical modifications and synthetic derivatives of TSN were not promising since these derivatives failed to prevent BoNT/A-mediated death in mouse lethality assays (Nakai et al. 2010).

Lc Catalytic Activity Inhibitors

The challenges involved in developing antidotes that counteract BoNTs at MNT which is the primary site for their action stimulated the quest for cell-permeable peptides, peptidomimetics, and small molecule synthetic drugs. These bioactive peptides and small molecules are aimed to interfere with the proteolytic activity of the neurotoxins. That is, these molecules are specifically designed to block the catalytic site of the Lc endoprotease that cleaves selective SNARE complex proteins to inhibit neurotransmitter release. The ideal qualities of an antidote include:

1. Target specificity and selectivity: Specifically to antagonize BoNT metalloendoprotease in cell-free, in vitro and in vivo model systems and accelerate recovery from neuroparalysis
2. Adequate bioavailability: Available, at appropriate concentrations, at the MNT, the primary target of BoNTs, to exert desired effects
3. Stable for site-specific delivery: Amenable to formulation requirements for site-specific delivery to counteract BoNTs
4. Minimal toxicity: Do not produce any undesired toxic effects
5. Amenable to structural modification/derivatization: Readiness for chemical modifications/derivative formation to improve physicochemical properties, target specificity, and stability in formulations as well as in the MNT and to inhibit BoNTs with specificity
6. Does not have any interaction with other drugs, food, and/or pharmaceutical excipients
7. Provide maximal benefits at minimal cost

Currently, no robust and reliable therapeutic measure is available to counteract BoNT intoxication. The available antitoxins have a limited window of opportunity since they cannot antagonize the toxin that has already entered MNT. The growing clinical use of BoNTs to treat neurological complications also provides opportunities for accidental or intentional misuse of the neurotoxin and warrants the need for therapeutics against BoNTs.

Developing cell-permeable antibodies (transbodies) that have specific half-life would potentially benefit the use of such an approach in clinical situations. Alongside the maxim, several research laboratories have begun to develop small molecule inhibitors, which vastly include peptide-based drugs, peptidomimetics, and organic synthetic drugs that abrogate the proteolytic activity of the neurotoxin. Such an approach to inactivate the neurotoxin will not only benefit by accelerating recovery from neuroparalysis but also prevent the undesirable consequences of the long-lasting and persistent toxicities of BoNT/A.

Small Molecule Inhibitors

Small molecule inhibitors that antagonize the catalytic activity of the neurotoxin are developed and evaluated by several research laboratories throughout the world. The effects of such inhibitors are mostly evaluated in cell-free systems where fluorogenic substrate cleavage assays were largely used. Development of high-throughput screening methods, cell-based enzymatic assays, structure-based drug design (Agarwal and Swaminathan 2008), and crystallographic and modeling studies have resulted in the identification of new chemical entities that inhibit Lc endoprotease activity. Efforts to determine the crystal structure of BoNT/B and its complex with sialyllactose at resolutions 1.8 and 2.6 A°, respectively, brought into light the catalytic and binding sites of the neurotoxin (Swaminathan and Eswaramoorthy 2000). This helped in the development of Lc catalytic activity inhibitors.

Peptide Inhibitors

It is well established that the L_C of BoNTs is a Zn^{2+} metalloprotease, which is released from the holotoxin into the neuronal cytosol (translocation step) to cause SNARE proteolysis and inhibition of neurotransmission. It is also recognized that of the seven serotypes of the neurotoxin, BoNT/A produces the longest duration of neuroparalysis. This has stimulated efforts toward development of peptide, peptidomimetics, and small molecule inhibitors, which have the capability to antagonize the neuroparalytic effects of BoNT/A (Willis et al. 2008). BoNT/A is a zinc metalloprotease, which cleaves only one peptide bond in SNAP-25. Therefore, single-residue changes in a 17-residue substrate peptide were introduced as first specific, competitive BoNT/A L_C inhibitors. Substrate analog peptides with P1 or P2 substitution (CRATKML analogs) had IC50 of 2 μM (Schmidt et al. 1998). The potency, however, was determined by in vitro assays using the substrate peptide consisting of residues 187–203 of SNAP-25. Results obtained from such assays did not necessarily translate into the effectiveness in in vitro or in vivo models of botulinum poisoning in the mammalian MNTs. This was evident from the experiments with testing CRATKML peptides (Cp) in the mouse MNTs poisoned in vitro with BoNT/A (10 pM) or in vivo by injection of the toxin locally

(3 μl of 6.67 pM) into EDL. Cps had neither a protective nor a therapeutic effect in vitro or in vivo (Potian et al. 2010b). The four Cp analogs tested were designed for optimal penetration and stabilization in the cell. To what extent the Cps permeated in the MNTs was not known. Other factors for the failure of Cps to protect MNTs from (Potian et al. 2010b) BoNT/A-induced paralysis could be binding without internalization, nonselective binding, and degradation of peptides once inside the MNT.

The in vitro inhibitor binding to the *CRATKML* peptide was further modified by replacing the cysteines with other sulfhydryl-containing compounds. One such peptide had K(i) of 330 nM and was claimed to be the best inhibitor available at the time. This peptide inhibitor was recently tested in a targeted drug carrier (TDC) system for transferring functional proteins or peptides into mouse MNT of hemidiaphragm muscles (Edupuganti et al. 2012). Essentially, the gene encoding a nontoxic BoNT/B protease-inactive mutant (BoTIM) was linked via core streptavidin (CS)-BoTIM/B to biotinylated liposomes for preferential transfer of cargo such as inactive peptides to inhibit BoNT/A paralysis. Such system acts as a targeted drug carrier system. When the modified *CRATKML* peptide inhibitor was included in CS-BoTIM/B-TDC at an effective bath concentration of 8.3 μM, the time for a 90 % block of MNT in mouse diaphragm was 133 min as compared to 80 min for 100 pM of BoNT/A alone (Edupuganti et al. 2012). Inefficient encapsulation (only 38 %) of inactive peptide and degradation of peptide (instability of peptide) at the nerve terminal could account for a marginal prolongation for the paralysis time.

Recently, three related peptide-like molecules (PLMs) were designed, synthesized, and assayed for in vitro using HPLC-based assay to calculate PLM inhibition against BoNT/A L_C. Crystal structure, along with structure activity data, of the zinc metalloprotease domain of BoNT/A bound to a potent peptidomimetic inhibitor (Ki = 41 nM) was reported (Zuniga et al. 2010). The ability of these inhibitors to antagonize BoNT/A at the mammalian MNT still remains to be evaluated. Yet another report of successful inhibition by a peptide inhibitor obtained from wasp bee venom was shown to antagonize BoNT/A in primary cultures of mouse spinal cord neurons (Ray et al. 2008). Potassium-evoked glycine release was measured. The botulinum-poisoned nerve cells were rescued by Mastoparan-7 (Mas-7), a peptide constituent of bee venom delivered through a drug delivery vehicle (DDV, constructed from the nontoxic fragment of BoNT itself). In spinal cord cultures incubated with 1 pM BoNT/A for 8 h at 37 °C, a complete inhibition of glycine release was observed, while only 28 % SNAP cleavage occurred indicating that complete SNAP-25 cleavage was not required for the transmitter release. DDV-Mas-7 treatment restored glycine release to the extent of 40 %. Additionally, it is interesting to note that in BoNT/A-poisoned neurons, DDV-Mas-7 treatment had no effect on SNAP-25 cleavage. As interesting as these data are, it must be noted that spinal cord neurons are not the target for BoNTs and secondly it is doubtful that the wasp venom peptides could be used for the treatment. Nevertheless, these results certainly shed some light on mechanisms other SNAP-25 cleavage caused by BoNT/A.

Peptide Inhibitors Designed to Block BoNT Inhibition

Several peptide-based BoNT/A LC inhibitors were developed and tested. The details are summarized in Table 1.

Hydroxamate-Based Lc Metalloendoprotease Inhibitors

Hydroxamate derivatives of drug molecules serve as excellent tools to antagonize metalloproteases. Hydroxamates are recognized therapeutically as histone deacetylase (HDAC) inhibitors to treat cancer. The inhibition of Zn^{2+}-dependent endoproteases by phosphoramidon and peptide hydroxamate was studied as early as 1995 (Deshpande et al. 1995). In vitro experiments performed to evaluate the effects of these compounds against BoNT/A and BoNT/B intoxication in phrenic diaphragm NMP showed that phosphoramidon but not peptide hydroxamate prolonged the time to 50 % inhibition of nerve-stimulus-evoked twitch tensions.

Following this, several non-peptide-based synthetic hydroxamates and their derivatives were evaluated for their ability to antagonize BoNTs in in vitro and in vivo models of BoNT intoxication (Eubanks et al. 2007). Their systematic analyses using high-throughput primary screening in cells for SNAP-25 cleavage protection and in vivo mouse lethality assay resulted in the identification of structures that prolonged time to death due to BoNT/A intoxication. However, these compounds did not prevent SNAP-25 cleavage by BoNT/A in vitro. This in vitro-in vivo disconnect between the effects of inhibitors provides new insight into the mechanisms by which these inhibitors antagonize BoNT intoxication.

Specifically, 2,4-dichlorocinnamic hydroxamic acid (2,4-DCH) and its methyl derivative (synthesized by Absolute Biosciences Inc, MA, USA, and designated as ABS-130) were tested for their effects to inhibit BoNT/A Lc endoprotease (Thyagarajan et al. 2010). The chemical structures of 2,4-DCH and ABS-130 are given in Fig. 6.

In vitro, the Ki for inhibition of BoNT/A Lc was 0.40 and 0.36 36 μM mu;M for 2,4-DCH and ABS-130, respectively. When isolated nerve muscle preparations were exposed to 2,4-DCH or ABS-130 following BoNT/A intoxication, an increase in the quantal content of acetylcholine release and a delay of 40–90 min in restoration of muscle twitches were observed. However, local in vivo hind limb injection of 2,4-DCH or ABS-130 neither prevented the toe spread reflex (TSR) inhibition nor improved muscle functions. These data envisage that the ability of hydroxamate-derived drugs to prevent or therapeutically antagonize BoNT intoxication can be attributed to the lack of selectivity, toxicity, or poor stability of these drugs.

More recently, a combination of crystallographic and modeling was used to design a series of hydroxamates derived from 1-adamantylacetohydroxamic acid (Silhar et al. 2013). This effort has identified compounds with improved potency against BoNT/A revealing these as most potent non-peptide small molecules to inhibit BoNT/A Lc.

Table 1 Peptide-based BoNT/A L_C inhibitors

BoNT/A L_c inhibitors	System/model tested	Effectiveness	Comments
Membrane-permeable *CRATKML* (Schmidt et al. 1998)	In vitro cell-free system	K_i 2 μM	Effectiveness tested for SNAP-25 cleavage
Four *CRATKML*-derived peptide (Cp) analogs (Potian et al. 2010b)	Mouse diaphragm nerve in vitro, mouse EDL in vivo injections, Neuro 2a cells in culture	Cp 20 μM mu;M preincubation/ postexposure to BoNT/A (10pM)	No protective or therapeutic effect. Effectiveness in cell-free systems against SNAP-25 cleavage does not necessarily translate into protection in BoNT/A-poisoned MNT
Mastoparan-7, a peptide from bee venom, delivered via a nontoxic fragment of BoNT (Ray et al. 2008)	Mouse spinal cord neurons, K^+-evoked glycine release	Mastopran-7 restored glycine release, rescue occurred independent of SNAP-25 cleavage	Evidence shows disconnect between SNAP-25 cleavage and transmitter release
Three synthetic peptide-like molecules (PLMs) (Zuniga et al. 2010)	In vitro HPLC-based assay	PLMs were found to be competitive inhibitors of the BoNT/A Lc protease with Ki values in the nanomolar range	Peptide inhibitors not tested in mammalian MNT
High-affinity peptide inhibitors of BoNT/A (2-mercapto-3-phenylpropionyl-RATKMLGSG) (Schmidt and Stafford 2002)	In vitro proteolysis of SNAP-25 by BoNT/A	K_i 330 nM	Effectiveness tested by hydrolysis of peptide
A targeted drug carrier (TDC) (2-mercapto-3-phenylpropionyl-RATKMLGSG) prepared by Schmidt and Stafford (2002) and used for transferring functional proteins or peptides into MNT (Edupuganti et al. 2012)	The gene encoding an innocuous BoNT/B protease-inactive mutant (BoTIM) expressed in *Escherichia coli* and the purified protein was conjugated to surface-biotinylated liposomes	Attenuated the neuroparalytic effects of BoNT/A or BoNT/F in mouse MNT	Liposomal delivery of peptides provides the proof of concept for targeted delivery. It should be noted that the time to 90 % paralysis was delayed marginally from 80 to 133 min. for 100 pM BoNT/A (note: high concentration of BoNT/A)

Cl Cl H N OH O **2,4 DCH** Cl Cl H N OH O **ABS 130**

Fig. 6 Structures of 2,4-DCH and ABS-130

N NH_2 **4-aminopyridine (4-AP)** N NH_2 NH_2 **3,4-diaminopyridine (3,4-DAP)**

Fig. 7 Structures of aminopyridines

The ineffectiveness of hydroxamates, especially the lead compound 2,4-DCH, evaluated at the mammalian MNT in vivo (Thyagarajan et al. 2010) warrants for future research work to determine the pharmacokinetic parameters and stability of hydroxamates to be used as effective antidotes against BoNT intoxication.

Nonetheless, the usefulness of these compounds cannot be ignored since adequate amount or concentration of these compounds may not have reached the active site to antagonize BoNT/A Lc.

Aminopyridines as BoNT Antidotes

The ability of aminopyridines (Fig. 7) to modify neurotransmission was recognized by several researchers (Harvey and Marshall 1977; Molgo et al. 1980). The advantage of ability of aminopyridines to block potassium channels (Van Lunteren and Moyer 1996; van Lunteren et al. 2001) and thereby increase presynaptic Ca^{2+} has been used to restore neurotransmitter release following BoNT/A intoxication. Compounds like 4-aminopyridine and 3,4-diaminopyridine were evaluated for their ability to restore neurotransmission following BoNT intoxication (Mayorov et al. 2010; Molgo and Thesleff 1984; Morbiato et al. 2007).

The clinical efficacy of aminopyridines for multiple sclerosis supported the concept to develop them as BoNT antagonists. Research work performed at the MNT demonstrated that treatment with 3,4-DAP following BoNT/A intoxication significantly restored the nerve muscle preparations from BoNT/A-mediated inhibition of stimulus-evoked muscle twitches and stimulus-evoked acetylcholine release (Adler et al. 2012). In vivo infusion of 3,4-DAP via systemic circulation was shown to antagonize the inhibitory effects of BoNT/A on neuromuscular transmission (Adler et al. 2000). Infusion of 3,4-DAP in rats showed higher tetanic tensions compared to BoNT/A-alone-injected animals. However, the benefits

conferred by 3,4-DAP infusion were not maintained when the drug infusion was stopped. This suggests that continuous presence of 3,4-DAP is indeed necessary for its antagonism of BoNT/A. Therefore, a sustained delivery of 3,4-DAP would help in the recovery following BoNT/A intoxication. However, the length of such sustained delivery following BoNT/A intoxication and the concentrations of 3,4-DAP that needs to be maintained to exert its therapeutic effects still remain to be evaluated.

Alternatively, a dual-effect prodrug approach was adapted to conjugate carbamate, a reversible inhibitor of acetylcholinesterase (an enzyme that metabolizes acetylcholine at the synaptic cleft), with 3,4-DAP (Zakhari et al. 2011) with the intention of prolonging the release of acetylcholine at the MNT. This resulted in new candidate with clinically relevant half-life and stability with less toxicity. However, future studies are needed to evaluate and validate its effects on in vivo BoNT/A-poisoned animals.

Efforts to combine hydroxamates and 3,4-DAP to antagonize BoNT/A intoxication were developed to evaluate the effects of dual-drug approach to antagonize BoNT/A intoxication (Thyagarajan et al. 2010). However, when applied to in vitro BoNT/A-poisoned nerve muscle preparations, following hydroxamate-based 2,4-DCH or ABS-130, 3,4-DAP temperature-dependently increased twitch tension. That is, additive effects of hydroxamates and 3,4-DAP on nerve-stimulus-evoked twitch tension were observed only in preparations that were poisoned with BoNT/A at room temperature (22 °C) but not in preparations poisoned with BoNT/A at 37 °C. Since 2,4-DCH or ABS-130 and 3,4-DAP did not produce additive or synergistic restoration of neuromuscular transmission due to their distinct mechanisms of action to antagonize BoNT/A, the lack of effects of 3,4-DAP following 2,4-DCH or ABS-130 at 37 °C raises interesting questions on the temperature-dependent restorative effects of these compounds. These findings suggest that the restoration of acetylcholine release from MNT that are acutely poisoned with BoNT/A is limited due to the proteolysis of SNAP-25 and this cannot be overcome by drugs with distinct mechanisms of action to antagonize BoNT/A.

Despite the promise that aminopyridines offered in restoring neurotransmission following BoNT/A, their use is abandoned due to their neurotoxicities. Aminopyridines have the ability to cross the blood–brain barrier to cause such effects. In order to circumvent this, a synthetic approach was adopted to evaluate structural analogs of 3,4-DAP that exhibited the ability to antagonize BoNT/A, similar to the parent compound, but lacked the ability to cross the blood–brain barrier to cause neurotoxicity (Mayorov et al. 2010). This research resulted in the identification of a small molecule potassium channel blocker with appreciable cell permeability and potency to counteract BoNT/A intoxication (Mayorov et al. 2010).

Although 3,4-DAP efficiently antagonizes BoNT/A intoxication, it does not restore neurotransmission following BoNT/B or BoNT/E poisoning. In experiments that were directed to evaluate the effects of 3,4-DAP on BoNT/A-, BoNT/B-, BoNT/E-, or tetanus toxin (TeTX)-poisoned rat phrenic nerve-hemidiaphragm

nerve muscle preparations, 3,4-DAP had greater effects against BoNT/A compared to BoNT/B, BoNT/E, or TeTX (Simpson 1986).

One important observation that is not well addressed mechanistically at the MNT is that 3,4-DAP could restore acetylcholine release and synaptic transmission despite the proteolytic effects of BoNT/A on SNAP-25. That is, the presynaptic Ca^{2+} rise due to 3,4-DAP-mediated prolongation of action potential triggers synaptic vesicle fusion and/or release in spite of the presence of BoNT/A and its ability to cleave SNAP-25. Even though the effects of 3,4-DAP are transient and can be maintained only as long as the drug is available at the MNT, how the Ca^{2+} rise helps in the restoration of neurotransmission is not clear until now. Also, the selectivity of 3,4-DAP to BoNT/A intoxication over other serotypes of BoNTs and TeTX raises an interesting question of how the rise in intracellular Ca^{2+}-triggered synaptic vesicle fusion and/or release overcomes the effects of BoNT/A and BoNT/C but not other BoNT serotypes or TeTX. Although BoNT/A, BoNT/C, and BoNT/E proteolyze SNAP-25 (albeit the difference in their cleavage sites), 3,4-DAP antagonizes BoNT/A and BoNT/C but not BoNT/E. Perhaps, a Ca^{2+}-stimulated SNARE complex formation via activation of Ca^{2+}-dependent protein kinases at the presynaptic nerve terminals specific for BoNT/A and BoNT/C may be involved in this process. Further research studies are, therefore, needed to address this question and to unravel the novel mechanisms by which presynaptic Ca^{2+} rise triggers neurotransmission at the MNT.

Although the role of several SNARE complex proteins, their phosphorylation states, and their Ca^{2+}-dependent association and activity modulation are studied at the central synapses, whether these mechanisms are conserved at the MNT still needs to be investigated.

Aptamers as Antidotes

Aptamers are oligonucleic acid or peptide molecules that bind to a specific target molecule. Aptamers can be used for both basic research and clinical purposes as macromolecular drugs. DNA or RNA aptamers consist of short strands of oligonucleotides, while peptide aptamers predominately consist of a short variable peptide domain, attached at both ends to a protein scaffold. They act as antagonists to inhibit protein-protein/receptor-ligand interactions. Using aptamers to inhibit Lc-mediated SNAP-25 cleavage highlights their potentials as BoNT antidotes. A single-strand DNA aptamer was used to target against BoNT's proteolytic activity (Tok and Fischer 2008). This work was followed by the development of RNA aptamers that bind strongly to the BoNT/A Lc endoprotease to inhibit its SNAP-25 proteolytic catalytic activity in low nanomolar range (Chang et al. 2010).

Bruno et al. (2012) developed several DNA aptamers to target against BoNT/A Lc and for diagnostic purposes. Two of the most responsive candidates responded

well to BoNT/A, BoNT/B, and BoNT/E holotoxins in a dilute soil suspension, but not in human serum. Although aptamers offer promise to target against BoNT intoxication, they are still under developmental stage and require validation and characterization for use against BoNTs in animal models of intoxication. Site-specific delivery of aptamers to treat botulism is a critical challenge in the development of aptamers as potential antidotes to counteract botulism. Future studies are needed to address this and develop aptamers as robust therapeutic measures to antagonize botulism. The use of aptamers to treat neurotoxin intoxication is discussed in this section.

UPS-Dependent Lc Degradation

The phenomenal potency of BoNT/A resides in the endoprotease activity of its Lc. The persistence of BoNT/A at the MNT presents a challenge to treat its toxic effects (Keller 2006; Kuo et al. 2011). The knowledge of signaling mechanisms that govern the persistence of the Lc endoprotease at the MNT and its clearance from the MNT is rudimentary. The surprisingly high stability and longevity of the proteolysis of SNAP-25 activity by BoNT/A at the MNT make it the most persistent endoprotease known so far to scientific community. Recent research work envisages degradation of BoNTs via ubiquitin proteasomal degradation pathways (Kuo et al. 2011; Tsai et al. 2010).

Proteasomes are large multisubunit proteases that are ubiquitously found in the cytosol free or attached to the endoplasmic reticulum, as well as in the nucleus. They recognize, unfold, and digest protein substrates that have been marked for degradation following ubiquitination of target proteins. Therefore, research strategy to enhance BoNT/A Lc ubiquitination and thereby accelerate its degradation is a good strategy to decrease the toxicities of BoNTs.

It has been shown recently that tumor necrosis factor receptor-associated factor 2 (TRAF2) directly mediates Really Interesting New Gene (RING)-dependent ubiquitination of BoNT/E L_C and promotes its proteasomal degradation (Tsai et al. 2010). They used chimeric SNAP-25-based ubiquitin ligases to accelerate intracellular degradation of BoNT/A L_C so that the longevity of the neurotoxin is decreased.

Another therapeutic strategy was used to develop intraneuronal BoNT proteases for accelerated degradation of BoNTs by the ubiquitin proteasome system (Kuo et al. 2011) to facilitate clearance of all serotypes. This approach consisted of a camelid heavy chain domain that is specific for selective BoNT protease. This domain was fused to an F-box domain that is recognized by an intraneuronal E3-ligase. This approach was developed to cause accelerated ubiquitination and degradation of the selective serotype. Although these approaches were used in experimental cellular models of BoNT intoxication, these methods have not yet been evaluated at the MNT.

Mutant Toxins as Investigational Tools

Designing and characterizing catalytic activity site mutants of BoNTs that are structurally similar to the holotoxin are a good start point to:

1. Understand the biological half-life and neuronal disposition of the neurotoxin so as to target antidotes to counteract the toxin
2. Develop vaccines using these genetically modified mutant proteins
3. Delineate the pharmacokinetics of BoNTs in cellular and whole animal model systems
4. Determine the interactions between BoNTs and novel signalsomes at the MNT

This knowledge will help in the development of selective antidotes to counteract BoNT intoxication. In this line of thoughts, the safety profile of a catalytically deactivated, nontoxic version of BoNT/A (DrBoNT/A) was evaluated at the MNT (Baskaran et al. 2013). DrBoNT/A is devoid of its endoprotease activity (SNAP-25 cleavage) even at very high concentrations. In vivo hind limb local injections of DrBoNT/A failed to cause neuroparalysis unlike the holotoxin. However, DrBoNT/A was shown to internalize and localize at the MNT similar to the wild-type BoNT/A. Nonetheless DrBoNT/A lacked the zinc binding ability and the catalytic activity to cleave its substrate (Baskaran et al. 2013). These data provide new opportunities not only to expand our knowledge on BoNT/A neurobiology but also to advance the development of novel countermeasure strategies based on the points explained above.

Conclusion and Future Directions

The challenges that impact the development of antidotes against BoNTs include:

1. Differences in concentration of pure holotoxin or its conjunction with neurotoxin-associated proteins (NAP) in research studies

   Experiments performed with BoNTs include a wide range of concentrations that include mouse LD50 units, simply units, and picomoles, which make it difficult to interpret the differences in results obtained. Further, the molarity of the neurotoxin will be different between 150, 300, 500, and 700 kDa toxins. It is important for developing antidotes to establish a consensus on the concentration of toxins to be used for in vitro and in vivo experiments.
2. Dyssynchrony among model systems that are employed in evaluating the efficacy of BoNT antidotes

   Several in vitro (cell-free system, cell-based assays, diaphragm twitch tension, EDL electrophysiology) and in vivo (TSR inhibition and mouse lethality assay) models are used to evaluate the efficacy of antidotes against BoNTs. Dyssynchronous data, gathered from these assays, make their interpretation and validation difficult.

3. Lack of site-specific delivery approach

   A critical factor that accounts for major differences between the effects of antidotes on in vitro and in vivo model systems is the ability of the antidote to reach the site of action to counteract the neurotoxin. Strategies to develop target-site-specific delivery of antidotes to antagonize BoNTs will dramatically improve the efficacy of such antidotes.
4. Knowledge on the pharmacokinetics and bioavailability of antidotes

   Determining the bioavailability and pharmacokinetics of antidotes will help in minimizing any artifacts that affect efficacy studies.
5. Exploring novel mechanisms that involve modulation of other neuronal proteins that retard the activity of the toxin or facilitate its degradation

   Research involved in the intoxication mechanisms of BoNTs has uncovered the role of several proteins that regulate the process of neurotransmitter release and its inhibition by BoNTs. Further, the role of the rise in presynaptic Ca^{2+} in the restoration of exocytosis following intoxication with BoNTs needs to be investigated.

Finally, a multidrug approach to antagonize botulism may arise due to the distinct mechanisms by which the available antidotes antagonize BoNTs. Therefore, it is important to develop research strategies to test the effects of combining drugs against BoNTs. Investigating the role of novel interactomes (proteins that interact with BoNT signaling at the MNT) that are modulated by antidotes will advance the development of a strategy to counteract botulinum intoxication.

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Immunoneutralization of Abrin

18

Shradha Bagaria and Anjali A. Karande

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Abstract

Abrin is a type II ribosome-inactivating protein obtained from the mature seeds of a subtropical plant named *Abrus precatorius*. It is a glycoprotein which arrests protein synthesis in eukaryotes by inactivating ribosomes irreversibly. The heterodimeric protein comprises of the toxic subunit, the A chain which is disulfide bonded to the B chain, a galactose-specific lectin, which helps in binding and trafficking of the toxin in cells. A single molecule of the abrin A chain which reaches the cytosol is sufficient to kill the cell. Owing to its extreme toxicity and ease of purification and dissemination, abrin is considered as a dreaded bioterror agent. In spite of several reports on abrin poisoning, there is no effective antidote or vaccine available against the lethal toxin. An active site mutant of the abrin A chain has been proposed as a potential vaccine candidate against abrin intoxication, though it might not be useful for public at large. However, passive administration of antibodies has served as the primary mode of therapy against a large number of toxins. To date, the monoclonal antibody D6F10 is the only known neutralizing antibody reported against abrin. The antibody can rescue cells as well as mice challenged with lethal doses of the toxin. A recent study has demonstrated that the epitope corresponding to the antibody is present in close proximity to the active site of abrin A chain and the antibody can neutralize abrin-mediated cytotoxicity intracellularly. Humanization of the antibody and a detailed understanding of the trafficking of the abrin-antibody complex are required for its development as therapy, pre- and post-abrin exposure.

List of Abbreviations

ABA	Abrin A chain
APA	*Abrus precatorius* agglutinin
BAT3	HLA-B-associated transcript3
DEAE	Diethylamino ethyl cellulose
EF-2	Elongation factor 2
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ICP	Intracranial pressure
Ig	Immunoglobulin
ITs	Immunotoxins
JNK	cJun N-terminal kinase
kDa	kilo Dalton
LD_{50}	50 % lethal dose
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MKK4	MAPK kinase 4
PCR	Polymerase chain reaction
PDB	Protein data bank

PDI	Protein disulfide isomerase
RCA	*Ricinus communis* agglutinin
RIPs	Ribosome-inactivating proteins
rRNA	Ribosomal ribonucleic acid
RTA	Ricin A chain
SAPK	Stress-activated protein kinase
SEK1	Stress signaling kinase1
TNF	Tumor necrosis factor

Introduction to Abrin

Abrin, obtained from the *Abrus precatorius* plant, is a glycoprotein toxin that arrests protein synthesis by inactivating ribosomes irreversibly (Stirpe 2004). It is a heterodimer comprising of the catalytically active A chain and a lectin-like B chain and is therefore classified as a member of the type II ribosome-inactivating proteins (RIPs). Type II RIPs are mostly of plant origin and differ from bacterial toxins which inhibit protein synthesis through mechanisms other than ribosome inactivation. The heterodimeric toxins like abrin, its well-known sister toxin ricin, shiga toxin of bacterial origin are also referred to as A/B toxins as they are made up of two chains, A (for active) and B (for binding), linked together by a single disulfide bond (Stirpe 2004). The toxic subunit, i.e., the A chain of abrin, is an RNA N-glycosidase which depurinates the universally conserved α-sarcin loop of ribosomal RNAs (Stirpe 2004). The A chain is known to depurinate only eukaryotic ribosomes among which mammalian ribosomes are most sensitive. On the other hand, the B chain is involved in the binding and transport of the toxins in cells (Stirpe and Battelli 2006).

History of Abrin

Abrus precatorius is derived from the Greek words *abrus* (meaning beautiful) and *precare* (means to pray) as the seeds have a nice red color glossy appearance with a black spot, as shown in Fig. 1, and were used to make rosaries (Olsnes 2004). Owing to the durability and uniformity of the hard shelled seeds, they were often used for weighing gold and making cheap jewelry. Humans and birds, who aid in dispersal of abrin seeds, are usually unaffected by the toxicity as the seeds possess a hard shell which remains undigested in the gut and is egested intact from the intestinal tract (Olsnes 2004). The toxicity of the seeds of *Abrus precatorius* (jequirity bean) or *Ricinus communis* (castor bean) is known since time immemorial (Olsnes 2004; Barbieri et al. 1993). It was later found that jequirity bean and castor bean consist of the toxins abrin and ricin respectively. These toxins are accumulated in the matured seeds of their respective plant source and constitute almost 5 % of the dry weight of the seeds (Lord and Spooner 2011). The earliest known reports on

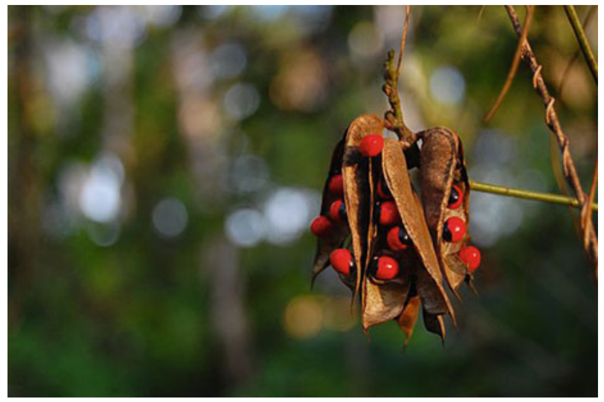

Fig. 1 A picture of the abrin seed pod. The *Abrus precatorius* plant produces multiple pods, each of which typically contains three to five abrin seeds. The ovoid-shaped seeds are *bright red* in color with a *black spot* at the base (Photo credit: Nirmalya Basu)

RIPs come from studies on these two toxins (Stirpe 2004). The term RIP was coined after the discovery that these toxins are proteins and act by inactivating ribosomes. In 1884, it was suggested that abrin is a protein. Later in 1891, Hellin purified abrin and demonstrated that the purified abrin preparation resulted in the agglutination of red blood cells (Olsnes 2004). Further work on RIPs revealed that the observed toxic and agglutination activities of these proteins are due to the presence of two different polypeptide chains. Olsnes and Pihl demonstrated that the A chain (~30 kDa) is the enzymatic subunit, while the B chain (~35 kDa) is a galactose-specific lectin (Olsnes 2004).

In the 1970s, two major events occurred in the history of RIPs that turned the attention of the world towards these toxins. The first was the finding that RIPs are more toxic to tumor cells in comparison to normal cells. Abrin and ricin were thus proposed as novel antitumor substances (Olsnes 2004), and the toxic A chain was coupled to antibodies to specifically target cancer cells (Kreitman 1999). These antibody conjugated toxins, referred to as immunotoxins (ITs), developed into a new class of agents for cancer therapeutics. The ITs inhibited protein synthesis and induced apoptosis when targeted to malignant cells. In a recent study, the recombinant abrin A chain was conjugated to an antibody raised against human gonadotropin-releasing hormone receptor, and the IT was shown to specifically induce cell death in cells expressing the receptor (Gadadhar and Karande 2013). Many studies in the field of ITs have yielded promising results and clinical trials have been carried out. It has been proposed that immunotherapy should be used as combinatorial therapy in conjunction with radiotherapy and chemotherapy (Kreitman 1999). However, it is also observed that ITs are less effective on solid tumors due to their poor penetration (Stirpe and Battelli 2006).

The second event was when ricin became infamous for its criminal use (refer to "▶ Chap. 3, The Biowarfare Agent Ricin"). Since then, several countermeasures have been developed against these deadly toxins (Rainey and Young 2004).

Ricin is the most studied type II RIP, and the findings with ricin are often extrapolated to other related toxins like abrin.

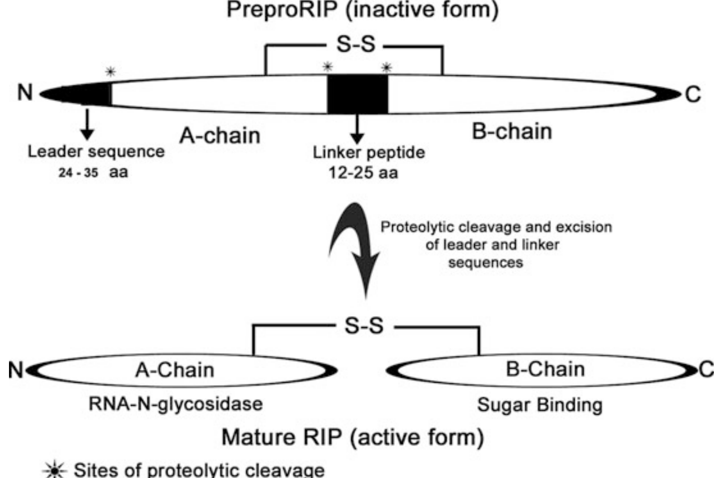

Fig. 2 Processing of prepro-RIPs into mature RIPs. The mature active form of type II RIPs is produced by the excision of the leader sequence/ER signal sequence and the linker peptide present in the precursor polypeptide. The ER signal sequence is removed during translation, while the linker peptide is cleaved by an endopeptidase present in vacuoles (Figure adapted from Ph.D. thesis of Dr. Kalpana Surendranath 2007, Indian Institute of Science, Bangalore)

Synthesis and Role of Type II RIPs in Plants

The type II RIPs are synthesized in plants as single-chain polypeptide precursors referred to as the prepro-form (Fig. 2). These precursors are inactive and consist of the endoplasmic reticulum (ER) signal peptide followed by the A chain, a linker peptide, and finally the B chain (Barbieri et al. 1993). The A and B subunits are connected by a disulfide bond formed between cysteine residues at the C-terminus of the A chain and the N-terminus of the B chain (Barbieri et al. 1993).

In 1991, the preproabrin gene sequence was characterized based on its similarity with ricin (Wood et al. 1991). It was found that preproabrin consists of an N-terminal signal sequence of 34 amino acids, followed by 251 amino acid long A chain, a 14 amino acid linker, and finally the B chain consisting of 263 amino acids (Fig. 2).

The role of RIPs in plants has been explored with respect to plant defense. The toxins are known to deter herbivory and possess antiviral, antifungal, and anti-insecticidal properties though the mechanisms of these effects still remain unresolved (Stirpe 2004). Intensive efforts were made to explore other activities of RIPs. It was found that in addition to the classical RNA N-glycosidase activity, RIPs also cleave adenine from DNA or poly(A) tail containing RNAs. The polyadenosine glycosidase activity was reported for many RIPs.

Intracellular Trafficking of RIPs

Type II RIPs take advantage of the cellular machinery to gain access to their target in cells (Sandvig and van Deurs 2000; Lord and Spooner 2011). The ricin B chain, a galactose-specific lectin, binds to cell surface glycolipids and glycoproteins which end in terminal galactose. The toxin is then endocytosed by clathrin-dependent and clathrin-independent mechanisms which contribute almost equally in the uptake process. In addition to the endosomes and lysosomes, a relatively small fraction (~5 %) of the internalized toxin is transported to the Golgi complex. Intracellular calcium is believed to be involved in regulating the transport of ricin from the endosomes to the Golgi apparatus. Henceforth, the toxin exploits the retrograde pathway in cells to reach the cytosol. In the absence of the KDEL sequence, required for transport to the ER, the toxin hitchhikes onto KDEL-tagged proteins like calreticulin via the galactose-binding property of the B chain. It is believed that the protein disulfide isomerase (PDI) in the ER lumen reduces the disulfide bond between the A and B chains and facilitates partial unfolding of the A chain. The disassembly of the two chains is essential for optimal enzymatic activity. Subsequently, the free A chain disguises as a misfolded protein and utilizes the ER-associated degradation (ERAD) pathway to translocate into the cytosol. The A chain partially unfolds and traverses through the Sec61p translocon to reach the cytosol. A significant portion of the ricin A chain escapes the normal degradative fate owing to its low lysine content. Lysine residues are potential ubiquitination sites, and hence low lysine content reduces the risk of proteasomal degradation of the ricin A chain. Introduction of four additional lysines in the ricin A chain dramatically reduces the cytotoxicity of ricin, and this effect is reversed on the inhibition of proteasomes. It is hypothesized that ribosomes aid the refolding of ricin A chain in the cytosol. After refolding, the toxic A chain inactivates ribosomes leading to inhibition of protein synthesis and finally leads to cell death (Stirpe 2004). It is still not clear whether the B chain is retained in the ER or traverses to the cytosol. Some reports indicate that the B chain might get translocated to the cytosol and exerts additional effects. It is observed that the B chain of shiga toxin can induce DNA cleavage and apoptosis.

Numerous reviews focus on the trafficking of ricin and shiga toxin inside cells (Sandvig and van Deurs 2000; Lord and Spooner 2011). It is assumed that abrin travels inside the cell in a similar manner (Fig. 3). Studies have reported that abrin enters cells via receptor-mediated endocytosis and is processed through the cell's vesicular system (Barbieri et al. 1993). Brefeldin A, a fungal metabolite which blocks Golgi function, was shown to block intoxication by abrin and ricin, confirming the involvement of Golgi in the intracellular processing of the toxins (Hudson and Grillo 1991).

Biological Activities of Abrin

There are two major effects of the toxin on cells: inhibition of protein synthesis and apoptosis.

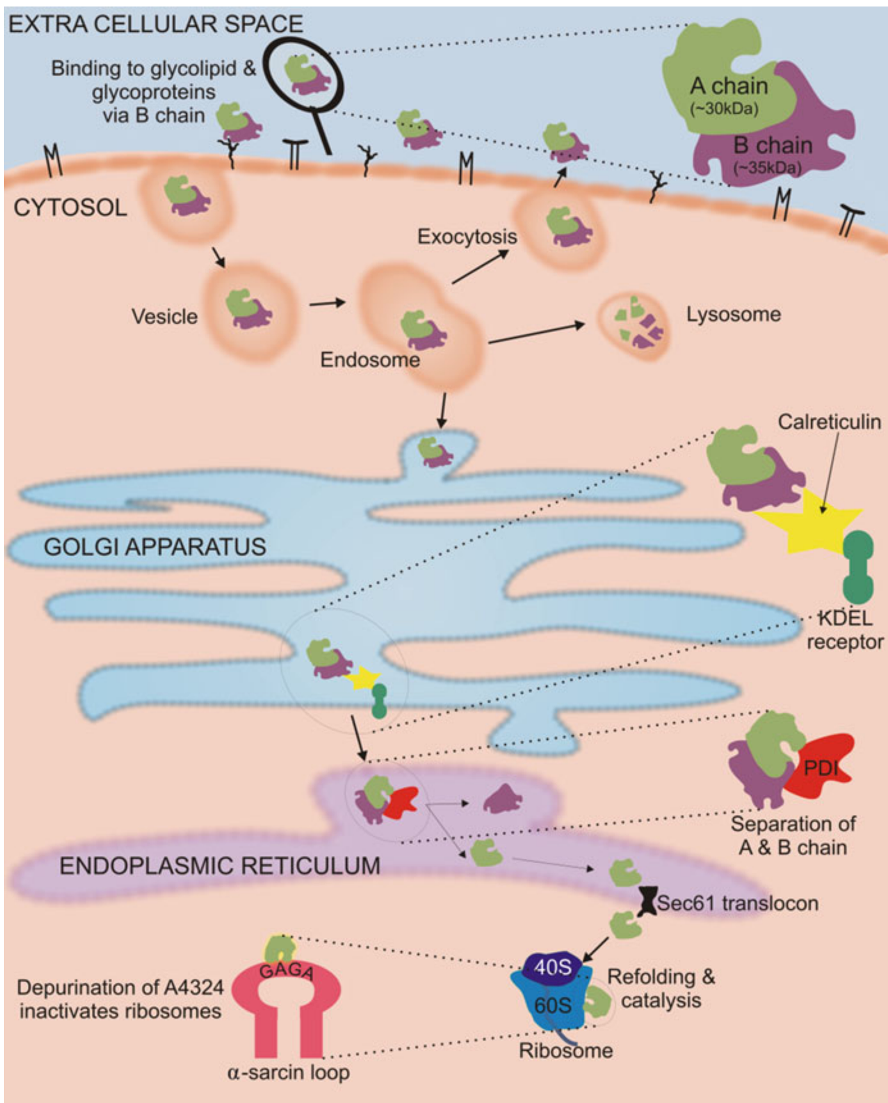

Fig. 3 Schematic representation of the binding and subsequent intracellular trafficking of type II RIPs inside a cell. The B chain binds to terminal galactose on cell surface receptors and the whole toxin is endocytosed. The toxin reaches the Golgi complex and exploits retrograde transport to translocate to the ER. The separation of the A and B chains in the ER by protein disulfide isomerase is followed by the escape of the A chain to the cytosol through the Sec61 translocon. In the cytosol, the A chain depurinates adenine 4324 of the α-sarcin loop in the 60S ribosomal subunit and thereby inhibits protein synthesis irreversibly

Inhibition of Protein Synthesis by Abrin in Cells

The B chain facilitates the trafficking of the toxic A chain to the cytosol where it inactivates eukaryotic ribosomes and thereby inhibits protein synthesis. The inhibition of protein synthesis by ricin in cells was demonstrated in 1971. The observation was later confirmed in vitro by Olsnes and Pihl (Stirpe 2004). The involvement of the elongation factor 2 (EF-2) and the kinetics of ribosomal depurination by abrin and ricin were also elucidated (Olsnes 2004). The mechanism of inhibition of protein synthesis was delineated by Endo et al. in 1987 by studying the action of the ricin A chain on rat liver ribosomes (Endo et al. 1987). The study indicated that the A chain recognizes a highly conserved region, a "GAGA" sequence in the 14 nucleotide long α-sarcin loop (Fig. 3). The α-sarcin loop is sometimes referred to as the "Achilles heel" of ribosomes and is the target for several toxins. It is found approximately 400 nucleotides upstream from the 3′ end of 28S rRNA of the 60S ribosomal subunit. The 28S rRNA is crucial for ribosome function as its sequence forms a part of the binding site for eukaryotic as well as prokaryotic EF-2. The A chain irreversibly cleaves the N-glycosidic bond between adenine and the sugar at position 4324 in the α-sarcin loop (Endo et al. 1987). Since the targeted adenine is present in the middle of the loop, its cleavage destabilizes the loop resulting in inhibition of binding of EF-2 and thus impairs protein synthesis. Several studies have been carried out to determine the kinetics and mechanism of catalysis of the A chain of ricin. It has been reported that one molecule of ricin can inactivate up to 2,000 ribosomes/min. Autoradiographic studies suggest that penetration of a single molecule of abrin is sufficient to kill a cell (Eiklid et al. 1980). The halt in protein synthesis by RIPs is suggested to culminate in apoptosis-like cell death, though the link between the two biological effects still remains elusive.

Abrin-Induced Programmed Cell Death

In addition to inhibition of protein synthesis, abrin can trigger apoptosis in cells. In 1980, a study on HeLa cells demonstrated that abrin, ricin, and modeccin toxins reach the cytosol and kill the cells (Eiklid et al. 1980). It was initially thought that the toxins ricin and abrin induce necrotic cell death. One of the earliest reports that RIPs are capable of inducing programmed cell death or apoptosis came from the studies carried out by Griffiths on rats (Griffiths et al. 1987). Typical markers of apoptosis like chromatin condensation, membrane blebbing, and DNA fragmentation have been observed in cells treated with the toxins (Hughes et al. 1996). Treatment with abrin results in cell lysis of several cells in a manner characteristic of apoptosis. Several studies have established that the type II RIPs like ricin, modeccin, and abrin induce cell death by apoptosis (Narayanan et al. 2005). The observations have been recorded in a variety of cell lines like U937, HeLa, and Jurkat (Narayanan et al. 2004). In HeLa and MCF7 cells, ricin was shown to trigger morphological changes characteristic of apoptosis by caspase-3-mediated

proteolytic activation of BAT3. Studies with shiga toxin also demonstrate enhanced Bax expression and activation of caspases. Similarly, abrin-induced apoptosis was observed to be caspase-3 dependent and followed the intrinsic pathway of apoptosis (Narayanan et al. 2004). It is not yet clearly understood whether the inhibition of protein synthesis by RIPs leads to apoptosis or whether these two are independent events. It was hypothesized that different regions of the A chain can be responsible for inhibition of protein synthesis and apoptosis. Experimental evidence does exist to support the speculation. In 2001, Shih et al. showed that abrin inactivates a thiol-specific antioxidant protein, thereby resulting in the generation of reactive oxygen species which are capable of participating in a wide variety of cellular functions including apoptosis (Shih et al. 2001). Furthermore, reports have shown that isolated B chain of ricin has cytotoxic effects against epithelial cells in rat intestine. Iordanov et al. proposed that the damage of 28S rRNA by ricin resulted in a novel pathway of kinase activation termed as the "ribotoxic stress response," resulting in the activation of SAPK/JNK and its activator, SEK1/MKK4 (Narayanan et al. 2005). It was also shown in the study that activation of SAPK/JNK1 is not just due to inhibition of protein synthesis but also due to signaling from 28S rRNA affected by RIPs. Ricin is also known to induce apoptotic cell death in the macrophage cell line RAW 264.7 with an increased secretion of TNF-α. Pretreatment with the p38 MAPK inhibitor, SB202190, resulted in appreciable drop in the secreted TNF- α levels and also cell death (Narayanan et al. 2005). Another study dealing with abrin treatment on a human B cell line, U266B1, revealed that abrin triggers programmed necrosis in U266B1 cells in a caspase-independent manner (Bora et al. 2010). In U266B1 cells, abrin-mediated necrosis involved production of reactive oxygen species leading to lysosomal membrane permeabilization, resulting in release of cathepsins and subsequently cell death. A recent report also revealed the involvement of the extrinsic pathway of apoptosis upon abrin treatment in Jurkat cells (Saxena et al. 2013). The Fas/Fas ligand pathway was shown to get activated leading to cleavage of caspase-8 and the downstream effector caspase-3. Thus, it should be noted that though abrin inhibits translation in all cell types, it can induce multiple signaling pathways resulting in cell death.

RIPs Obtained from *Abrus precatorius* Plant

A subgroup of type II RIPs, commonly referred to as four-subunit toxins, is tetrameric proteins comprising of two A chains and two B chains (Fig. 4). The four subunit type II RIPs have also been found to be less toxic and better agglutinins when compared to the heterodimeric type II RIPs. Most of the studies on RIPs have been conducted on ricin and abrin, whereas their corresponding agglutinins, namely, *Ricinus communis* agglutinin (RCA) and *Abrus precatorius* agglutinin (APA), have been ignored due to their lower toxicity. In terms of whole animal toxicity, ricin is 2,000 times more cytotoxic to mice than RCA. The 50 % lethal dose (LD_{50}) for abrin is 20 μg/kg body weight, whereas it is 5 mg/kg body weight

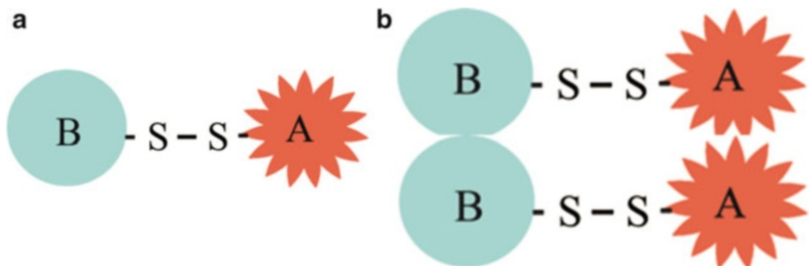

Fig. 4 **Types of type II RIPs**. Type II RIPs are heterodimers (**a**) or heterotetramers (**b**) composed of the catalytically active A chain (*A*) and the lectin-like B chain (*B*)

for APA (Bagaria et al. 2006). It was hypothesized that the lower animal toxicity of the agglutinins might arise due to their aggregation with serum galactoproteins and subsequent precipitation and destruction.

Various isoforms of abrin and the agglutinin (APA) have been purified from the seeds of *Abrus precatorius* plant (Hegde et al. 1991). Several methods like ion exchange, gel chromatography, and Sepharose affinity chromatography have been employed for the isolation of the isoforms. Lin et al. in 1981 isolated four isoforms of the heterodimeric abrin and one agglutinin (APA) and tested for their toxicity. Out of the four isoforms of abrin named abrin-a, abrin-b, abrin-c, and abrin-d, abrin-a was found to be the most toxic, while abrin-b was least active (Lin et al. 1981). The B chains of the isoabrins show substantial conservation, whereas as many as 46 amino acid substitutions were reported in the A chains (Hung et al. 1993). The complete amino acid sequences of the A chain of abrin-a and B chains of abrin-a and abrin-b have been reported (Funatsu et al. 1988; Kimura et al. 1993). Three isoforms of abrin A chains were cloned and expressed in *E. coli* in 1994 (Hung et al. 1994). However, APA which is a heterotetramer was not studied extensively owing to its reduced activity (Lin et al. 1981).

Structure and Mode of Action of Abrin

The active site residues of most of the RIPs are very well conserved and so is their architecture. The structure of ricin was the first to be solved in the family of RIPs. The crystal structure of abrin-a was solved at 2.14 A° resolution by Tahirov in 1995 (Tahirov et al. 1995) (Fig. 5). The overall architecture of abrin was found to be very similar to ricin. Abrin is composed of two polypeptide subunits, the 30 kDa toxic A chain and the 33 kDa lectin-like B chain (Olsnes 2004). The two subunits of abrin are disulfide bonded by the Cys-247 residue of the A chain with Cys-8 of B chain. The B chain of abrin is glycosylated at two sites unlike the A chain which is not glycosylated (Tahirov et al. 1995). The B chain binds to cell surface oligosaccharides ending in terminal galactose and aids the internalization and intracellular trafficking of the toxin inside the cell. The abrin A chain (ABA) is an RNA N-glycosidase and is proposed to have a well-defined active site cleft like ricin

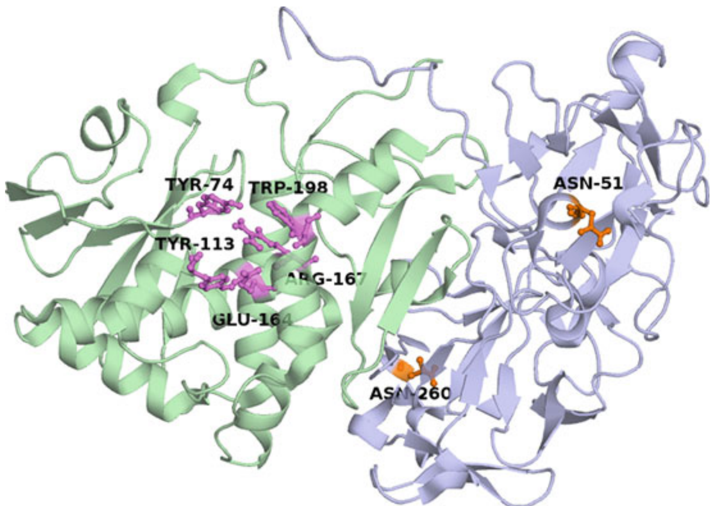

Fig. 5 **Crystal structure of abrin showing the heterodimeric configuration comprising of the A chain (*green*) and the B chain (*purple*)**. The active site residues of the A chain – Tyr-74, Tyr-113, Glu-164, Arg-167, and Trp-198 – are represented as *ball* and *sticks* in *pink* color, while the residues Asn-51 and Asn-260 near the galactose-binding pockets of the B chain are represented in *yellow*. The figure was generated using the open-source PyMOL software (PDB code for abrin: 1ABR)

(Tahirov et al. 1995). Once inside the cytosol, the A chain cleaves the C-N bond of an adenine at position 4324 of the α-sarcin loop (Endo et al. 1987). Depurination of the adenine destabilizes the α-sarcin loop in the 28S ribosomal RNA and results in loss of binding to the elongation factor 2 thereby stalling protein synthesis irreversibly (Endo et al. 1987).

Architecture of the Abrin A Chain

The three-dimensional structure of ABA is divided into three folding domains in accordance with the structure of ricin (Tahirov et al. 1995). The domain 1, present towards the N-terminus, is composed of the residues 1–109. It is rich in β- sheets and consists of a six stranded β- sheet and two helices (Fig. 6). The domain 2, composed of residues 110–197, is the most conserved part between abrin and ricin A chains and comprises of five α- helices. The amino acids 198–251 towards the C-terminus of the protein form the domain 3. A small β- sheet composed of two antiparallel β -strands and two α- helices is present in the third folding domain of ABA (Tahirov et al. 1995).

The A chain of abrin consists of five invariant active-site residues, namely, Tyr-74, Tyr-113, Glu-164, Arg-167, and Trp-198 (Fig. 5) (Chen et al. 1997). These residues are clustered together around the active site cleft and are known to be conserved throughout the wide range of RIPs. Hung et al. in 1994

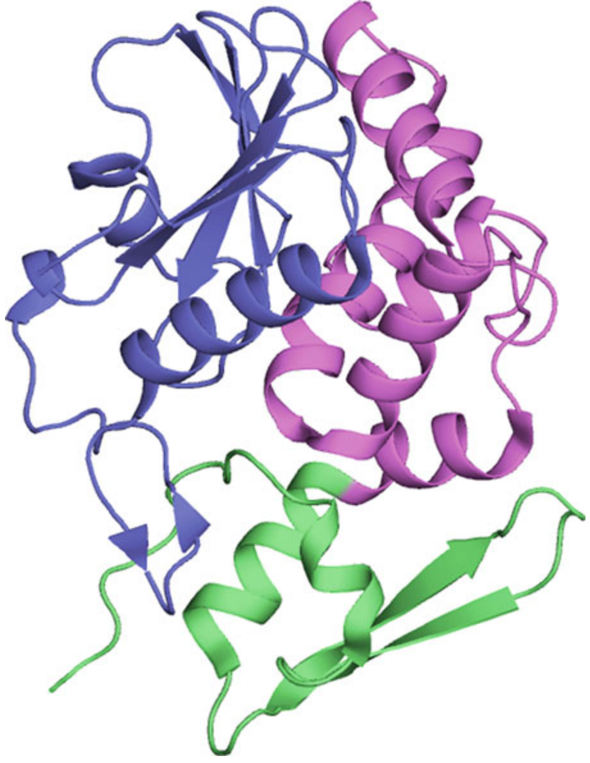

Fig. 6 The three folding domains of ABA. Domain 1 of ABA composed of the amino acids 1–109 is colored *blue*, domain 2 composed of the amino acids 110–197 is colored *pink*, and the domain 3 composed of the amino acids 198–251 is colored *green*. The figure was generated using the open-source PyMOL software

demonstrated that Glu-164 and Arg-167 are essential for catalysis. The protein biosynthesis inhibitory activity of the mutants E164A, R167L, and the double mutant reduced to 25-fold, 625-fold, and 1,250-fold respectively in comparison to wild-type ABA (Hung et al. 1994). The corresponding amino acids Glu-177 and Arg-180 in ricin are hydrogen bonded to each other and involved in coordination of a water molecule, important for the N-glycosidation reaction. The amino acids stabilize the transition state, i.e., the oxycarbonium ring in the catalytic reaction. Furthermore, the residues Tyr-74, Tyr-113, and Trp-198 are involved in substrate binding. The aromatic rings of both tyrosines lie almost parallel in a position appropriate for sandwiching the planar adenine substrate (Tahirov et al. 1995). Mutagenesis of Tyr-74 or Tyr-113 increases the Km significantly, while Kcat drops only slightly, confirming their role in substrate binding (Chen et al. 1997). The authors also analyzed the mutants of the residues Tyr-74, Tyr-113, Glu-164, and Trp-198 for their protein synthesis inhibitory activity and effect on conformation of the protein structure (Chen et al. 1997). It was found that protein synthesis inhibitory activity of Y74F, Y113F, and W198F decreased moderately but was almost abolished completely in case of E164Q. Moreover, the mutants Y74F, Y113F, and E164Q attained almost similar conformation as the wild-type ABA and were not involved in reassociation with the B chain of abrin. On the other hand, a change in conformation was observed for W198F, and it could not reassociate with abrin B

chain. The observations clearly suggested that Trp-198 is involved in substrate binding and is required for the structural integrity of the protein (Chen et al. 1997).

Three-Dimensional Structure of Abrin B Chain

The complete amino acid sequence of abrin B chain was obtained in 1993 (Kimura et al. 1993). The B chain is 267 amino acids long and consists of two homologous globular domains (Chen et al. 1992). Each domain can bind one galactoside and comprises of four subdomains λ, α, β, and γ (Tahirov et al. 1995). The main hydrophobic cylindrical core of the domains is formed by α, β, and γ subdomains, while the λ subdomain connects the two domains. The α subdomain has all the basic elements for galactose binding, and an evolutionary theory for ricin B chain states that the αβγ domain evolved by gene duplication events (Tahirov et al. 1995). The two galactose-binding pockets for ricin have similar folding topologies. Each pocket consists of the conserved tripeptide, Asp-Val-Arg, which are is the key residues involved in hydrogen bonding to the sugar. The authors reported that simultaneous mutations of these residues in both the domains were required to abolish lectin activity of the B chain. It was observed that certain other residues, namely, Lys-40, Asn-46, and Asn-255, aid in hydrogen bonding to the galactose moiety. Studies with the site-directed mutant, N255A of ricin B chain showed ~99 % decrease in binding and toxicity to cells. Chen et al. in 1992 also commented that abrin B chain has two potential galactose-binding sites comprising of the amino acids Asn-51 and Asn-260 (Chen et al. 1992).

Structural and Functional Details of *Abrus precatorius* Agglutinin

Abrus precatorius agglutinin or APA is a homologue of abrin obtained from the same plant source (Lin et al. 1981). Unlike abrin, which is a heterodimer, APA is a heterotetramer comprising of two abrin-like heterodimers. A study in 1991 documented the purification and characterization of APA using lactamyl-Sepharose affinity chromatography followed by gel filtration and DEAE-Sephacel column chromatography (Hegde et al. 1991). The overall folded architecture and all the catalytic residues are completely conserved between abrin and APA A chains (Liu et al. 2000) (Fig. 7). However, the reported LD_{50} dose for abrin is 20 μg/kg body weight as compared to 5 mg/kg in case of APA (Bagaria et al. 2006). In spite of ~70 % sequence identity between the A chains, APA exhibits at least 200-fold lower toxicity when compared to abrin (Bagaria et al. 2006). The primary structure and function analysis of APA A chain suggested that substitution of Asn-200 by Pro-199 in APA can induce bending of the helical structure. This conformational change could presumably lower the binding to the substrate, resulting in weak inhibitory activity (Liu et al. 2000). However, the crystal structure of the ~60 kDa APA heterodimer suggested that the decreased toxicity of APA was not because of a kink induced in the helix. Rather,

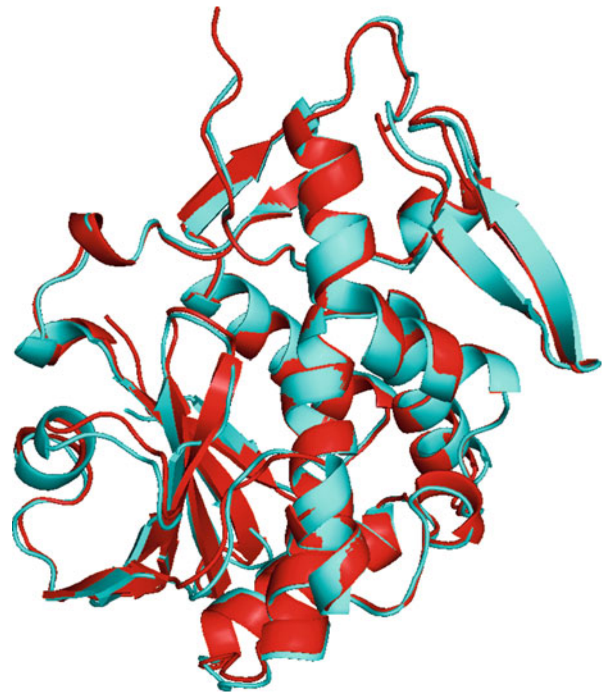

Fig. 7 Superposition of A chains of abrin (*blue*) and APA (*red*). The figure represents the almost identical structures of abrin and APA A chains. The figure was generated using the open-source PyMOL software (PDB code for APA A chain: 2ZR1_A)

the presence of Pro-199 instead of Asn-200 would result in fewer interactions with the substrate (Bagaria et al. 2006). The ~120 kDa APA heterotetramer crystallized in 2010 confirmed that unlike Asn-200, the hydrophobic proline residue at position 199 is unable to form critical hydrogen bond with G4323 of the 28S rRNA (Cheng et al. 2010). The observation explained the lower activity of APA in comparison to abrin.

Abrin as a Potential Biowarfare Agent

Until 2000, it was argued that the use of biological weapons is an unlikely event and public funds should be spent elsewhere. The debate ended with the bioterror attack of anthrax in the USA soon after 9/11 (Atlas 2002). The use of anthrax laden letters shook the world and brought the realization that bioterrorism was not just a threat but could be turned into reality. Since then, an array of literature dealing with development of agents against several plant and bacterial toxins has been published. Various biological weapons which kill people but do not harm infrastructure have been described as “poor man’s neutron bomb” (Atlas 2002). The Centers for Disease Control and Prevention categorized bioterror toxins into A, B, and C based on their ability to cause mass casualties in case of bioterror attacks. The category A toxins include small pox, anthrax, and botulinum toxin and are considered of the “highest priority,” owing to the high mortality and ease of infection.

Abrin and ricin are considered moderately lethal and fall under category B, while the "least priority toxins" constitute the category C.

The plant toxins abrin and ricin are of particular concern as biowarfare agents due to their low cost of isolation and ease of use either as aerosols or contaminants in food or water (Bhaskar et al. 2012). The toxins are quite stable and effective at low doses. Also, there is currently no treatment available for individuals poisoned with these toxins (refer to "▶ Chap. 3, The Biowarfare Agent Ricin"). Several cases of abrin poisoning have been reported from India (Sahni et al. 2007; Sahoo et al. 2008). The diagnosis, clinical features, and prophylaxis in cases of poisoning with jequirity seeds (has abrin and APA) are the same as ricin intoxications. Since both abrin and APA are present in equivalent amounts in large quantities inside the mature seeds of *Abrus precatorius* plant, there is always a high probability of their simultaneous use in biowarfare.

Cases of Poisoning by Abrin and Related Symptoms

Abrin has often been ingested as a means of suicide in India (Subrahmanyan et al. 2008). The toxin is fatal by ingestion, inhalation, or injection, and the lethal dose varies according to the route of administration. The estimated human fatal dose for abrin is 0.1–1 μg/kg and ingestion of one or two crushed seeds is sufficient to cause death (Dickers et al. 2003). Several reports suggest that abrin causes death in cases of accidental and intentional poisoning (Bhaskar et al. 2012; Subrahmanyan et al. 2008; Pillay et al. 2005; Fernando 2001; Sahni et al. 2007). Persons who ingest abrin are known to suffer with acute abdominal pain, multiple episodes of vomiting and watery diarrhea followed by renal failure and seizures (Subrahmanyan et al. 2008). Pulmonary edema, hypertension, and hemolysis of red blood cells have also been reported (Fernando 2001). Death of patients with symptoms of acute demyelinating encephalopathy and raised intracranial pressure (ICP) along with gastrointestinal bleeding due to ingestion of abrin seeds was reported in India (Sahni et al. 2007; Sahoo et al. 2008; Subrahmanyan et al. 2008). In the absence of a drug, supportive and symptomatic treatment was provided to the intoxicated individuals (Fernando 2001). The first line of treatment includes gastric lavage and use of activated charcoal followed by measures to lower ICP (Subrahmanyan et al. 2008).

Recently a study was performed on dose- and time-dependent transcriptome profiling of mouse brain after abrin exposure (Bhaskar et al. 2012). Abrin was injected intraperitoneally and the whole genome microarray data was analyzed. The results revealed significant regulation of various pathways like MAPK pathway, calcium signaling pathway, Jak-STAT signaling pathway, and natural killer cell-mediated toxicity. Neurological symptoms of abrin exposure included delirium, hallucinations, reduced consciousness, and generalized seizures (Bhaskar et al. 2012).

At the biowarfare front, the likely mode of administration of the toxin would be through aerosols. Several studies were conducted to understand the effects of

challenging animals with aerosolized ricin. Rhesus monkeys challenged with ricin present in aerosols died of respiratory distress. Symptoms like acute inflammation of airways, pulmonary edema, necrosis, and lesions in thoracic cavity were observed in rats and monkeys after inhalation of ricin (Brown and White 1997).

Detection and Diagnosis

A comprehensive account of the diagnosis of several toxins like anthrax, ebola, plague, and ricin has been reported (Burnett et al. 2005). Detection of ricin is mostly based on immunoassays. Studies with array biosensors have also been carried out for rapid and simultaneous detection of multiple toxins like cholera toxin, ricin, trinitrotoluene, etc. The technique employs the use of immobilized antibodies to perform sandwich and competitive immunoassays based on fluorescence. Anti-ricin single-domain antibodies have been developed towards better diagnosis of ricin contamination in food matrices. A detection limit as low as 1.6 ng/ml has been achieved through these diagnostic methods. Simultaneous detection of ricin and abrin DNA in contaminated food matrices has also been demonstrated using real-time PCR (Felder et al. 2012). In 2011, a study on llama-derived single-chain antibodies was conducted to detect crude preparation of abrin and APA (Goldman et al. 2011). Monoclonal antibodies with detection limit of 7.8 ng/ml for abrin-a have also been reported recently (Li et al. 2011).

Overview of Antitoxins Reported Against RIPs

Since the small-scale attack with anthrax caused much panic and levied a heavy economic burden, the fear of catastrophic large-scale attacks has driven researchers to focus on developing therapeutics and diagnostics against such bioterror agents (Burnett et al. 2005). Several recombinant vaccines have been developed against leading biowarfare toxins. Earlier studies in the field focused on testing formaldehyde-treated toxoids of ricin and abrin. Several antitoxins have been studied against ricin which include either substrate analogs of toxins or antibodies which work by either preventing toxin processing or translocation (Rainey and Young 2004). The reported substrate analogs of ricin include polynucleotide RNA-based inhibitors which block the rRNA depurination activity of ricin A chain in vitro (Fig. 8) (Burnett et al. 2005). Alternatively, chemical inhibitors which block intracellular trafficking of ricin like Brefeldin A or Golgicide A protected cells from the cytotoxic effects of ricin or shiga toxin, but owing to issues relating to toxicity and delivery of these chemical inhibitors in vivo, they fail to protect animals exposed to these lethal toxins (Burnett et al. 2005). Despite the understanding of the molecular details of the toxins ricin and abrin, the development of effective antidotes against these lethal toxins has proven to be elusive. Monoclonal antibodies have always proven to be promising in immunotherapy, and passive administration of neutralizing antibodies has been employed for the

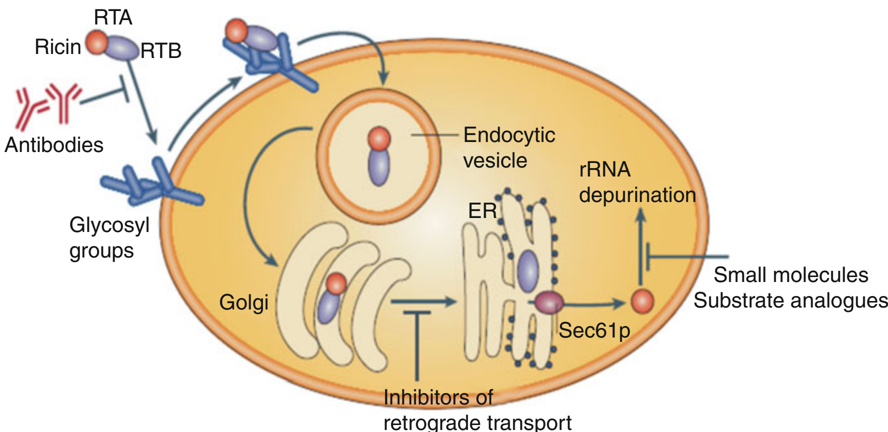

Fig. 8 **The antitoxins tested against ricin intoxication**. The figure represents the mode of action of two main classes of inhibitors tested against ricin. These include substrate analogs of ricin A chain which inhibit the catalytic function of the A chain or antibodies which prevent cell attachment of the B chain (Figure adapted from Rainey and Young 2004)

management of several toxins (Burnett et al. 2005). Pincus in 2011 discussed the available passive and active vaccination strategies to prevent ricin poisoning (Pincus et al. 2011). The most recent advance in the development of vaccination for ricin is RiVax. The vaccine is a double mutant of the recombinant ricin A chain and is already in phase I clinical trials (Pincus et al. 2011). Furthermore, a recent study published in June 2012 claimed that consumption of milk at room temperature can possibly reduce ricin toxicity (Rasooly et al. 2012). While many detection or treatment modalities have been developed for ricin, none have been reported for abrin (Pincus et al. 2011). Moreover, no chemical antidotes have been developed against abrin to date (Bhaskar et al. 2012).

Immunotherapy to Counteract Toxins

In the past, more than 150 agents including cellular membrane effectors, reducing agents, antioxidants, effectors of endocytosis, and ricin analogs have been screened for protection against ricin toxicity (Rainey and Young 2004). However, none of these compounds showed protective efficacy when administered in mice. On the other hand, neutralizing antibodies have proven to be effective against ricin and several other toxins. Monoclonal antibodies (mAbs) have gained increased importance in therapeutics as more number of antibodies are now being humanized. Stress should be laid on stockpiling neutralizing antibodies to protect the human population against biological warfare threats. Passive administration of antibodies is an effective and specific mode of defense against poisoning by various biological toxins like snake venom. Passive protection by antibodies dates back to 1890 when for the first time serum-mediated protection from lethal doses of diphtheria and

tetanus was described. It was also demonstrated that antibodies against fragment B of diphtheria toxin (produced by *Corynebacterium diphtheria*) were neutralizing and acted by preventing cell attachment of the toxin. Since then monoclonal antibodies have routinely been explored for countering several toxins like tetanus and shiga toxin, an A/B toxin produced by *Shigella dysenteriae*. Several studies have suggested that a combination of anti-A chain and anti-B chain neutralizing antibodies could potentially be useful for immunotherapeutic purposes in case of ricin poisoning or even prevention (Pincus et al. 2011; O'Hara et al. 2012).

Immunoneutralization of Abrin

Abrin is known to be 75 times more potent than ricin with an LD_{50} dose of only 2.8 μg/kg body weight of mice (Stirpe and Battelli 2006). Neutralizing antibodies against ricin and *Ricinus communis* agglutinin were reported as early as 1975. Since then numerous neutralizing antibodies have been well characterized for ricin (O'Hara et al. 2012), but up to date, only one neutralizing antibody named D6F10 has been reported against abrin (Surendranath and Karande 2008). It was shown to rescue cells as well as mice from the toxicity of abrin. The antibody rescued abrin-mediated inhibition of translation in human breast cancer cell line, MCF7. Complete inhibition of abrin-induced apoptosis was observed in Jurkat cells treated with abrin in the presence of the antibody. Further, high molar concentrations of the antibody prevented abrin binding on Jurkat cells. These observations suggested that the neutralizing effect of the antibody was due to interference with abrin binding on the cell surface. However, the actual mechanism of the neutralizing effect of the mAb D6F10 has been resolved only recently (Bagaria et al. 2013). The protective effect of the antibody was also observed in vivo. Hundred percent survival was observed in mice administered with 250 μg of the antibody, 1 h prior to challenge with lethal doses of abrin (1.25 μg), injected intraperitoneally (Surendranath and Karande 2008).

Epitopes of Neutralizing Antibodies Reported Against Ricin and Abrin

Delineating epitopes of neutralizing antibodies is important to gain a better understanding of the mechanisms involved in the immunoneutralization of toxins by the antibodies and also for developing better rationale for design of vaccines against toxins. A study aimed at predicting a conserved neutralizing epitope in ribosome-inactivating proteins suggested that owing to high sequence variability, RIPs would possess distinct neutralizing epitopes and their antibodies might not cross-react. The neutralizing mAb D6F10, raised against the recombinant abrin A chain, was also found to be specific for abrin as it failed to recognize ricin or APA (Surendranath and Karande 2008). Knowledge of the epitopic regions would also help in developing strategies for improved immunotherapeutics by

immunoconjugates of antibodies and A chain of A/B toxins. In a study published in 2004, a dominant linear B cell epitope of ricin A chain (spanning amino acids L161-I175) was reported to be the target for neutralizing response in Hodgkin's lymphoma patients treated with anti-CD25 immunotoxin (O'Hara et al. 2012). O'Hara et al. provided a detailed study of the protective antibodies targeting different folded domains of RTA. They suggested that 88 % neutralizing epitopes were conformation dependent or discontinuous as they failed to react with the 12-mer peptide library spanning entire length of RTA. In spite of the pressing need for more information on neutralizing epitopes of toxins like ricin or abrin, very few discontinuous epitopes have been reported till date. GD12, a murine monoclonal IgG1 specific to residues T163 to M174 (TLARSFIICIQM) of ricin A chain (RTA), was shown to protect mice from intraperitoneal and intragastric ricin challenge (O'Hara et al. 2012). Another hybridoma named UNIVAX 70/138 has been reported to produce mAb that neutralized ricin toxicity in vitro and in vivo. The neutralizing mAb R70 has been characterized well and is shown to recognize the amino acids 97–108 of RTA (O'Hara et al. 2012). In contrast to the available reports of anti-A chain antibodies to ricin, there are hardly any reports on ricin B chain-specific epitopes (O'Hara et al. 2012). Furthermore, it has been suggested that epitope specificity and not isotype or antibody affinity or Fc-mediated clearance is the main determining factor in immunoneutralization of ricin (O'Hara et al. 2012). Also, subtle differences in epitope specificity are known to determine whether an antibody is neutralizing or not. These studies stress the importance of mapping epitopes of neutralizing antibodies in order to gain a better understanding of immunoneutralization of lethal toxins such as abrin and ricin.

The only known neutralizing mAb to abrin, D6F10 prevented binding of abrin to cells. Thus, it was hypothesized that the antibody might bind towards the C-terminal domain of ABA, which is present in close proximity to the B chain and would thereby sterically hinder the binding of the B chain to the cell surface (Surendranath and Karande 2008). In order to gain insights into the molecular basis of neutralization of abrin by mAb D6F10, the epitope corresponding to the antibody was mapped (Bagaria et al. 2013). A recent study employed the use of recombinant chimeric proteins composed of parts of ABA and APA A chains to delineate the epitopic region of mAb D6F10. It was revealed that the epitope corresponding to the mAb D6F10 is conformation dependent and the amino acids 74–123 of abrin A chain contain the core epitope for the antibody. The residues Thr112, Gly114, and Arg118 of ABA were found to be crucial for binding to the antibody (Bagaria et al. 2013). In silico analysis of the position of the epitope revealed that it is present in close proximity to the active site cleft of ABA.

Mechanism of Neutralization of Abrin by the mAb D6F10

A recent study focused on delineating the mechanism of immunoneutralization of abrin by the mAb D6F10. The epitope corresponding to the antibody was found to be present in close proximity to the active site cleft of abrin A chain (Bagaria

et al. 2013). The observation suggested that the binding of the antibody near the active site might occlude the entry of the substrate, thereby blocking the enzymatic activity of abrin A chain and rescuing the inhibition of protein synthesis by the toxin in vitro. Further, the epitope was found to be spatially far from the B chain, indicating that binding of the antibody to the A chain should not prevent the B chain from binding to the cell surface (Bagaria et al. 2013). Interestingly, the inhibition of cell surface binding of abrin was observed only at very high molar concentrations of the antibody (at least 50- to 100-fold molar excess). It has been hypothesized that the glycans of the antibody might interfere with cell attachment of abrin via its B chain. Also, at 1:10 molar concentration of abrin-antibody, the antibody D6F10 rescued cells from abrin-mediated inhibition of protein synthesis but did not prevent cell attachment of abrin. The internalization of the antibody D6F10 bound to abrin was also observed in HeLa cells, suggesting that the antibody might rescue abrin-mediated cytotoxicity intracellularly (Bagaria et al. 2013). It is possible that the antibody directly blocks the active site of abrin or interferes with the intracellular trafficking of the toxin. The study is the only available report on the neutralizing epitope for abrin and provides mechanistic insights into the poorly understood mode of action of anti-A chain antibodies against several toxins including ricin. A very recent study has reported that an antibody against ricin A chain delays internalization and hinders intracellular routing of ricin. These studies conclusively demonstrate that the major site for neutralization of the toxin is intracellular and stress the importance of anti-A chain antibodies for toxin neutralization.

Potential Vaccine Candidates Against Abrin

Passive administration of antibodies has served as the primary mode of therapy against a large number of toxins. Though, a key concern is whether the administration of antibodies, post-toxin exposure would provide protection or not (Pincus et al. 2011). On the other hand, active immunization against potential biowarfare agents might not be useful for public at large but can prove to be beneficial for individuals at high risk of exposure of these lethal toxins.

In spite of the high toxicity of abrin and its potential threat in biowarfare, there are hardly any reports for countermeasures against abrin intoxication (Rainey and Young 2004). In 2011, an active site mutant of ABA was proposed as an effective vaccine as it protected mice challenged with abrin (Han et al. 2011). However, the vaccine was developed in the absence of prior knowledge regarding the immunodominant regions of ABA. The key catalytic active site residues of ABA, Glu164, and Arg167 were mutated which dramatically reduced the cytotoxicity, but immunogenicity of the mutant protein remained same as wild-type ABA. Three vaccinations with the double mutant resulted in 100 % protection of mice, administered intraperitoneally with 10 times the lethal dose of native abrin (Han et al. 2011). A chimera between ABA and APA A chain, $ABA_{1\text{-}123}APA_{124\text{-}175}$, composed of first 123 amino acids of ABA and the amino acids 124–175 of APA A

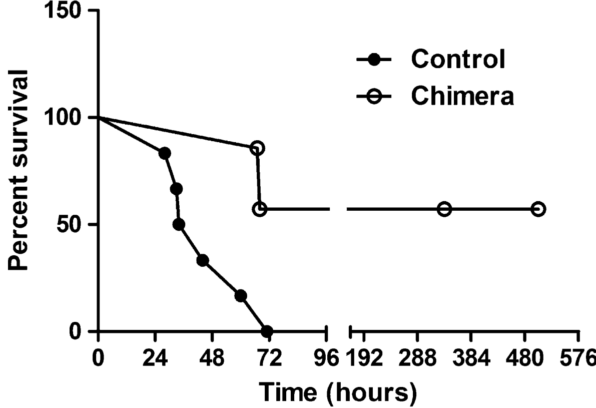

Fig. 9 **Survival of control and immunized mice with the chimeric protein $ABA_{1\text{-}123}APA_{124\text{-}175}$ post-abrin challenge**. Six mice each were injected with adjuvant alone or adjuvant along with the recombinant protein $ABA_{1\text{-}123}APA_{124\text{-}175}$. After administration of two booster doses, all mice were injected intraperitoneally with 5 X LD_{50} dose of abrin and their survival time post-abrin challenge was recorded up to 3 weeks

chain (in accordance with sequence alignment with ABA), has also been proposed as a vaccine candidate against abrin intoxication (Bagaria and Karande, unpublished data). The recombinant protein comprises of primarily the first two folding domains of the protein, known to harbor most of the neutralizing epitopes on ricin. Also, the chimera possesses at least 50-fold lower catalytic efficiency than wild-type abrin A chain and is soluble unlike recombinant wild-type ABA composed of the first 175 amino acids. In preliminary studies, 50 % survival was seen in mice administered with two doses of the vaccine (Fig. 9). Studies are underway to refine the chimera as a vaccine candidate.

Conclusions and Future Directions

Abrin is 75 times more potent than the well-studied toxin ricin and its potential use in biowarfare is a cause of major concern. Dozens of antitoxins have been reported against ricin, but there are hardly any reports for countermeasures against abrin intoxication. Stockpiling of effective neutralizing antibodies and vaccines might serve as deterrents against misuse of lethal toxins like abrin and ricin. The mAb D6F10 rescues abrin toxicity completely in cells and mice when administered before abrin exposure or along with the toxin. Studies dealing with the mechanism of immunoneutralization by the antibody suggest that it rescues abrin-mediated toxicity intracellularly which highlights the possibility of its use post-abrin exposure. The antibody could be delivered inside the cells by encapsulating in liposomes for postexposure treatment of abrin intoxication. Better understanding of the trafficking of the abrin-antibody complex and determination of the effective window for administration of the mAb D6F10 pre- and post-abrin exposure is essential for

its development as therapy. The murine antibody can possibly be humanized for improved efficacy in therapeutics. However, the stressing need for developing alternative modes of therapies for neutralization of cytotoxicity of abrin post-intoxication cannot be ignored. Active immunization could prove to be beneficial for individuals at high risk of exposure of these lethal toxins. Clinical trials for the active site mutant of ABA should be performed to develop it as a vaccine against abrin. Comparative analysis of the efficacy of neutralization of abrin/mixtures of abrin and APA in mice immunized with the chimera $ABA_{1\text{-}123}APA_{124\text{-}175}$ versus full-length active site mutant of ABA needs to be carried out. It is possible that the chimera would provide better protective efficacy against intoxication with whole seed extracts containing mixtures of abrin and its homolog APA.

Cross-References

► Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment
► The Biosecurity Threat Posed by Biological Toxins
► The Biowarfare Agent Ricin

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19 Combating the Insidious Enemy: Epidemiology, Pathophysiology, and Treatment of Clostridial Gas Gangrene

Biplab Giri and Labanyamoy Kole

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Abstract

Clostridial gas gangrene is a life-threatening condition having dreaded features like myonecrosis, gas production, and sepsis and is usually caused by *Clostridium perfringens* which exists in the soil and as part of the gastrointestinal flora of humans. The disease is a noncommunicable one and the infection generally occurs in traumatic wounds with soil contamination and surgery involving the bowel; nonetheless, a spontaneous gangrene is also reported. Common characteristics of inciting events include contamination of the site with *Clostridium* sp. and devitalization of tissue. Areas with low oxygen concentration are ideal for these infections; anaerobic conditions impel *Clostridium* sp. to convert itself from spore to the vegetative form which produces toxins, say α-toxin and θ-toxin which in a row are responsible for tissue damage and systemic manifestations. On successful infection, it destroys RBCs, platelets, and polymorphonuclear leukocytes (PMNs), causing widespread cell membrane and capillary damage which establish the typical pathophysiology. Initially the wound becomes swollen and the affected skin primarily appears to be blistered with hemorrhagic bullae, red and feels warm with pain upon touching before progressing to a bronge, brown or black color. Foul-smelling brown-red or bloody discharge drip from the affected tissues or wound and with time signs of sepsis, toxemia, septic shock, and multiorgan failure become evident. For managing the infected patients, an early diagnosis is crucial. Other successive indispensible measures include aggressive resuscitation, surgical debridement, antibiotic therapy, and supportive intensive care. Delays in doing so increase tissue loss and mortality which are directly proportional to time of intervention.

Introduction

Gangrene is a serious and life-threatening condition that occurs when a considerable mass of body tissue dies by means of necrosis. It is caused by a loss of blood supply due to an underlying illness, injury, and/or infection. Fingers, toes, and limbs are affected most often, but gangrene can also occur inside the body, damaging organs and muscles. There are different types of gangrene with different symptoms, such as dry gangrene, wet gangrene, gas gangrene, internal gangrene, and necrotizing fasciitis (Carol 2007).

Gas gangrene is a bacterial infection that produces gas within tissues. It is a deadly form of gangrene usually caused by a bacterial exotoxin producing clostridial species, mostly found in soil, and other anaerobes (e.g., *Bacteroides* and anaerobic streptococci). The three most common species responsible for myonecrosis are *Clostridium perfringens*, *Clostridium septicum*, and *Clostridium novyi* (Fernandez and Gluck 1994). These environmental bacteria may enter the

muscle through a wound and subsequently proliferate in necrotic tissue and secrete powerful toxins. These toxins destroy nearby tissue, generating gas (5.9 % hydrogen, 3.4 % carbon dioxide, 74.5 % nitrogen, and 16.1 % oxygen), which was reported in one clinical case (Chi et al. 1995).

These infections occur in both military and civilian settings. The disease can be traumatic/postoperative, the most common form accounting for 70 % of the total cases worldwide or spontaneous/nontraumatic gangrene. This life-threatening local and systemic complication frequently occurs mainly due to bacterial infection at the site of penetrating and perforating wounds. Infection spreads rapidly as the gases produced by bacteria expand and infiltrate healthy tissue in the vicinity. The hallmarks of this disease are rapid onset of myonecrosis with muscle swelling, severe pain, gas production, and sepsis. Progression to toxemia and shock is often very rapid (Moustokas et al. 1985). Because of its ability to quickly spread to surrounding tissues, gas gangrene should be treated as a medical emergency. Treatment options include debridement (or, in severe cases, amputation) of the affected body parts, antibiotics, vascular surgery, maggot therapy, and hyperbaric oxygen therapy.

Types of Gangrene

In general, clostridial soft-tissue infections may be categorized into three classes: gas gangrene, anaerobic cellulitis, and superficial contamination. The most fatal is gas gangrene that requires prompt, often severe treatment. Frequently encountered clostridia infections are much less acute and require much less radical treatment; however, they may share some similarities with gas gangrene which complicates differential diagnosis. Gas gangrene producing clostridia primarily damages muscle and impairs blood supply resulting in discolored and edematous tissues with a pungent foul smell and gas bubbles. On accumulation of exudates and increased gas production, the clostridia multiply within the necrotic tissue, releasing more toxins into the tissue and the systemic circulation. On the other hand, anaerobic cellulites is a condition where clostridia invade only tissue that is already dead and the infection does not spread to healthy, undamaged tissue. The least severe type of clostridia infections is the superficial contamination, which involves infection of necrotic tissue where the pain experienced is minimal and wound healing is rapid. Typically, two major types of gangrenes are encountered among patients: dry gangrene and wet gangrene.

Dry gangrene: More common in people with diabetes and autoimmune diseases, dry gangrene usually affects the hands and feet. It develops when blood flow to the affected area is impaired, usually as a result of poor circulation. Unlike other types of gangrene, infection is typically not present in dry gangrene. However, dry gangrene can lead to wet gangrene if it becomes infected.

Wet gangrene: Unlike dry gangrene, wet gangrene almost always involves an infection. Injury from burns, or trauma where a body part is crushed or squeezed, can rapidly cut off blood supply to the affected area, causing tissue death and increased risk of infection. It is called “wet” because of pus formation. Infection from wet gangrene can spread quickly throughout the body, making wet gangrene a very serious and potentially life-threatening condition if not treated quickly.

Types of wet gangrene include the following:

Internal gangrene: If gangrene occurs inside the body, then it is referred to as internal gangrene. This is usually related to an infected organ such as the appendix or colon. Colon cancer is one of the predisposing factors for developing internal gangrene.

Fournier’s gangrene: Also a rare condition, Fournier’s gangrene is caused by an infection in the genital area. Men are affected more often than women. If the infection gets into the bloodstream, a sepsis-like condition develops which can be life threatening.

Gas gangrene: Gas gangrene is rare, but dangerous. It occurs when infection develops deep inside the body, such as inside muscles or organs, usually as a result of trauma. The bacterium that causes gas gangrene, called clostridia, release dangerous toxins or poisons that wreak havoc throughout the body along with gas which can be trapped within body tissue. Gas gangrene warrants immediate medical treatment. Without treatment, death can occur within 48 h.

Etiology

In humans, few clostridia species normally exist in the gastrointestinal tract and in the female genital tract, although they occasionally are isolated from the skin or the mouth. Of the known species of the genus *Clostridium*, at least 30 have been isolated from human infections. Like several other pathogenic anaerobic bacteria species, clostridia are quite aerotolerant, but they do not grow on artificial media in the presence of oxygen. Clostridia characteristically produce abundant gas in artificial media and form subterminal endospores. *Clostridium perfringens*, one of the most important species, is encapsulated and nonmotile and rarely sporulates in artificial media; the spores can usually be destroyed by boiling. Clostridia are present in the normal colonic flora at concentrations of 10^9–10^{10}/g. Of the ≥30 species that normally colonize humans, *C. ramosum* is the most abundant and is followed in frequency by *C. perfringens*. These organisms are universally present in soil at concentrations of up to 10^4/g. *C. perfringens* strains are classified (on the basis of their production of several lethal toxins) into five types, designated A through E. Type A predominates in fecal flora of humans as well as in soil, whereas the habitats of types B through E are thought to be the intestinal tracts of other animals. Although clostridia are gram-positive organisms, many species may

appear to be gram-negative in clinical specimens or stationary-phase cultures. Therefore, the result of Gram's staining of cultures or clinical material should be interpreted with great care. *C. perfringens* is the most common clostridia species isolated from tissue infections and bacteremias; next in frequency are *C. novyi* and *C. septicum*. In the category of enteric infections, *C. difficile* is an important cause of antibiotic-associated colitis, and *C. perfringens* is associated with food poisoning (type A) and enteritis necroticans (type C) (Borriello 1995; Lorber 2000). The complex etiology of this disease may also be confounded by multiple types of strains that play different roles during the disease process. There still remains many questions regarding these complicated diseases and the organisms that cause them.

Predisposing Risk Factors

Age – Gangrene is much more common in older people. People older than 60 years of age are diagnosed with gangrene more often than younger people. As people gets older, their heart and blood vessels change. Blood may have a harder time getting to the tissues that need it most. This raises the risk for gangrene.

Diabetes – The high blood sugar levels, which are common in diabetes, may eventually damage the nerves, especially in the feet. When the nerves are damaged, the patient does not feel pain and will not know if he/she has an injury. The patient may continue walking without protecting the wound. The wound may get worse and develop into a foot ulcer. High blood sugar levels may also damage blood vessels, resulting in poor blood supply to the area. Less blood means less nutrients and oxygen for the tissue cells and fewer white blood cells and T cells to fight off infection. The ulcer becomes infected; the infection grows rapidly and gangrene develops. The oxygen-/nutrient-deprived cells are weak and rapidly die.

Obesity – Obesity is linked to health factors related to gangrene including diabetes and vascular disease. The stress of extra weight may constrict arteries which also reduces blood flow. Reduced blood flow increases the risk of infection and causes wound to heal poorly.

Vascular diseases – Diseases of the blood vessels, such as atherosclerosis (narrowed arteries), and blood clots can result in poor blood flow to various parts of the body.

Injury or surgery – Anything which wounds the skin and tissues below will raise the risk of gangrene. People with underlying conditions which may affect blood flow who also wound their skin run an even higher risk. Approximately 40 % of wet gangrenes are caused by infections that occur during surgery and about 50 % are caused by serious traumatic injuries. Gangrene from frostbite and gunshot wounds are less common than from automobile accidents, crush injuries, burns, and industrial accidents.

Weakened immune system – People with weakened immune systems, such as those with AIDS, patients receiving chemotherapy or radiotherapy, as well as organ transplant recipients who are on immune suppressants, are more susceptible to the complications of infection, which include gangrene.

Smoking – Smoking causes the blood vessels to narrow, resulting in less blood flow.

Other factors – Reynaud's disease, trauma, blood clots, appendicitis, hernia, animal bites, compound fractures, foreign bodies, frostbite, thermal or electrical burns, subcutaneous or intravenous drug use, drinking alcohol, pressure sores, motor vehicle crashes, postoperative, gastrointestinal tract surgery, genitourinary tract surgery, abortion, amputation, tourniquets, casts, bandages, or dressings applied too tightly may also add to the risk of developing gangrene. The disease process must include tissue inoculation and a low oxygen tension environment. More than 50 % of cases are preceded by trauma. Other cases occur spontaneously or in patients after operative procedures (Davoudian and Flint 2012).

Pathogenesis

Mode of Transmission

Bacterial infection of the soft tissues occurs in a variety of roots, viz., soil contamination of the wounds, surgery involving the bowel or biliary system, unhygienic injection of medications, or by the intake of contaminated food. Food-borne illness acquired by ingestion of large number of *C. perfringens* vegetative cells present in the food. Food sources are usually cooked meat, contaminated pork meat, vegetables, fish, or poultry dishes which have been stored at ambient temperatures for a long time after cooking. Infection can occur through contamination of wounds (fractures, bullet wounds) with dirt or any foreign material contaminated with *C. perfringens* with an incubation period of 8–24 h (Fig. 1) (Ryan 2004). It is not communicable from person to person but can be transmitted from animals to humans only through food. Spores can survive in soil, crevices, food, decaying vegetation, marine sediments, internal cavities, and in the anaerobic conditions inside the meat rolls, animal carcasses, feces, and dehydrated and cooked food. Spores are resistant to most disinfectants, heat, and gamma-irradiation; when susceptible, they require longer contact time. Enterotoxin is heat labile and can be inactivated by heat treatment at 60 °C for 5 min. Vegetative cells can be rapidly killed by dry heat at 160–170 °C for 1–2 h or moist heat at 121 °C for 15–30 min. There is no known vector for the transmission of the disease (Songer 2010).

Bacteriology of Pathogenicity

The microbiology of gas gangrene is an exceptionally intricate problem, far from being entirely implicit till date. *Clostridium perfringens* was first described by Welch and Nuttall in 1892 and is at once the commonest, the most important, and the best known of the gas gangrene clostridia. In a smear made from an acute case, *C. perfringens* appears as a short, plump, gram-positive rod with rounded ends and a well-marked capsule (MacLennan 1962). Histotoxic clostridium is a

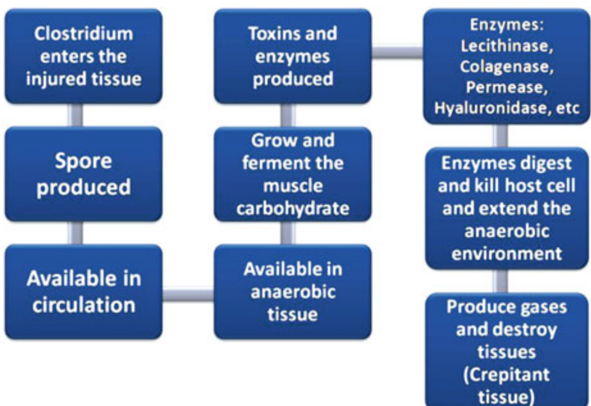

Fig. 1 Mode of bacterial entry into the host tissue and its pathogenicity. The causative form of gas gangrene enters into the body through the mechanically injured site and enters in the tissue. It produces a large number of spores in tissue mass and subsequently enters into the blood stream. In deep tissue, an anaerobic physiological environment will help them to start fermentation using muscle carbohydrate. At this stage, it produces a large number of toxins with different enzymatic activities which digest and kill the adjacent cells and tissues simultaneously with producing gas and enhancing the anaerobic environment

Table 1 Involvement of *C. perfringens* types and its connection with animal and human diseases

Type	Major toxins	Disease link
A	α, θ	Animal and human gas gangrene, fowl necrotic enteritis, bovine enterotoxaemia, human food poisoning, horse colitis, hemorrhagic gastroenteritis
B	α, β, ε	Dysentery, enterotoxaemia of lamb, sheep, goats
C	α, β	Human necrotic enteritis, sheep and calves enterotoxaemia
D	α, ε	Enterotoxaemia of sheep, goats, cattle, and human
E	α, ι	Enteritis of rabbit, enterotoxaemia of calves and lambs

spore-forming, saprophyte, nonmotile organism which is invariably found where decaying organic matter is present, commonly found in soil and in the intestines of humans and other animals. The species has been divided into five distinct types, A–E. Of these subgroups, *C. perfringens* type A causes the majority of human infections. Although classified as an anaerobe, *C. perfringens* is somewhat aerotolerant, one may say it a facultative type of bacterium. Under optimal conditions, its generation time can be as little as 8–10 min, and growth is accompanied by abundant gas production (Stevens and Bryant 2002). The ability of the bacterium to cause infection is ascribed chiefly to the production of an array of potent extracellular protein toxins. The so-called major toxins (a-, b-, e-, and i-toxins) are not necessarily produced in large quantities, but the differential production of these toxins is used to assign strains into one of five biotypes. These biotypes are associated with different diseases of humans and animals (Table 1) (Titball 2005).

Mode of Pathogenicity

In spite of the isolation of clostridia species from many serious traumatic wounds, the prevalence of severe infections due to these organisms is low. Two factors that appear to be essential to the development of severe disease are tissue necrosis and a low oxidation-reduction potential. *C. perfringens* requires about 14 amino acids and at least six supplementary growth factors for optimal growth. These nutrients are not found in appreciable concentrations in normal body fluids but are present in necrotic tissue. When *C. perfringens* grows in necrotic tissue, an area of tissue damage due to the toxins elaborated by the organism allows progressive growth. In contrast, when only a few bacteria leak into the bloodstream from a small defect in the intestinal wall, the organisms do not have the opportunity to multiply rapidly because blood as a medium for growth is relatively deficient in certain amino acids and growth factors. Therefore, in a patient without tissue necrosis, bacteremia is usually benign.

C. perfringens possesses at least 17 possible virulence factors, including 12 active tissue toxins and enterotoxins. The enterotoxins include four major lethal toxins: α, β, ε, and ι. The α-toxin is a phospholipase C (lecithinase) that splits lecithin into phosphatidylcholine and diglyceride (Fig. 2). It has been associated with gas gangrene and is known to be hemolytic, to destroy platelets and polymorphonuclear leukocytes (PMNs), and to cause widespread capillary damage. When injected intravenously, it causes massive intravascular hemolysis and damages liver mitochondria. The α-toxin may be important in the initiation of muscle infections that may progress to gas gangrene. Experimentally, the higher the concentration of α-toxin in the culture fluid, the smaller the dose of *C. perfringens* required to produce infection. The protective effect of antiserum is directly proportional to its content of α-antitoxin. Studies suggest that θ-toxin, a thiol-activated cytolysin that is also called perfringolysin O, may also play an important role in pathogenesis by promoting vascular leukostasis, endothelial cell injury, and regional tissue hypoxia. The resulting perfusion defects extend the anaerobic environment and contribute to rapidly advancing tissue destruction. A characteristic pathologic finding in gas gangrene is the near absence of PMNs despite extensive tissue destruction. Experimental data indicate that both α- and θ-toxins are essential in the leukocyte aggregation that occurs at the margins of tissue injury instead of the expected infiltration of these cells into the area of damage. Genetically altered strains induce less leukocyte aggregation when α-toxin is absent and none when θ-toxin is missing. The other major toxins, β, ε, and ι, are known to increase capillary permeability (Kasper and Madoff 2005; Stevens and Bryant 2002).

Epidemiology

Clostridia species are ubiquitous and widely distributed in the soil, especially in cultivated land. The density of clostridia in the soil is a contributing factor in the development of trauma-related gas gangrene. In the USA, civilian cases of gas

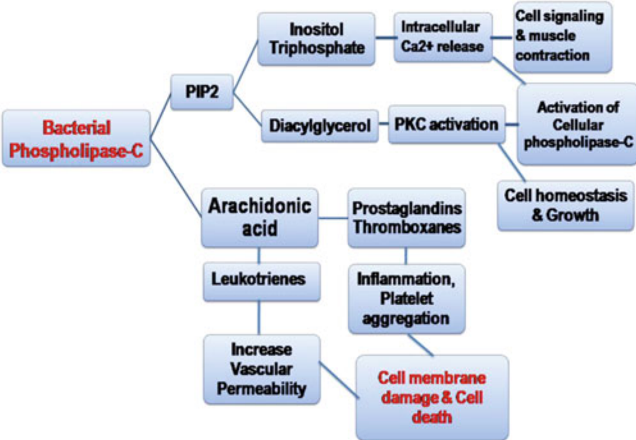

Fig. 2 Role of alpha-toxin on eukaryotic cell signaling and cell death. At sublytic concentrations, *C. perfringens* alpha-toxin causes accumulation of diacylglycerol leading to activation of protein kinase C (*PKC*) which subsequently activates cellular phospholipase C and ultimately can lead to partial cell membrane damage. Clostridial phospholipases C also able to stimulate the arachidonic acid cascade. Arachidonic acid in one hand produces leukotrienes which increase vascular permeability; it also produces prostaglandins and thromboxanes which start inflammatory cascade of reactions which ultimately leads to cell damage and cell death

gangrene are more common, with approximately 3,000 cases per year. Gas gangrene can be classified as posttraumatic, postoperative, or spontaneous. Posttraumatic gas gangrene accounts for 60 % of the overall incidence; most cases involve automobile collisions (Brown and Kinman 1974). Approximately 50 % of wet gangrene cases are the result of a severe traumatic injury, and 40 % occur following surgery. Car and industrial accidents crush injuries, and gunshot wounds are the most common traumatic causes. Because of prompt surgical management of wounds with the removal of dead tissue, the incidence of gangrene from trauma has significantly diminished. Approximately two-thirds of cases affect the extremities, and the remaining one-third involves the abdominal wall. According to the US Agency for Healthcare Research and Quality, 45,400 Americans were hospitalized for gangrene in 2003, compared to 21,000 in 1991. From 1998 to 2002, *C. septicum* was implicated in causing serious infections in recipients of contaminated musculoskeletal-tissue allograft (Fisher et al. 2005).

Several users of injection drugs in Scotland, Ireland, and England developed serious clostridia infections (*C. novyi* and *C. perfringens*) complicated by a high mortality rate (97 %). Most of these patients reported injecting heroin intramuscularly within the previous 2 weeks. With more than 200,000 liposuctions performed in Germany in 2003, several serious complications had been reported. Necrotizing fasciitis and gas gangrene were the most frequent, major, and lethal complications observed in a review of 72 cases of complications caused by liposuction performed in Germany between 1998 and 2002 (Lehnhardt et al. 2008). A tsunami ravaged Indonesia in December 2004 and killed more than 200,000 Indonesians. Soaking in

contaminated water, several injured persons later died of tetanus or gas gangrene. In May 2008, the Sichuan earthquake in China caused more than 70,000 deaths and approximately 400,000 injuries; several injured persons developed gas gangrene and later underwent amputations. Among 2,131 survivors admitted to a public hospital in the Sichuan area, at least 19 patients (0.9 %) developed gas gangrene (Wang et al. 2010).

Gangrene occurs equally in men and women. Although age is not a prognostic factor in gas gangrene, advanced age and comorbid conditions are associated with a higher likelihood of mortality. Type I necrotizing fasciitis occurs most commonly in patients with diabetes and patients with peripheral vascular diseases. It is the most common form of necrotizing fasciitis in the general population. Type II necrotizing fasciitis has an annual incidence of 5–10 cases per 100,000 in the USA. *C. perfringens* is one of the most common causes of food poisoning in the USA and Canada. Contaminated meats contained in stews, soups, and gravies are usually responsible for outbreaks in developed countries and cause about 250,000 cases of food-borne illness every year in the USA. Approximately, half of the cases of streptococcal necrotizing fasciitis occur in young and previously healthy people. The incidence of gas gangrene in the USA is nearly 3,000 cases annually. Severe penetrating trauma or crush injuries associated with interruption of the blood supply are the usual predisposing factors. *C. perfringens* is the most common cause of trauma-associated gas gangrene with very high mortality rates (Stevens et al. 2005). Spontaneous gas gangrene caused by *Clostridium septicum* may be more common than trauma-associated gangrene, caused by other *Clostridium* species. Disease frequency is common among drug abusers and patients with atherosclerosis, antiphospholipid syndrome (9 %), and malignancy affected by paraneoplastic acral vascular syndrome. The annual incidence of atheroembolism that leads to ischemic gangrene ranges from 0.3 % to 3.5 % overall, although after a vascular procedure it can rise to 30 %. Most patients with Reynaud's phenomenon are women aged between 20 and 40 years, in whom lesions develop during the cold months (Baker and Bick 2008; Hirschmann and Raugi 2009). Gas gangrene is undoubtedly an infection that carries a very high mortality rate. The reported mortality rates vary widely, with a rate of 25 % in most recent studies. The mortality rate approaches 100 % in individuals with spontaneous gas gangrene and in those with delayed treatment (Stevens et al. 2005).

Retrospective population-based surveillance for clostridia bacteremia was conducted among all residents of the Calgary Health Region (population 1.2 million) during 2000–2006. One hundred and thirty-eight residents had incident *Clostridium* species bacteremia (1.8 per 100,000/year); 45 (33 %) were nosocomial, 55 (40 %) were healthcare-associated community onset, and 38 (28 %) were community acquired. Older age and a number of underlying conditions were risk factors for acquiring *Clostridium* species bacteremia most importantly hemodialysis (relative risk (RR) 212.3; 95 % confidence interval (CI) 106.5–385.5), malignancy (RR 40.2; 95 % CI 27.6–58.1), and Crohn's disease (RR 11.2; 95 % CI 3.0–29.4). *Clostridium perfringens* was most commonly identified with 58 (42 %)

isolates followed by *Clostridium septicum* (19; 14 %), *Clostridium ramosum* (13; 9 %), *Clostridium clostridioforme* (8; 6 %), and *Clostridium difficile* (7; 5 %). Reduced susceptibility to penicillin occurred in 14/135 (10 %), to metronidazole in 2/135 (1 %), and to clindamycin in 36/135 (27 %) isolates. The median length of stay was 12.7 days and 39/130 (30 %) patients died in hospital for mortality rate of 0.5 per 100,000/year (Leal et al. 2008).

Gas gangrene was responsible for around 6 % of open fractures and 1 % of all open wound infections during World War I and 0.7 % during World War II. Early recognition and adequate therapy lowered the mortality rate in a high percentage of cases. On the other hand, this observation of low reports can be delineated only to urban areas and developed countries. Though a few gas gangrene cases have been reported from many parts of developing and underdeveloped countries, these reports merely suggest an approximate estimate of the disease incidence; the actual numbers may significantly be higher, as, very often, many cases go unreported due to lack of awareness among the people, inadequate surveillance, and poor epidemiologic and pathologic expertise.

Pathophysiology

Gangrene is necrosis and subsequent decay of body tissues caused by infection or thrombosis or lack of blood flow. Gas gangrene fabricates a severe life-threatening pathophysiological condition which has the following features: muscle necrosis, gas production, sepsis, and ultimately death. It is usually the result of critically insufficient blood supply sometimes caused by injury and subsequent contamination mainly with clostridium class of bacteria.

Clostridia are gram-positive, anaerobic, spore-forming bacilli commonly found throughout nature (with the exception of the North African desert). Cultivated rich soil has the highest density of organisms. In addition, clostridia have been isolated from normal human colonic flora, skin, and the vagina. More than 150 *Clostridium* species have been identified, but only six have been demonstrated to be capable of producing the fulminant pathological condition known as clostridial gas gangrene. A study by Méndez et al. suggests that sugar may inhibit the production of alpha- and theta-toxins that trigger the gas generation (Méndez et al. 2012). *Clostridium perfringens*, previously known as *Clostridium welchii*, is the most common cause of clostridial gas gangrene (80–90 % of cases). Other clostridia species responsible for the condition include *Clostridium novyi (40 %)*, *Clostridium septicum (20 %), Clostridium istolyticum (10 %), Clostridium bifermentans (10 %)*, *and Clostridium fallax (5 %).* The two most commonly isolated species have been *C. perfringens and C. septicum. Clostridium perfringens* is ubiquitous in nature, commonly found in the soil and gastrointestinal tracts of warm-blooded animals. *C. septicum* is also virulent, sometimes referred to as the malignant edema bacillus, but poorly understood pathogen that is recognized as the causative agent of atraumatic myonecrosis. It also produces α-toxin, distinct from *C. perfringens*, which acts as a pore-forming

Table 2 Different key exotoxins produced by *Clostridium perfringens* and their biological effects

Key exotoxins	Biological effects
Alpha-toxin	Lecithinase, necrotizing, hemolytic, cardiotoxic
Beta-toxin	Necrotizing
Epsilon-toxin	Permease
Iota-toxin	Necrotizing
Delta-toxin	Hemolysin
Phi-toxin	Hemolysin, cytolysin
Kappa-toxin	Collagenase, gelatinase, necrotizing
Lambda-toxin	Protease
Mu-toxin	Hyaluronidase
Nu-toxin	Deoxyribonuclease, hemolytic, necrotizing

cytolysin and is essential for virulence (Kennedy et al. 2005). *Clostridium perfringens* gas gangrene is, without a doubt, the most fulminant necrotizing infection that affects humans. Infections are characterized by a very low level of host inflammation in response to organism-associated exotoxins. In fact, it is more of a response to the exotoxins than a classic immune response to invading organisms. Purulence is often absent. The process of myonecrosis can spread as fast as 2 cm/h. This results in systemic toxicity and shock that can be fatal within 12 h. Overwhelming shock with accompanying renal failure usually leads to death (Stevens and Bryant 2002).

Infection requires two conditions to coexist. First, organisms must be inoculated into the tissues. Second, oxygen tension must be low enough for the organisms to proliferate. These organisms are not strict anaerobes; 30 % oxygen tension in the tissues allows for free growth of these bacteria, but 70 % oxygen tension restricts their growth. Inoculation of organisms into low oxygen tension tissues is followed by an incubation period that usually ranges from 12 to 24 h. However, this period can be as brief as 1 h or as long as several weeks. The organisms then multiply and produce exotoxins that result in myonecrosis. Although not very well understood, exotoxins appear to be tissue-destructive soluble antigens produced by clostridia. They include lecithinase, collagenase, hyaluronidase, fibrinolysin, hemagglutinin, and hemolysin toxins (Table 2). *C perfringens* produces at least 17 identifiable exotoxins that are used for species typing (e.g., type A, type B, type C) (Titball 2005).

Theta-toxin causes direct vascular injury, cytolysis, hemolysis, leukocyte degeneration, and polymorphonuclear cell destruction. These effects on leukocytes may explain the relatively minor host inflammatory response that is observed in tissues of patients with clostridial myonecrosis. Kappa-toxin, also produced by *C perfringens*, is a collagenase that facilitates the rapid spread of necrosis through tissue planes by destroying connective tissue. Alpha-toxin is produced by most clostridia and has phospholipase C activity. This potent lecithinase causes lysis of red blood cells, myocytes, fibroblasts, platelets, and leukocytes. It also may

decrease cardiac inotropy and trigger histamine release, platelet aggregation, and thrombus formation (Monturiol-Gross et al. 2012).

Considerable variation exists among clostridia species as to the mechanism of action of the alpha-toxin. In *C. septicum*, the alpha-toxin forms pores and induces necrosis by causing the rapid loss of intracellular potassium and depletion of adenosine triphosphate (ATP). Strains that do not produce alpha-toxin are less virulent, underscoring its importance (Knapp et al. 2010). In mice models, alpha-toxin-induced lethality was inhibited by the pre-administration of erythromycin. Erythromycin resulted in a reduction of the release of cytokines tumor necrosis factor-alpha (TNF-alpha), interleukin 1 (IL-1), and interleukin 6 (IL-6). Additionally, TNF-alpha-deficient mice were resistant to *C. perfringens* alpha-toxin, suggesting that TNF-alpha is an important contributor to the toxic effects of clostridial proteins (Oda et al. 2008). Moreover, very recently, it has been found that, at lytic concentrations, *C. perfringens* PLC (α-toxin) causes membrane disruption, whereas at sublytic concentrations, this toxin causes oxidative stress and activates the MEK/ERK pathway, which contributes to its cytotoxic and myotoxic effects. The results demonstrate that the toxin induces reactive oxygen species (ROS) production through PKC, MEK/ERK, and NFκB pathways, the latter being activated by the MEK/ERK signaling cascade. Inhibition of either of these signaling pathways prevents cytotoxic effect of α-toxin (Monturiol-Gross et al. 2014).

Genetic regulation of clostridia exotoxin production is under the control of several different regulatory systems, including the global VirR/VirS 2-component signal transduction system and the RevR. The VirR, a membrane-bound external sensor, and the VirS, a gene response regulator, together transmit and receive signals from the environment to the inside of the cell. The VirR/VirS system uses RNA intermediates to control 147 distinct genes and their associated operons (Ohtani et al. 2010). The phi-toxin is a hemolysin. Even though it does not directly suppress myocardial function in vitro, it contributes to myocardial suppression in vivo, possibly by increasing the synthesis of secondary mediators that do suppress myocardial function in vitro. At higher concentrations, the phi-toxin can cause extensive cellular degeneration and direct vascular injury. The kappa-toxin produced by *C perfringens* is a collagenase responsible for destruction of blood vessels and connective tissue. Other toxins include a deoxyribonuclease and hyaluronidase (Monturiol-Gross et al. 2012).

Traumatic gas gangrene and surgical gas gangrene occur through direct inoculation of a wound. With a compromised blood supply, the wound has an anaerobic environment that is ideal for *C. perfringens*, the cause of 80–95 % of cases of gas gangrene. Spontaneous gas gangrene is most often caused by hematogenous spread of *C. septicum* from the gastrointestinal tract in patients with appendicitis or colon cancer or through other gateway. Neutropenic and immunocompromised patients are also at risk. The organism enters the blood via a small break in the gastrointestinal mucosa and subsequently seeds muscle tissue. Unlike *C. perfringens*, *C. septicum* is aerotolerant and can infect normal tissues.

Glucose fermentation causes gas production in gas gangrene where the major components are nitrogen (74.5 %), followed by oxygen (16.1 %), hydrogen (5.9 %), and carbon dioxide (3.4 %). Production of hydrogen sulfide and carbon dioxide gas begins late and dissects along muscle bellies and facial planes. These all provide favorable conditions for spread of infection. Low oxidation/reduction and the necessary enzymes provided by the necrotic tissue result in the spore germination. Spores germinate to form the vegetative cells which produce exotoxins which result in tissue necrosis, thrombosis, and edema. With *C. perfringens*, the local and systemic manifestations of infection are due to the production of potent extracellular protein toxins by the bacteria. These are most notably alpha-toxin (a phospholipase C) and theta-toxin (a thiol-activated cytolysin). These toxins hydrolyze cell membranes, cause abnormal coagulation leading to microvascular thrombosis (further extending the borders of devascularized and thus anaerobic tissue), and have direct cardio-depressive effects. Furthermore, the products of tissue breakdown, including creatine phosphokinase, myoglobin, and potassium, may cause secondary toxicity and renal impairment (Titball 2005; Moustoukas et al. 1985).

Significant and refractory anemia may also be present in patients with gas gangrene. This effect is a direct consequence of toxin-mediated hemolysis of RBCs when significant amounts of alpha-toxin are released into the bloodstream. Alpha-toxin has negative inotropic effects on cardiac myocytes contributing to the severe, refractory hypotension seen in some cases of gas gangrene. Theta-toxin causes a cytokine cascade, which results in peripheral vasodilation similar to that seen in septic shock. These secreted exotoxins might also result in hemolysin which causes low hemoglobin and hypertension and may cause acute tubular necrosis and renal failure. It has been reported that alpha-toxin and theta-toxin which are lipophilic in nature are bound to tissue plasma membrane and may result in secretion of secondary metabolites (Fiorentini et al. 1999; Moustokas et al. 1985).

Role of Alpha- and Theta-Toxin

Among all the exotoxins produced by this organism, the alpha-toxin, α (phospholipase C, PLC), and theta-toxin, θ (perfringolysin O, PFO), are its major virulence factors. Although the role of PFO in the pathogenesis of gas gangrene is somehow controversial, the part played by the α-toxin is beyond doubt in causing the disease. However, on the other hand, the PFO have been shown to facilitate the growth of its corresponding bacterium within mammalian phagocytic cells. Furthermore, experimental animal studies have demonstrated the protective efficacy of several antibody preparations against these toxins (Bryant and Stevens 1997). PLC and PFO each contribute to the morbidity and mortality of gas gangrene by uniquely different mechanisms. PLC is hemolytic, is cytotoxic to platelets and leukocytes, and increases capillary permeability effects that are likely related to its ability to cleave sphingomyelin and the phosphoglycerides of choline,

ethanolamine, and serine present in eukaryotic cell membranes. PLC requires calcium for optimal activity. It gets that Ca^{2+} by hydrolyzing the intracellular phosphoinositide (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG), a potent second messenger that in turn activates several other cell signaling mechanisms including the one leading to the generation of thromboxane A2 (Fig. 2).

As mentioned earlier, the A-type *C. perfringens* chiefly produces the exotoxin-α that has a hemolytic activity and is the main causative agent of clostridial myonecrosis or gas gangrene. The toxin is a 370-residue, zinc metalloenzyme that has phospholipase C activity and can bind to membranes in the presence of calcium. In fact, this was the first bacterial toxin to be identified as an enzyme. During that time people started to accept a convention that all phospholipase C's are toxic. Further studies proved, however, that not all PLCs are toxic (*Clostridium novyi* PLC, e.g., is toxic, while that *Bacillus cereus* PLC is not) and that therefore enzymatic activity alone is not sufficient for toxicity. Studies of α-toxin have shown that it is the key virulence determinant of *C. perfringens* in gas gangrene. Specific mutants of *C. perfringens* that do not produce α-toxin are unable to cause disease, and vaccination with a genetically engineered toxoid has been shown to induce protection against gas gangrene (Williamson and Titball 1993). An emerging theme is that toxicity can be ascribed to the ability of the enzyme to interact with membrane phospholipids, thereby perturbing host cell metabolism and promoting the effects outlined above which allow the spread of the bacteria into otherwise healthy tissues. To fully understand the molecular basis of toxicity, the crystal structure of α-toxin has been determined (Naylor et al. 1998). This reveals a two-domain protein. The α-helical amino-terminal domain contains three zinc ions, located within a cleft. These zinc ions appear to play roles both in stabilizing the structure of the protein and in the phospholipase C catalytic activity (Titball and Rubidge 1990) (Fig. 3). There are several pieces of evidence indicating that this cleft is the phospholipase C active site. Firstly, when the surface of the protein is modeled, a phospholipid molecule can be accommodated within the charged cleft (Naylor et al. 1998). More importantly, when recombinant amino-terminal domain is produced in E. coli, the purified polypeptide retains phospholipase C activity. However, the amino-terminal domain lacks the toxicity and the cytolytic activity of the holotoxin (Titball et al. 1991).

Although the amino-terminal domain alone is nontoxic, it is clear that its enzymatic activity is required for toxicity. The site-directed mutagenesis of zinc-binding ligands in the amino-terminal domain simultaneously abolishes phospholipase C activity and other toxic and cytolytic activities (Nagahama et al. 1997). This finding suggests that the carboxy-terminal domain confers cytolytic and toxic activities on the enzyme. The isolated carboxy-terminal domain has no detectable toxic or cytolytic activity, but mixing the purified amino- and carboxy-terminal domains together in solution restores hemolytic activity, presumably because these domains can associate via the hydrophobic face between these domains in the holotoxin (Titball et al. 1993).

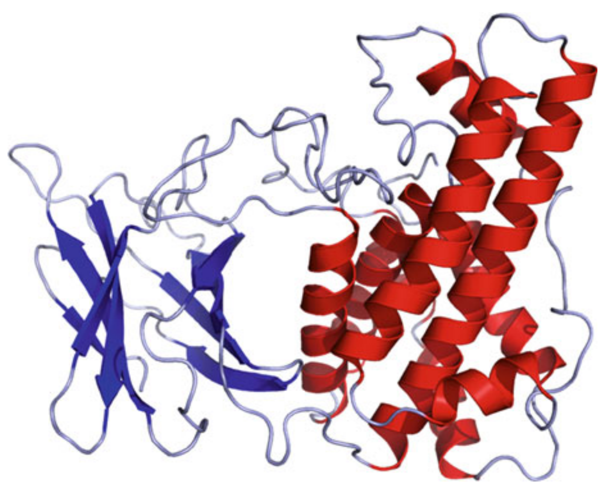

Fig. 3 The crystal structure of "*Clostridium perfringens*" alpha-toxin published in the Protein Data Bank (*PDB*: 1CA1). Alpha-helical amino-terminal domain (*red*) contains three zinc ions binding cleft. The carboxy-terminal domain (*blue*) binds calcium and allows the toxin to bind to the phospholipid head groups on the cell surface (Source: http://en.wikipedia.org/wiki/File:Clostridium_perfringens_Alpha_Toxin.rsh.png; Created from PDB 1CA1 and rendered by Ramin Herati (2006) using Pymol)

Precise Mechanism of Action of Alpha-Toxin

The alpha-toxin is a zinc metalophospholipase, requiring zinc for activation. First, the toxin binds to a binding site on the cell surface. The C-terminal C2-like PLAT domain binds calcium and allows the toxin to bind to the phospholipid head groups on the cell surface (Fig. 3). The C-terminal domain enters the phospholipid bilayer. The N-terminal domain has phospholipase activity. This property allows hydrolysis of phospholipids such as phosphatidylcholine, mimicking endogenous phospholipase C. The hydrolysis of phosphatidylcholine produces diacylglycerol, which activates a variety of second messenger pathways. The end result includes activation of arachidonic acid pathway and production of thromboxane A2 and production of IL-8, platelet-activating factor, and several intercellular adhesion molecules (Fig. 2). These actions combine to cause edema due to increased vascular permeability that in turn allows the growth of the obligatory anaerobe, although it is capable of growing in slightly aerobic condition (Titball et al. 1993; Titball 2005).

Symptoms and Complications

Gas gangrene may result generally from predisposing factors like blood loss, trauma, hemorrhage, or bone fracture near the wound and by accidental contact of soil or fecal matter in wound. The earliest symptoms of gas gangrene are intense pain, low-grade fever, edema, and a sweet-odorous discharge which occurs several

hours to a few days (6 h to 3 days) after injury. Within few hours, the entire region may become markedly edematous. Gas produced by clostridia may produce a crackling sensation when the affected area is pressed. Crepitus follows gas production; at times, crepitus may not be detected with palpation owing to brawny edema. It becomes swollen and the swollen skin may initially be blistered with hemorrhagic bullae, red, and warm to the touch before progressing to a bronze, brown, or black color. Foul-smelling brown-red or bloody discharge drips from the affected tissues or wound. In advanced stages, all the signs of profound damage-like severe pain in the affected area are followed by numbness, rapid breathing (tachycardia), a grayish/yellowish complexion, etc. if the toxins spread into the bloodstream. Symptoms typically begin suddenly and quickly worsen. If the patient is not treated at the initial stage, the infection from gangrene gets into the blood; it may develop sepsis and go into septic shock which is life threatening. Symptoms of sepsis may include low blood pressure, rapid heartbeat (tachycardia), shortness of breath, change in body temperature, light headedness, body pain and rash, confusion, and cold, clammy, and pale skin (Kasper and Madoff 2005).

If the patient remains untreated for long time, in the advance stage, few other medical complications like massive hemolysis, disseminated intravascular coagulation, acute renal failure, jaundice with liver damage, acute respiratory distress, coma, delirium, and shock are very common. Gangrene can lead to scarring which sometimes requires reconstructive surgery. Sometimes, the amount of tissue death is so extensive that the body parts or extremities may need to be surgically removed.

Diagnosis

The diagnosis of clostridial disease, in association with positive cultures, must be based primarily on clinical findings. Because of the presence of clostridia in many wounds, their mere isolation from any site, including the blood, does not necessarily indicate severe disease. Smears of wound exudates, uterine scrapings, or cervical discharge may show abundant large gram-positive rods as well as other organisms. Cultures should be placed in selective media and incubated anaerobically for identification of clostridia. The diagnosis of clostridial myonecrosis can be established by frozen-section biopsy of muscle. Histopathological findings in gas gangrene consist of widespread myonecrosis, destruction of other connective tissues, and a paucity of neutrophils in the infected area. Leukocyte aggregates are found in the border regions. The urine of patients with severe clostridial sepsis may contain protein and casts, and some patients may develop severe uremia. Profound alterations of circulating erythrocytes are seen in severely toxemic patients. Patients have hemolytic anemia, which develops extremely rapidly, along with hemoglobinemia, hemoglobinuria, and elevated levels of serum bilirubin. Spherocytosis, increased osmotic and mechanical red blood cell fragility, erythrophagocytosis, and methemoglobinemia have been described. In patients with severe sepsis, Wright's or Gram's staining of a smear of peripheral blood or buffy coat may demonstrate clostridia. X-ray examination sometimes provides an

important clue to the diagnosis by revealing gas in muscles, subcutaneous tissue, or the uterus. However, the finding of gas is not pathognomonic for clostridial infection; other anaerobic bacteria too, frequently mixed with aerobic organisms, may produce gas (Kasper and Madoff 2005).

CT scanning is also helpful, especially in abdominal cases of gas gangrene. A recent study with the new-generation CT scanners reported 100 % sensitivity to detect necrotizing soft-tissue infections; however, it excluded patients taken to surgery prior to CT scanning and did not explore surgically all clinically suspected cases (Zacharias et al. 2010). Studies on MRI to detect necrotizing soft-tissue infection have reported lower sensitivity (80–90 %) and limited specificity. In addition, MRI is time consuming and is not always available. Ultrasound, although attractive as rapid bedside test, has not been well studied in this clinical scenario. In a cadaveric model of soft-tissue gas, it showed excellent sensitivity in detecting gas and its localization (Butcher et al. 2011). Rapid detection of alpha-toxin or sialidases or neuraminidases in infected tissues through enzyme-linked immunosorbent assay (ELISA) is not widely available but represents a potential diagnostic tool. ELISA can provide results in as little as 2 h when the test is applied to wound exudate, tissue samples, or serum.

Treatment

Treatment for gangrene involves removing the dead tissue, treating and preventing the spread of infection, and treating the condition that caused gangrene to develop. Owing to fast progression of the disease, early management of gas gangrene plays a critical role in saving the patient. Three main classes of therapy are recommended in general: surgical debridement of necrotic tissue, use of antibiotics, and hyperbaric oxygen therapy. The combination of aggressive surgical debridement and effective antibiotic therapy is the determining factor for successful treatment of gas gangrene. In case of advanced bacterial toxemia, blood transfusions and supportive therapy may also be required to manage pain and secondary pathophysiological conditions.

Debridement

Several studies have shown that the most important factor affecting mortality is timing and adequacy of initial surgical debridement. Delayed or inadequate debridement dramatically increases mortality. Radical debridement may necessitate limb amputation. It removes the source of infection and toxins, and furthermore, removal of infarcted tissue improves the subsequent penetration of antibiotics. Profuse washing should be performed with sterile normal saline solutions and/or 3 % liquid hydrogen peroxide. Debridement of all wounds should be performed as soon as possible, with removal of badly damaged, contaminated, and necrotic tissue, especially in patients who might have been contaminated by soil,

farm land, or dirty water. The infection is rarely eradicated after a single debridement, and serial debridements are almost always needed. Optimally, three debridements spaced 12–36 h apart are required to obtain control of gross infection. Debridement may result in significant intraoperative blood loss and inability to close surgical wounds. Vacuum-assisted dressings and skin expansion devices may have a role. Reconstructive surgery should be considered only when the patient has been stabilized and the infection fully eradicated (Davoudian and Flint 2012).

Antibiotic Therapy

In animal models, prompt treatment with antibiotics significantly improves survival rates. Historically, penicillin G in dosages of 10–24 million U/day was the drug of choice. Currently, a combination of penicillin and clindamycin is widely used (Stevens et al. 2005). A number of studies showed that protein synthesis inhibitors (e.g., clindamycin, chloramphenicol, rifampin, tetracycline) may be more effective because they inhibit the synthesis of clostridial exotoxins and lessen the local and systemic toxic effects of these proteins. In spite of increasing clindamycin resistance among anaerobes, cases of clindamycin-resistant *C. perfringens* are exceptional (Khanna 2008). A combination of clindamycin and metronidazole is a good choice for patients allergic to penicillin. A combination of penicillin and metronidazole may be antagonistic and is not recommended. Because other nonclostridial bacteria are frequently found in gas gangrene tissue cultures, additional antimicrobial coverage is indicated. Although approved for treating complicated skin and soft-tissue infections, newer antibiotics such as daptomycin, linezolid, and tigecycline have not been studied in patients with gas gangrene; therefore, they should not be used as primary antibiotics for treating this condition (Stevens et al. 2005). Supportive medication should be given to manage pain and other secondary pathophysiological conditions.

Hyperbaric Oxygen (HBO) Therapy

Since the 1960s, HBO therapy has been used in the USA for the treatment of gas gangrene; however, its use remains controversial. Controlled prospective studies on human subjects have not evaluated the impact of this treatment on survival. One reason for this is the low number of patients with gas gangrene. In addition, the therapeutic effect of HBO is difficult to reliably assess because of a lack of well-designed comparative studies. Many retrospective studies report increased survival in patients when HBO therapy is added to treatment with surgery and antibiotics. However, HBO therapy failed to show a survival advantage in two retrospective multicenter studies of the treatment of major necrotizing infections (George et al. 2009). Studies of animal models show conflicting reports about enhanced survival associated with HBO therapy. Studies indicate that HBO therapy has a direct bactericidal effect on most clostridia species, inhibits alpha-toxin production,

and can enhance the demarcation of viable and nonviable tissue prior to surgery. For these reasons, some authors recommend the use of HBO therapy before the initial debridement, if possible. The most common regimen for HBO therapy involves administration of 100 % oxygen at 2.5–3 absolute atmospheres for 90–120 min 3 times a day for 48 h and then twice a day as needed. In view of the frequent catastrophic outcomes in patients with gas gangrene, HBO therapy is an important adjunct to surgery and antimicrobial therapy, despite the lack of convincing clinical efficacy. Potential risks in patients undergoing HBO therapy include pressure-related trauma (e.g., barotraumatic otitis, pneumothorax) and oxygen toxicity (e.g., myopia, seizures). Other common adverse effects include claustrophobia. Most adverse effects are self-limiting and resolve after termination of HBO therapy (Tibbles and Edelsberg 1996; Kindwall 1993).

Intensive care should be taken as the patients with gas gangrene frequently have end-organ failure and other concomitant serious medical conditions that require intensive supportive care. Monitoring serum calcium may need special attention when large areas of necrotic fat may lead to its deposition (Ustin and Malangoni 2011).

Antitoxin and Vaccines

Gas gangrene has a relatively shorter incubation period and hence antitoxin is of little benefit in treatment. If the target animal is pre-immunized continually with formalinized antigens (toxins), it may be helpful to prevent infection. Chiefly, antitoxins may be administered to prevent the growth and to neutralize the trace amounts of toxins that are latent in the tissue/wound in a longer duration. The usefulness of gas gangrene antitoxin is currently a disputed matter. Some healthcare experts suggest that the advanced medical care currently available would be sufficient to beat any uses of the polyvalent antitoxins while some believe that the latter should be administered systematically and locally in the salvaged tissue for a complete cure. Currently there are no licensed vaccines suitable for use in humans which protect against gas gangrene. However, vaccines being developed for use in animals have the potential to be developed for use in humans in the future (Titball 2009). In 2005, *Clostridium perfringens* type A toxoid was developed by a US-based company and was the first commercial product for cattle to receive a conditional license by the US FDA to aid in the control of disease syndromes caused by the alpha-toxin of *C. perfringens*. But this toxoid is strictly prohibited for human utilization.

Fragments of the alpha-toxin of *C. perfringens*, a major virulent factor responsible for gas gangrene, were produced using genetic manipulation techniques, and the recombinant proteins were immunized into mice. Anti-Cpa_{1-249} serum neutralizes phospholipase C activity but not hemolytic activity of the toxin. Anti-$Cpa_{247-370}$ serum neutralizes both the phospholipase C and hemolytic activities (Williamson and Titball 1993). Chen et al. developed a new strategy using group II intron-based Target-Tron technology and inactivated the *plc* gene (alpha toxin gene) in *C. perfringens* (Chen et al. 2005). Stevens et al. immunized mice with

C-terminal of alpha-toxin and they found in histopathological studies demonstrating limited muscle necrosis reduced microvascular thrombosis and enhanced granulocytic influx to the infected site (Stevens et al. 2004).

Maggot Therapy (Biotherapy)

Maggot therapy is a nonsurgical way of debridement to remove dead tissue. When used to treat gangrene, certain type of maggots from fly larvae (specially bred in a sterile laboratory conditions) are placed on the wound, where they consume the dead and infected tissue without harming healthy tissue. They also help fight infection and speed up healing by releasing substances that kill bacteria. During maggot therapy, the tiny maggots are put on to the wound and covered with gauze, under a firm dressing. After a few days, the dressing is cut away and the maggots, often 10 times bigger after eating the dead tissue, are then flushed away. A number of medical studies have shown maggot therapy can achieve more effective results than surgical debridement. However, due to the nature of this type of treatment, many patients are reluctant to try it (Dumville et al. 2009; Opletalova et al. 2011).

Conclusion and Future Directions

Considering the multiple toxins and organisms involved in gas gangrene etiology and the ever-increasing abuse of antibiotics and unavailability of world-class medical setup and disproportionate illiteracy rate in developing countries can raise serious concerns regarding the cure of this disease. Explicit major threats, posed by gas gangrene, are as follows: firstly, the involvement of multiple pathogens in the onset of the disease can be a problem in early detection and treatment of the disease. Coinfection of multiple pathogens like *E. coli* and *S. aureus* along with clostridia in the wounds would mask the isolation of the latter as the former pathogens grow rapidly on synthetic and semisynthetic media that are generally used to isolate clostridia. The second concern is due to the extensive antibiotic abuse in developing countries. As a result of this problem, many clostridia already gained antibiotic resistance genes and the spectrum of this resistance pattern is ever increasing. Due to this unsettled problems, a nonantibiotic therapeutic molecule or vaccine is to be developed; it should be a subunit vaccine incorporating multiple protective domains. Effective antibody development against the toxins is essentially important for not only fighting against the clostridial exotoxin but also limiting the systemic cytokine release associated with systemic inflammatory response syndrome. Care should be taken to consider all the possible variant of antigens (alpha-toxins) from different necrotizing clostridia species of different geographical sources during multivalent antibody development which should be working effectively and universally. Careful structure-function relationship and functional domain analysis and interactions with bodily available natural ligands should be helpful to develop perfect antitoxin molecules which could have the

ability to neutralize these toxins at multiple sites. The calcium binding is important at C-terminal domain of alpha-toxin which allows the toxin to bind to the phospholipid head groups on the cell surface, and N-terminal domain has phospholipase activity which hydrolyses the phospholipids of the membrane. Therefore, it is a time to understand all the possible structure-function using modern bioinformatics tools in such a challenging situation. Due to debridement of necrotic tissues, the portions that require regeneration may need stem cell therapy and regenerative medicine that will be helpful for complete tissue recovery. The viral vector-based antitoxin gene therapy may be another future option. Lastly, the scientific world should acquire sufficient global antitoxin weapons against the deadly clostridia toxins to get ready in fighting such bioterrorism.

Cross-References

► Challenges in Developing Biotoxin Inhibitors
► Evolutionary Traits of Toxins
► Immunosensors: Using Antibodies to Develop Biosensors for Detecting Pathogens and Their Toxins
► The Public Health Response to Potential Bioterrorism by Toxin Attack

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Animal Vaccine Technology: An Overview 20

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Abstract

This chapter introduces the principles of vaccines and discusses recent advances for the various forms of vaccine. Live and attenuated vaccines remain the most effective at eliciting robust immune responses, but reversion to virulence poses great safety concern. Since mutations are inherent for living pathogens, the development of nonliving, exogenous protein components as vaccines provides a safe alternative. However, protein components do not provide sufficient "danger" signals required for strong immune response; therefore, adjuvants are generally needed. DNA vaccines can induce endogenous expression of immunogenic components, but the risk of chromosome integration and the optimal site

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of antigen expression still need to be addressed. While vaccines have contributed enormously to the control of infectious diseases in the past, even more sophisticated vaccines may be necessary to combat ever-emerging, new pathogens.

Introduction

Since the discovery of the smallpox vaccine by Dr. Edward Jenner in the nineteenth century, advances in vaccination technology have helped eradicate numerous infectious diseases. However, there are currently no effective vaccines available for infectious agents such as the human immunodeficiency virus, malaria *Plasmodium*, tuberculosis bacteria, and dengue virus. For domesticated animals, a host of pathogens continue to threaten their health and welfare. Vaccination involves artificial induction of immunity by exposing individuals to modified pathogenic or toxic components. Significant improvements have been made to increase vaccine safety while maintaining effectiveness, or antigenicity, of the vaccine components. Advances in immunology and molecular biology have also paved the way for developing sophisticated forms of vaccine.

This chapter will cover the principles of the various forms of vaccine and introduce recent advances for each vaccine category. Live and attenuated vaccines, generally eliciting strong immune responses, will be discussed first. Inactivated or killed vaccines, with better safety profile, will then be introduced. Recombinant DNA technology has allowed the development of subunit vaccines, and production of these protein vaccines through bacterial and insect systems will be covered. Finally, since DNA encoding various antigens has been used as a form of vaccine with notable success, the principles of DNA vaccine and the challenges of development will be discussed. Except for live and attenuated vaccines, the other forms of vaccine usually require adjuvants to enhance immune responses. The appropriate adjuvants for each vaccine category will be covered under each section, while encapsulation technology as an adjuvant form will be discussed separately.

Live Vaccine

Live vaccines contain low virulent or attenuated microorganisms. They are altered so that they cannot cause disease but retained their ability to induce protective immunity.

As the live organism can still infect target cells, these vaccines can replicate and are thought to better simulate natural infections and produce a broad immune response including both cellular and humoral immunity and generally do not require an adjuvant to be effective.

Attenuated microorganisms including particularly bacteria and viruses may be found naturally, or they may be attenuated via passage of the virus through a foreign host such as tissue culture, embryonated eggs, and live animals. But some viruses

are difficult to passage in nonhost materials. Reverse genetics can be used to rapidly develop a live-attenuated vaccine based on currently circulating virus. For example, NDV genotype VII is difficult to attenuate. Recurrent outbreaks of ND in vaccinated birds is due to an antigenic mismatch between the genotype II commercial vaccine strains and the genotype VII virulent strains that are presently circulating. The reverse genetics NDV genotype VII vaccine significantly reduced virus shedding from vaccinated birds when compared to B1 vaccine (Xiao et al. 2012). Live vaccine can produce a broad immune response including cellular immunity, humeral immunity system, and local immunity.

Live vaccines provide stronger, longer-lasting, and more rapid protection, and thus boosters are required less frequently. They are low cost and may require only one dose to be effective. Additionally, live vaccines can easily be administered by various routes, such as injection, drinkable water, or inoculation into the nasal cavity or eyes.

However, they still have a residual virulence or a risk of reversion to virulent wild types. As an example, in 1996, a porcine respiratory and reproductive syndrome (PRRS) vaccine program was initiated in Denmark for the control and prevention of PRRS (Botner et al. 1997; Grebennikova et al. 2004). Following the implementation of the vaccination program, epizootics of PRRS-like disease were reported in some of the vaccinated herds and PRRSV was isolated from clinically affected swine. In some cases, genetic analysis indicated the PRRSV was closely related to the vaccine virus (North American genotype) and not the Danish field viruses (European genotype) (Madsen et al. 1998). In conclusion, live vaccines have played a major and effective role in animal disease control, but are not without drawbacks.

Inactivated Vaccine

An inactivated vaccine (or killed vaccine) consists of microorganisms killed using methods and agents such as chemicals (formalin, binary ethyleneimine), heat, radioactivity, or antibiotics. The inactivated microorganisms are destroyed and cannot replicate in a vaccinated host, but the capsid and outer membrane proteins are intact enough to be recognized by the immune system. Inactivated vaccines are exogenous antigens, which activate CD4 + helper T cells to induce immune response. It cannot revert to virulent forms capable of causing diseases, but incomplete inactivation can result in intact and infectious particles.

Inactivated microorganisms generally provide a weaker immune response than live microorganisms. In particular, immune response elicited by inactivated vaccines lacks cell-mediated immunity. For this reason, inactivated vaccines are administered with adjuvants, and booster shots are required to elicit sufficient response and a long-term immunity. It is important to note that field virulent microorganisms should be used as vaccine antigen to provide good immune protection.

Subunit Vaccine

Subunit vaccine became a reality in human and animals due to the DNA recombination technique. The subunit vaccine is produced from specific subunit protein of a pathogen and therefore has less risk of adverse effects than whole pathogen vaccines. While there are many subunit vaccines used in animal (Kushnir et al. 2012), the human hepatitis B virus surface protein (HBV S protein) was the first subunit protein in humans (Kniskern et al. 1989). The primary advantage of subunit vaccine is safety. It is safe to use even in immunocompromised humans. The vaccine contains only the important immunogen or epitope and not the genetic material of pathogens. On the other hand, subunit vaccines must be combined with an adjuvant, and repeated immunization is required to enhance humoral and cellular immune responses. Subunit vaccine can be expressed by *Escherichia coli*, yeast, baculovirus, plant, and mammalian cell systems.

Bacterially Expressed Subunit Vaccine

Bacterial expression systems are excellent candidates for mass production. There are many bacterial species used for foreign genes expression. The bacteria *E. coli* is extensively used for expression in subunit proteins development. See Table 1 for the comparison of the advantages and disadvantages between prokaryotic and eukaryotic systems. There are several advantages in using bacterial expression for subunit vaccine. The production cost of the bacterial system is lower than other systems and the production equipment and processes are easy to handle. The low cost is the main reason for choosing the *E. coli* expression system, especially when the vaccine is being developed for veterinarian use. Moreover, with *E. coli*, the protein overproduction system is available and easy to control. The lac promoter is a common overexpress system induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), and the resulting expressed protein is abundant. However, protein overexpression will kill the bacteria or the protein might become included in *E. coli*'s cytoplasm. In an overexpressed system, the bacterial-related secretion, when fused with target genes, can reduce inclusion body formation. Many signaling peptides have been used in the *E. coli* system to increase protein solubility. Unfortunately, there is not a universal signaling sequence for all recombinant proteins. Selecting an optimal signal sequence is important for the efficient secretion of recombinant proteins. The selection is done through trial and error (Choi and Lee 2004).

The primarily disadvantage of *E. coli* expression system for subunit vaccine is that there are few posttranslational modification systems of proteins in *E. coli*. The bacteria protein glycosylation is rare and relatively few bacterial glycoproteins are known. In *E. coli*, only a few glycoproteins have been described (Sherlock et al. 2006; Benz and Schmidt 2001). Glycosylation is very important for protein's tertiary and quaternary structure. The B cell epitope conformation is different between pathogens and the expressed un-glycosylated subunit protein. Because of

Table 1 The advantage and disadvantage between different protein expression systems

System	Cost	Time	Yield	Posttranslational modification	Risk
Bacterial	Low	Short	High	No	Endotoxin
Yeast	Medium	Medium	Medium	Partial	Low risk
Mammalian cell	High	Long	Very low	Fully	Virus, prion, and oncogene
Baculovirus system	High	Long	Low	Fully	Virus, prion, and oncogene
Plant cell	Medium	Long	Medium	Partial	Low risk

the tertiary and quaternary structure, un-glycosylated proteins cannot induce property neutralization antibody against the pathogens. In a research, the author engineered a synthetic pathway in *E. coli* for the production of eukaryotic trimannosyl chitobiose glycans. The pathway also allowed for the transfer of glycans to specific asparagine residues in the target proteins (Valderrama-Rincon et al. 2012). These genetically modified bacteria could then produce glycosylated protein for subunit vaccine development. The gene codon usage bias is different between eukaryotic and prokaryotic systems. There are many rare codons in *E. coli* and it is difficult to express a eukaryotic gene. According to the bioinformatics and computational biology, many statistical methods have been proposed and used to analyze codon usage bias in *E. coli* (Comeron and Aguade 1998).

Several studies have demonstrated that subunit vaccines require repeated vaccination in order to establish and prolong sufficient immune response against viral, bacterial, and protozoal infections. The adjuvant is the key factor in subunit vaccine efficiency. The word "adjuvant" is derived from the Latin word "adjuvare," which means "to help." There are many kinds of adjuvants. Aluminum salts have frequently been incorporated as an adjuvant in vaccines licensed for use in the wild. Aluminum hydroxide, $Al(OH)_3$, has been approved by the FDA as a vaccine adjuvant for more than 70 years. It is used to boost immune response to vaccines by adsorbing the antigen. Oil-based adjuvants are commonly used in many commercial vaccines. Their major purpose is to hold the antigens at the injection site and release them slowly into the bloodstream and lymph node for longer-lasting immunity. Microparticle encapsulation also prolongs antigen release to enhance the immune response and will be discussed later in this chapter. Recently, some bacterial toxins were expressed as fusion protein with the main antigen and used to increase the immune response. The *E. coli* heat labile toxin (HT), *Clostridium perfringens* exotoxin (CPE), and Shiga-like toxin (Stx) were fused to the antigen to target the immune cell. The fusion increased the antibody level, T cell responses, and cytokines concentrate. Due to the adjuvant, same level of immune response can be achieved with a lower antigen dosage and fewer vaccinations. After taking into account the advantages and disadvantages of subunit vaccines and also the boost by an adjuvant, *E. coli*- expressed protein remains a good candidate for vaccine development.

Insect Cell-Expressed Subunit Vaccine

Baculoviruses are a diverse group of large, rod-shaped DNA viruses capable of infecting more than 500 insect species. Their double-stranded, circular genomes are 80–180 kbp in size depending on the viral species. Among the numerous baculoviruses, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and, to a lesser extent, the *Bombyx mori* nucleopolyhedrovirus (BmNPV) are most commonly utilized. The former infects insect-cell lines for protein expression, and the latter infects the silkworm for the production of recombinant protein. Both viruses can express foreign genes under the control of highly expressed, very late promoters, including polyhedrin (polh) and p10 promoters. Protein production in the BEVS is easily achieved by infecting cultured insect cells. The most common insect-cell lines include Sf-9 and Sf-21, derived from *Spodoptera frugiperda* and BTI-TN-5B1-4 (commercially known as High-Five™). Other cell lines in use include Tni PRO (Expression Systems) and Express SF (Protein Sciences). These insect cells grow optimally at 27 °C and do not require CO_2, making cell cultures feasible for most laboratories. The cells can grow in serum-free media and the cultures can easily be scaled up. Various serum-free media, desired for biopharmaceutical protein production, have been developed for Sf-9 (e.g., Ex-Cell 420, SF-900 II, and HyQ-SFX-Insect) and High-Five™ cells (e.g., Express Five®). Furthermore, these cell lines can be easily cultivated and infected in the monolayer or suspension cultures, making the scaling up from bench to industrial feasible (Ikonomou et al. 2003). The parameters in the expression and the process scale-up depend on various bioreactor types (e.g., stirred tank reactor, airlift reactor, packed-bed reactor, etc.), the biological parameters (e.g., cell density upon infection, dissolved oxygen in media, multiplicity of infection (MOI) of infection, harvest timing, etc.), and the environmental parameters (e.g., shear stress, agitation speed, etc.). These conditions influencing the production yield have been extensively studied and reviewed (O'Reilly and Luckow 1992; Taticek and Shuler 1995).

Compared to other expression systems, BEVS has a number of advantages:

(i) The strong p10 or polyhedrin promoter enables high levels of expression of heterologous genes compared to mammalian cell expression systems.
(ii) Baculoviruses are insect pathogens and are unable to replicate in vertebrates, including humans, and is therefore safer to work with than mammalian viruses. The lepidopteran insect cells used are also free of human pathogens.
(iii) Recombinant baculoviruses can be generated rapidly within 1 week. Several commercially available BES kits (e.g., BaculoGold™; FlashBAC, BacPAK™, Bac-to-Bac®) use the conventional method of homologous recombination in vivo or in *E. coli*.
(iv) The large baculoviral genome makes it possible to insert multiple gene expression cassettes and then to express multiple proteins simultaneously in a single infection.

(v) Insect cells permit multiple posttranslational modifications such as folding, glycosylation, phosphorylation, acylation, proteolytic cleavage, and so on. These modifications are similar or identical to those occurring in mammalian cells. Thanks to these attributes, BEVS has become one of the most popular systems for routine production of recombinant proteins (Hitchman et al. 2009).

Currently, a number of biologicals, including interferons, antigens, and vaccines produced in BEVS, are already approved or are in various phases of clinical trials (Hitchman et al. 2009). The proteins produced are used for functional studies, vaccine preparations, or diagnostics. The first commercialized vaccine produced in the baculovirus expression system is directed against the classic swine fever or hog cholera (Intervet). Among these products was GlaxoSmithKline's Cervarix™, a human papillomavirus vaccine that contained BEVS-produced virus capsid protein. Cervarix™ prevents cervical cancer and was approved for human use in the States in 2009 (Aucoin et al. 2010). In addition, a trivalent recombinant hemagglutinin (HA) vaccine (FluBlok®, Protein Sciences) exhibited high efficacy against seasonal influenza for human adults and was approved by the FDA in January 2013. There were also several BEVS-derived and licensed subunit vaccines for veterinary use. Porcilis® Pesti (Intervet) and Bayovac® CSF E2 (Bayer) are the first two subunit vaccines for the classic swine fever, which is one of the most important porcine contagious diseases. Porcilis® PCV(Merck) and CircoFLEX®(B. Ingelheim) are the second veterinary vaccines for PCV2 produced by BEVS (Cox 2012).

The disadvantages of the baculovirus expression system are as follows:

There are drawbacks associated with the BEVS. First, polyhedron and p10 are late promoters, and baculovirus infection results in cell death and lysis a few days postinfection. Protein production reaches a maximum near the death of the infected cells, so some posttranslational modification machinery might be suppressed at that time. Hence, the quantity and quality of the expressed protein could be suboptimal then. In order to overcome the drawback, early baculovirus promoters (e.g., IE-1) were used in either transiently or stably transformed cells systems. These systems provide a more suitable environment for protein production, but the expression level is lower than those obtained with the lytic baculovirus system.

The second disadvantage of BEVS is its inefficiency in properly processing large inactive precursor proteins, such as peptide hormones, matrix metalloproteases, and fusogenic viral envelope glycoproteins.

Thirdly, although BEVS has become a well-established platform for the production of recombinant glycoproteins, the glycoproteins produced by insect cells have a clearly different N-glycans than those produced by mammalian cells. In particular, insect cells lack sufficient processes such as sialyltransferase activities and metabolic enzymes such as CMP-sialic acid (Hooker et al. 1999). These are necessary for generating complex-type proteins. N-Glycans from insect cells are not usually processed into sialylate recombinant N-glycoproteins. Some have attempted to "mammalianize" or "humanize" the N-glycosylation capacity of insect cells (Jarvis et al. 2004).

Another limitation of BEVS is that up to 40 % of the genomic DNA, which also includes the inserted foreign gene, might be autonomously deleted during the baculovirus propagation (Kool et al. 1991). This problem becomes important in large-scale biotechnological applications since defective baculovirus might be formed and accumulated in a continuous production or scale-up of AcMNPV.

Baculovirus Transduction of Mammalian Cells

For a long time, the baculovirus was thought to be capable of infecting only insect and invertebrate cells, not mammalian cells. In 1983, however, the virus was found capable of entering mammalian cells. Subsequently, Hofmann et al. showed that recombinant baculoviruses harboring a mammalian expression cassette were able to transduce human hepatocytes (Hofmann et al. 1995). They also efficiently expressed the reporter genes in the mammalian cells under the control of cytomegalovirus (CMV) promoter. Since then, numerous cell types of human, rodent, porcine, bovine, fish, and chicken have been found permissive to baculovirus transduction (Hu 2006). Recombinant baculoviruses containing mammalian system promoters (e.g., CMV or Rous sarcoma virus (RSV) promoter) can perform high-level transgene expression after delivering foreign genes into mammalian cells. The efficient transduction of baculovirus into many cell types has made baculovirus a promising vector for in vivo gene delivery. It will also contribute to the study of gene functions, the development of non-replicative vector vaccines, and the increase in gene therapy strategies.

DNA Vaccine

In 1990, Wolff injected animals with vectors that contained CAT or lactase gene, resulting in the expression of CAT protein and lactase for a short period (Wolff et al. 1990). Subsequently, in 1993, Ulmer and others performed intramuscular injection of a vaccine containing viral DNA. The vaccine proved successful against the flu virus by simultaneously generating humoral and cellular immune responses (Ulmer et al. 1993). DNA vaccine is a new technique that combines an existing vaccine with plasmid DNA or RNA of specific pathogen antigen gene within the bacterial plasmid. After the injection of the immune plasmid DNA, the host cells produced antigen protein and induced humoral and cellular immune responses that protected the host (Wolff et al. 1990; McDonnell and Askari 1996). This type of vaccine is also known as the nucleic acid vaccine, the DNA vaccine, the gene vaccine, or the third-generation vaccine.

DNA Vaccine

Vaccines train the immune system to identify pathogens by treating exogenous protein as an immunogen. DNA vaccines primarily leverage mammalian expression vectors. The muscle cells express protein antigens after immunization.

Subsequently, the antigen-presenting cells (APC) activate helper T cells (Th) through phagocytosis of the protein antigens. The resulting release of cytokines stimulates B cells to produce antibodies. This process is known as humoral immunity. Alternatively, cell-mediated immunity occurs when the cytotoxic T lymphocytes (CTLs) destroy the pathogens directly. Once cleared of pathogens, some B cells and T cells turn into memory cells. In a future invasion by the same pathogen, the pathogen is immediately identified and the immune response triggered.

In recent years, studies have pointed out that some DNA vaccines such as the ones for hepatitis B virus or influenza can cause a very strong immune response (Ulmer et al. 1993; Chow et al. 1998). The results of this study showed that animals vaccinated with one dose of the DNA vaccine can produce persistent immune response but not all antigens produce the same effect. Currently, the mechanism with which DNA vaccine induces immune response is not known. However, there are three hypotheses (Gurunathan et al. 2000).

The first hypothesis describes the immune response as follows. An intramuscular injection or gene gun immunization of the DNA vaccine into muscle cells or skin cells turns them into antigen-presenting cells. Antigenic protein and proteasome are produced through intracellular transcription and translation. Proteolytic enzymes then turn proteasome into digested peptides. Small fragments of these peptides bind to major histocompatibility complex class I (MHC class I) molecules in the endoplasmic reticulum and get sent to the Golgi complex. They are then transferred to the cell surface, forming the MHC class I-peptides complexes. At this time, cytotoxic T cells use T cell receptor (TCR), combined with MHC class I-peptides complex, to activate an immune response. This process is similar to the response induced by a viral infection. However, there are some unanswered questions posed by this hypothesis. For example, in order to activate the T cells, the MHC class I-peptides complex binding of TCR by itself is not sufficient. The antigen-presenting cell surface of B7-1 and B7-2 binding of CD28 T cells to transmit secondary signal is also needed. However, skin cells or muscle cells do not express B7-1 or B7-2 molecules. Furthermore, the cell surface does not have MHC class II molecules and therefore cannot activate T helper cells.

The second hypothesis theorizes that the DNA enters the antigen-presenting cells directly. Examples of these types of cells are the macrophage and dendritic cells in muscle tissue and the Langerhans cells in the skin. The antigen-presenting cells express the antigen. The antigen then combines with MHC class I or MHC class II molecules and gets sent to the cell surface in order to activate T cells for the immune response.

The final hypothesis begins with the muscle or skin cells translating the antigenic proteins by themselves. The intracellular materials bind to heat shock protein (HSP) and then get secreted out of the cell. The antigen-presenting cells engulf the protein antigen to form lysosome through phagocytosis. In an acidic environment, antigens are digested into peptides by enzymes. Endosomal lysosome is formed through endosome and lysosome fusion. The endosome of MHC class II molecules then binds to the pathogen peptides, forming MHC class II-peptides complex.

The complex is then transferred to the cell membrane. The antigen-presenting cells activate $CD4^+$ T cells to provide the signal. They also activate B cells to secrete antibodies and cytokine, which in turn activates $CD8^+$ T cells to eliminate pathogen. For antigen proteins without phagocytosis, they are recognized by the membrane-bound IgM on B cell surface, triggering the first signal. When the cells are released, hormones stimulate B cells to transmit the second signal and also activate them to produce antibodies. When Iwasaki and other scholars initiate intramuscular and gene gun immunization, they find that the activated T cells caused CTL response by the bone marrow. The responses were all derived from antigen-presenting cells rather than the immune position. At this point, the second and third hypotheses remain under investigation, while the first hypothesis was disproved.

Influence Factors of DNA Vaccines

Studies have indicated that some DNA virus vaccine for viruses such as the common flu virus or the hepatitis B virus (HBV) can elicit a good immune response. However, some DNA vaccines do not offer protective immunity. The effectiveness of DNA vaccines is determined by several factors.

The first factor is DNA’s ability to enter cells. Although some type of cells, such as muscle or skin cells, are easy to enter, others are not so. The biggest challenge is the cell membrane. Since both the DNA and the membrane surface are negatively charged, they repel each other. The plasmid DNA has a hard time entering the membrane and requires mechanical or chemical methods to get past it.

The second factor concerns antigen expression and intracellular DNA characteristics. Factors affecting gene expression include gene promoter, gene enhancer, intron, etc. Currently, the most commonly used vector is the cytomegalovirus immediate-early promoter (pCMV). Antigen variety and transgenic cells result in promoters having different specificity and induced transcription efficiency. For example, pCMV in muscle cells has a higher expression of the antigen protein (Cheng et al. 1993). Additionally, promoter of plasmids can only be induced and expressed on specific type of cells.

Finally, the effectiveness of DNA vaccines can be enhanced with adjuvants. Adjuvants such as CpG motif, cytokine, and chemokine can be added to DNA vaccines to increase immune response.

Advantages of DNA Vaccines

DNA vaccines can make use of plasmid RNA or DNA for immunization. The advantage of using RNA is that there is no danger posed by embedded chromosomes. However, the disadvantage is that the vaccine will not be stable and the expression of antigen is brief. As a result, RNA vaccines are rarely used in recent vaccines. Conversely, DNA vaccines have proven effective in the study of various diseases. DNA vaccines have been studied extensively in the context of infectious diseases, cancer, allergies, and autoimmune diseases. Advantages of DNA vaccines include the elicitation of cellular and humoral immune responses and natural antigen expression. The absence of purified proteins distress improves cross protection and avoids the possibility of toxic recovery and virus strains mutation.

The vaccine can also be combined with various genes under the same vector and become a multivalent vaccine. DNA vaccines are more effective and their quality easier to control. Lastly, vast amounts of plasmid DNA can be produced quickly.

DNA Vaccine Safety

Although DNA vaccines in clinical applications have many advantages, safety is still the most important consideration. The use of DNA vaccines may lead to these three types of unintended effects.

The first consideration is whether the plasmid DNA will cause anti-DNA antibody response related autoimmune disorders such as systemic lupus erythematosus. While the first phase of clinical testing did not show any anti-DNA antibody response, this is a possibility that we have to watch out for.

The second unintended effect is the increased risk of cancer. When a retrovirus or plasmid DNA enters the host cell chromosome, it might lead to gene mutations or alter the proto-oncogene and tumor-suppressor gene expression. This increases the risk of cancer. In 1992, Wolff and other scholars find that after the plasmid DNA enters into the cells, it forms a complete ring and is stable inside the nucleus (Wolff et al. 1992). The DNA is not inserted into the host cell chromosome. For DNA vaccine development, this is good news.

The third unintended effect is that the plasmid DNA might be expressed in a host over the long term, causing intolerance or toxicity. Since vaccination is usually applied after birth to a baby whose immune system is not yet mature, it may be mistakenly targeted by the baby's immune system, leading to antigen tolerance. Once tolerance occurs, additional vaccination no longer produces protection. Studies have been conducted on a day-old mouse injected with DNA influenza vaccine. The mouse turns into an adult mouse; a booster shot still produces memory response, proving that antigen tolerance does not occur (Hassett et al. 2000). Although tolerance is not observed in newborn mice, we need to look for it in human trials. Additionally, long-term antigen expression, such as cytokine or chemokine gene expression, might cause toxicity.

DNA Vaccine Preparation

DNA vaccines are created by cloning the pathogen gene into the selected vector, and then the constructed vector is injected into animals. After animals have been vaccinated, protein is expressed in vivo, thereby achieving immunization.

Plasmids of DNA Vaccine

Plasmids are the most used vectors for DNA vaccines. The vectors are usually double-stranded loop from microorganisms with antigenic genes. The plasmid's promoter should be sufficiently strong as this is a prerequisite for DNA vaccines. Different promoters have different protein expression capability. High and stable expression promoters, such as the cytomegalovirus (CMV) or the Rous sarcoma virus (RSV) promoter, are currently the most often used DNA vaccine promoters. Additionally, plasmids must have replication origin and the resistance gene. A large number of copies can be made from plasmids.

Viral Vectors

Viral vectors are obtained from infected and invaded host cells. Recombinant vectors are embedded in viral gene vectors and injected into the host. To facilitate entry into the host cell, DNA segments were used. Recombinant vectors do not normally cause disease onset in the host and at most cause low morbidity rate. When the viral vector infects human or animals, DNA is inserted into cells, followed by the expression of the unit antigen and the triggering of immune response. The most frequently used viral vectors are retroviruses such as the adeno-associated virus. Infectious viral vectors are difficult to prepare and purify. In addition, the viral vector may also induce the immune response of the host so its clinical applications are limited. However, since viral vectors are used in DNA vaccines and gene therapy, it is still worth attention.

Bacterial Vector

Using genetic engineering technology, we can insert genes that express specific antigens into the genome of bacteria that can grow in the cells of human or animals. The host is then infected with the bacteria. The type of bacteria selected for infection does not cause significant pathogenic disease. Upon infection, DNA is inserted into the cell and its expression induces an immune response.

DNA Vaccines via Gene Gun

Gene guns are most frequently used in the mammalian DNA vaccination. Its principal is similar to that of an air gun that uses high-pressure helium gas to shoot DNA-coated gold particles into the target tissue for transfection. In a study, gene gun immunization with DNA vaccines in poultry elicited good immune effect (Kodihalli et al. 2000). However, the gene gun method is expensive. In 1993, Fynan and others compared different application methods in chickens: intramuscularly (IM), intravenously (IV), intratracheally (IT), intrabursally (IB), intraperitoneally (IP), subcutaneously (SC), and intraocularly (IO). The specificity of the IgA antibody titer in chicken tears and bile was higher in IO vaccination versus IM vaccination. However, the specificity of IgG antibody titers was significantly higher in the IM vaccination (Fynan et al. 1993).

DNA Vaccine Doses

With a gene gun vaccination, protection can be conferred with as little as 0.25 μg of plasmids. For intramuscular application, 100 μg should be used. The dose of vaccination correlates with antibody production. Antibodies, however, do not increase significantly beyond 100 μg when applied intramuscularly.

Future Development of DNA Vaccine

Recently, many scholars have committed to the development of DNA vaccines. Vaccines are under development for the human immunodeficiency virus, malaria, tuberculosis, measles virus, B-type hepatitis, C-type hepatitis virus, and dengue fever. Vaccines are also under development for allergies, autoimmune diseases, and

tumor (Gurunathan et al. 2000). DNA vaccines are being developed for animals such as cattle, sheep, pigs, dogs, fish, and shrimp. DNA vaccines are currently in clinical trials and will become widely used in the future.

CpG Adjuvant

The Immunostimulatory Effects of CpG ODNs

Adjuvants are important components of vaccine formulations. Effective adjuvants line innate and adaptive immunity by signaling through pathogen recognition receptors. Synthetic cytosine-phosphate-guanine (CpG) and oligodeoxynucleotides (ODNs) have been shown to have potentials as adjuvants for vaccines. Experiments have shown that the stimulatory effects of CpG ODNs can be influenced by several factors, including the number of motifs, the space between motifs, the flanking bases, the presence of motifs other than CpG (poly-G), and also the backbone (Hartmann and Krieg 2000). In addition, the effective motifs of CpG ODNs are species specific. For example, the optimal motif for mice, GACGTT, showed very weak activities in human cells. The most optimal motif for humans, GTCGTT, has been shown to elicit different levels of lymphocyte proliferation in many species of domestic animals, including chickens. A recent study demonstrated that a CpG ODN containing three copies of GACGTT motifs enhanced antibody production and lymphocyte proliferation in SPF chickens. The same CpG ODN, when being used as the adjuvant, also increased the protection of a Newcastle disease virus vaccine. In other words, it appears that both GACGTT and GTCGTT can interact with chicken cells.

CpG Adjuvant in Waterfowls Vaccine

In one study, the immunostimulatory effects of CpG ODNs on waterfowls, such as ducks and geese, were investigated (Lee et al. 2010). Two types of CpG ODNs (each containing three copies of GTCGTT or GACGTT motifs, respectively) were selected to test their effects on duck lymphocyte proliferation. Recombinant parvovirus VP2 (rVP2) was formulated with different types of adjuvant, including aluminum adjuvant and CpG oligodeoxynucleotides (ODNs), and the immunological responses in ducks were examined. Compared with the control group, the production of rVP2-specific antibodies, the expression of cytokines in peripheral blood mononuclear cells (PBMC) stimulated by rVP2, and the percentage of $CD4^+$/$CD8^+$ cells in PBMC were all significantly increased in ducks immunized with rVP2. The rVP2 was formulated with CpG ODNs containing three copies of GACGTT motif. The result revealed that the motif GACGTT was more stimulatory to duck lymphocytes and might be used to improve the efficacy of vaccines for ducks (Lee et al. 2010).

CpG Adjuvant in Bovine Vaccine

In another study, the adjuvant activity of CpG ODNs was studied in cattle vaccinated with a model antigen, keyhole limpet hemocyanin (KLH) (Chu et al. 2011).

Results showed that the CpG ODNs containing three copies of GACGTT motif resulted in the highest lymphocyte stimulation index. Additionally, CpG ODN significantly increased the mRNA expression of interferon (IFN)-γ, interleukin (IL)-12, and IL-21 in bovine PBMC. Production of KLH-specific antibodies in the serum and in the milk whey was enhanced. CpG ODNs regulated cytokine gene expression in bovine PBMC and enhanced the production of opsonic antibodies and the secretion of IFN-γ. These preliminary data are still inconclusive. Application of this CpG ODN as an adjuvant requires further investigations and may have positive effects on vaccines for dairy cows (Chu et al. 2011).

Microparticle Vaccine

Significant information obtained recently indicates that future investigations on vaccine development will have to include adjuvants for enhancing the protective immune responses against pathogenic infections in animals and humans (Schijns and Lavelle 2011). Different adjuvants capable of improving immunity and protection have been described in numerous studies. However, the safety concern of an adjuvant is still a crucial issue in adjuvant development (Tomljenovic and Shaw 2011). Therefore, vaccine antigens formulated with safe and potent adjuvants that promised to induce “appropriate” immune responses seem more likely to be approved for use.

Microparticles derived from different polymers, including polylactide-co-glycolide (PLG), alginate, starch, and other carbohydrate polymers, can be designed as carriers for proteins or drugs (Heegaard et al. 2011). Particularly, in the last 10 years, biodegradable and biocompatible PLG polymers, approved by the US Food and Drug Administration (FDA), have become safe and potent adjuvants. This adjuvant delivery system encapsulates vaccine antigens for the development of controlled-release microparticle vaccines (Jain et al. 2011). Through hydrolysis, the biodegradable microparticles that are made from PLG polymers break down into biocompatible metabolites and also lactic and glycolic acids. These produce little inflammatory activity and are excreted from the body via natural metabolic pathways (Eldridge et al. 1991). In addition, PLG polymers have been extensively used as sutures and drug carriers for many years due to their biodegradability and biocompatibility. Different forms of PLG polymers can be obtained according to the ratio of lactide to glycolide used for the polymerization (Eldridge et al. 1991). PLG polymers are soluble in some organic solvents, such as dichloromethane and ethyl acetate.

PLG polymers provide a number of practical advantages in acting as vaccine adjuvants or delivery systems. Adjuvant effects of the PLG microencapsulation can protect antigens from unfavorable degradation, allow the sustained and extended release of antigens over a long period (Lim et al. 2009), and enhance antigen uptake by antigen-presenting (APC) cells. The APC cells include macrophages and dendritic cells in specific lymphoid regions. These effects in turn reinforce the

antigen immunogenicity to favorably generate strong immune responses, particularly Th1 cell-mediated immunity. In other words, microparticle vaccines made from PLG polymers may fulfill the need for induction of a functional cell-mediated immune response. The response is required in order to eliminate intracellular pathogens in host cells. In addition, antigen-loaded PLG microparticles can enhance the antigen uptake by APC cells that migrate to other lymphoid compartments, such as the spleen and mesenteric lymph nodes. The cells then stimulate immunocompetent cells to induce antigen-specific immunity (Singh and O'Hagan 2003). These APC cells containing microparticles have been demonstrated to travel to specialized mucosal lymphoid compartments, known as mucosa-associated lymphoid tissues (MALTs). These sites stimulate potent immunity following intranasal vaccination. Thus, antigen from PLG MPs may also fulfill the need for the development of an effective mucosal vaccine that would induce strong immunity via the intranasal route of administration (McNeela and Lavelle 2012).

The sustained antigen release appears to substantially enhance antigen-specific immune responses, achieving long-term protection (Lim et al. 2009). The release of an antigen from biodegradable PLG microparticles is governed by the degradation rate of the PLG copolymer. The rate is influenced by the polymer molecular weight, polymer hydrophilicity, the ratio of lactide/glycolide, and the processing conditions employed during microparticle preparation. The processing conditions include the type of organic solvents used, the acidity, and the temperature. Sustained antigen release of antigen-loaded PLG microparticles has been achieved in the development of various potent microparticle vaccines. In two previous studies, long-lasting effect of the PLG encapsulation was possible and anti-SAG1 and anti-SAG1/2 immunity against *Toxoplasma gondii* was maintained (Chuang et al. 2013a, b). The microencapsulated proteins, SAG1 and SAG1/2, were released in vitro in a triphasic programmed manner consisted of an initial burst release of protein through fast diffusion, then a gradual release by slow diffusion, and finally a fast release of protein. The pattern mimics the multi-injection of an antigen during a conventional vaccination process (Rajkannan et al. 2006). Therefore, antigen-loaded PLG microparticles capable of sustaining triphasic release of an antigen can be designed as a single-dose vaccine without the need for booster doses. Additionally, for future studies, the design of a single-dose vaccine should consider the following factors: the protein load in PLG microparticles, the rate of protein release, the antigen amount for immunization, as well as the immunization route used (Gupta et al. 1998).

According to previous studies, a strong cell-mediated immune response elicited by PLG microparticles appears to be largely a consequence of their uptake into antigen-presenting cells and the subsequent delivery of these microparticle-containing APCs to specific lymphoid compartments (Grebennikova et al. 2004; Choi and Lee 2004; Sherlock et al. 2006). The particle size used for vaccination in animals is an important parameter in enhancing the uptake of antigen-presenting cells (Sherlock et al. 2006). Particles smaller than 10 μm in diameter are appropriate for direct uptake by antigen-presenting cells, such as macrophages and dendritic

cells (Sherlock et al. 2006). The proper size thus stimulates APCs to facilitate the microparticle uptake. Following the uptake, the APCs then effectively process and present the epitopes of microencapsulated antigen to T lymphocytes, especially Th1 and Tc, thereby inducing strong antigen-specific Th1 cell-mediated immunity (Men et al. 1999). Therefore, better uptake and delivery of antigen-loaded PLG microparticles by APCs can lead to a more effective inducement of cell-mediated immune responses (Jain et al. 2011).

Antigenicity retention following encapsulation and during the release of antigen from microparticles is critical. Although a number of proteins have been successfully entrapped in PLG microparticles without losing structural integrity, immunogenicity, or bioactivity, antigen denaturation due to organic solvent exposure in the encapsulation process is still a concern (Heegaard et al. 2011). Numerous studies have explored different encapsulation procedures to decrease contact between the antigen and the organic solvent containing the PLG polymers (Ye et al. 2010).

As mentioned previously, PLG polymers serve both as a potent adjuvant and as a delivery system, allowing antigen to gain access to specific lymphoid compartments. Future applications of the PLG adjuvant are likely to include the development of a more site-specific delivery system for both mucosal and systemic administration. Mucosal surfaces, such as the gastrointestinal tract, are principal sites of entry for many pathogens. Therefore, the development of effective mucosal vaccines formulated with potent adjuvants such as PLG polymers promises to elicit long-lasting protective immunity. It is also a critical step forward in the enduring control of mucosal infections. The use of antigen-loaded polymeric PLG MPs capable of sustaining the antigen release is a significant platform for the induction of long-lasting mucosal immunity and, consequently, long-lasting protection in animals (McNeela and Lavelle 2012).

Conclusion and Future Directions

Live and attenuated vaccines remain the most effective at eliciting robust immune responses, but reversion to virulence poses great safety concern. Since mutations are inherent for living pathogens, the development of nonliving, exogenous protein components as vaccines provides a safe alternative. However, protein components do not provide sufficient “danger” signals required for strong immune response; therefore, adjuvants are generally needed. DNA vaccines can induce endogenous expression of immunogenic components, but the risk of chromosome integration and the optimal site of antigen expression still need to be addressed. While contributing enormously to the control of most infectious diseases, vaccines may not be the silver bullet due to the evolution of pathogens and the nature of host-pathogen interaction. Nevertheless, continued effort in vaccine development and advances in immunology and disease pathogenesis will allow the most efficient use of vaccines for disease control.

Cross-References

▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
▶ Immunoneutralization of Abrin
▶ Immunosensors: Using Antibodies to Develop Biosensors for Detecting Pathogens and Their Toxins

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Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures

21

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Abstract

Botulinum neurotoxins (BoNTs) produced by the *Clostridium* family of bacteria are the deadliest of all naturally occurring toxins and are listed as the highest risk biothreat agents by the Centers for Disease Control and Prevention (CDC). They are the causative agents of botulism, a severe neuroparalytic disease which can be fatal in the absence of medical care. While natural cases of botulism are rare, by virtue of their extreme toxicity, easy production, and dissemination, BoNTs could potentially be employed as biowarfare agents and hence pose great threat to national security and public health. Nevertheless, the ability of these toxins to disrupt neurotransmission has been put to good use, and this potentially lethal toxin is now being used therapeutically in an ever expanding list of medical disorders and cosmetic treatments. A combination of features including neuronal target sites, efficient cellular entry, and unique enzymatic activity contributes to the extreme toxicity of BoNTs. Botulinum neurotoxins have a multi-domain structure with each domain contributing to a specific role in the toxin action. Recent advancements in deciphering the molecular mechanism of action of BoNTs and their structure-function relationship have facilitated a greater understanding of the potential use of individual domains of the toxin in the development of potent antidotes against botulism. Numerous biodefense efforts have been directed toward the development of improved technologies for botulism detection/diagnosis and for developing effective countermeasures, including vaccines, peptides, and small-molecule inhibitors.

Introduction

Toxins that have detrimental effects on humans are produced by a diverse variety of plants, animals, and microorganisms (Rappuoli and Montecucco 1997). Organisms synthesize toxins as a means of survival to acquire food or defend themselves against predation and to help infect potential hosts, with the ultimate goal of proliferation and reproduction. Among the Eubacteria, the Clostridia produce more protein toxins than any other bacterial genus (Johnson 1999). Clostridial neurotoxins (CNTs) are produced by a diverse group of rod-shaped, anaerobic, gram-positive, spore-forming bacteria of the genus *Clostridium*. These neurotoxins are the most toxic proteins known to humankind with mouse LD_{50} values ranging between 0.1 and 1 ng/kg body weight (Schiavo et al. 2000), which makes it about 330 million-, 5 million-, and 100 million-fold more toxic than diphtheria toxin, cobra toxin, and sodium cyanide, respectively (Singh 1996). Botulinum neurotoxins are produced by Clostridia species such as *C. botulinum, Clostridium argentinense*, as well as the rare strains of *Clostridium butyricum* and *Clostridium baratii*, whereas tetanus neurotoxin is produced only by *Clostridium tetani*. *C. botulinum* produces seven serotypes of botulinum neurotoxins (BoNTs) named A to G according to the order of their discovery (Sakaguchi 1983). They have mutually exclusive immunological properties but shared pharmacological characteristics as all cause flaccid paralysis in the botulism disease (Simpson 1988).

Clostridial neurotoxins act as metalloproteases that enter peripheral cholinergic nerve terminals and cleave proteins that are crucial components of the neuroexocytosis apparatus, causing a persistent but reversible inhibition of neurotransmitter release. Botulinum neurotoxins are the causative agents of botulism, a potentially fatal neurological disease characterized by flaccid muscle paralysis, resulting from BoNT-mediated blockage of acetylcholine release at the nerve-muscle junctions (Singh 2006). Tetanus neurotoxins (TeNTs) cause tetanus, a disease characterized by spastic paralysis (Montecucco and Schiavo 1995).

Overview of the Mode of Action of BoNTs and Neurotransmitter Release

All botulinum neurotoxins are synthesized in the bacterial cytosol as single, inactive polypeptide chains with a molecular mass of 150 kDa. They are subsequently cleaved by bacterial proteases to generate the fully active neurotoxin, composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) that remain linked through a disulfide bond (Singh 2000). One exception is BoNT/E, which remains as an intact polypeptide until it reaches the victim’s gut where the proteases in the gut activate the toxin by splitting it into light and heavy chains (Aoki and Guyer 2001). During the intoxication process, the interchain disulfide bond is reduced which is a necessary prerequisite for the intracellular action of the toxins (Humeau et al. 2000).

BoNT serotypes share a high degree of sequence homology, but they differ in their toxicity and molecular site of action. The three-dimensional structures of BoNTs reveal that they are folded into three distinct domains which are functionally related to their cell intoxication mechanism. The N-terminal domain is the 50 kDa light chain, a Zn^{2+}-dependent endopeptidase belonging to the thermolysin family of metalloproteases. The 100 kDa HC contains an N-terminal translocation domain and a C-terminal receptor-binding domain (Singh 2006) (Fig. 1). Such structural organization is functionally related to the fact the CNTs intoxicate neurons via a multistep mechanism. Biochemically, BoNT/A is a complex structure with the chemical formula $C_{6760}H_{10447}N_{1743}O_{2010}S_{32}$ and a molecular weight of 150 kDa (Berry and Stanek 2012).

The exact molecular mechanism of BoNT action is still not completely understood, but based on existing experimental evidence, BoNT intoxication is believed to occur through a multistep process involving each of the functional domains of the toxin (Montecucco et al. 1994). These steps include (a) passage of the toxin through

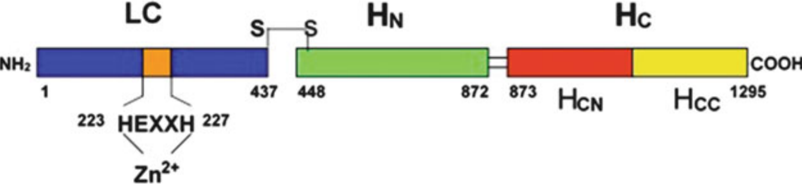


Fig. 1 Schematic representation of different domains of BoNT/A

the gut epithelial layer into the blood stream, (b) binding to specific receptors at the presynaptic nerve terminal, (c) internalization into the nerve cell and translocation across the endosomal membrane, and (d) intracellular endopeptidase activity against proteins crucial for neurotransmitter release. Each of these processes is described in detail below.

Absorption of Botulinum Neurotoxins Through the GI Tract

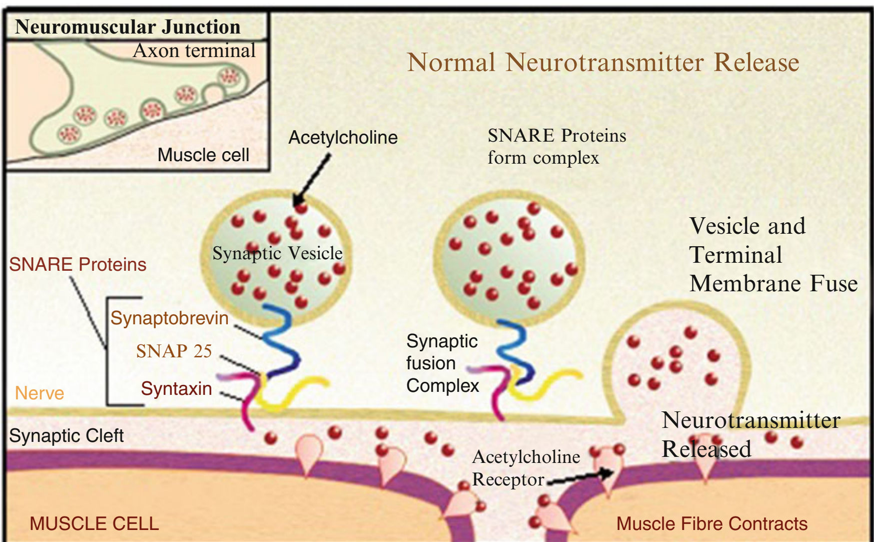
The most common mechanism of botulism poisoning is through the ingestion of preformed neurotoxin in food contaminated with *C. botulinum* or by ingestion of spores or bacteria, which may colonize the gut and produce the neurotoxin in situ. In either case, BoNT must escape the gastrointestinal (GI) tract to reach the blood and lymph circulation en route to the target cholinergic nerve endings where it exerts its neuroparalytic effects (Maksymowych et al. 1999). Penetration of the neurotoxin through epithelial cell barriers is the first essential step of botulinum intoxication (Maksymowych and Simpson 2004; Couesnon et al. 2008). One of the major reasons why BoNT is active by the oral route is because it is secreted by the bacteria as a complex comprised of the holotoxin and a group of neurotoxin-associated proteins (NAPs). The NAPs are known to protect the toxin in adverse environmental conditions such as low pH and proteases in the GI tract. BoNTs bind to polarized epithelial cells of the gastrointestinal system and undergo receptor-mediated endocytosis and transcytosis and are thus carried from the lumen of the gut into the general circulation.

Neurospecific Binding at the Neuromuscular Junction

The toxin from the general circulation eventually reaches the cholinergic nerve terminal, its principle site of action, to which it binds specifically. BoNTs bind to the peripheral cholinergic nerve endings through their heavy chain domain. A proposed double-receptor model suggests that BoNTs first bind to gangliosides on the neural membrane and then with a protein receptor through the H_C domain (Singh 2000) (Fig. 2). Complex polysialo-gangliosides, particularly G_{T1b} and G_{D1b}, interact with the receptor-binding domains of BoNTs (Nishiki et al. 1994; Rummel et al. 2004; Rummel 2013). In BoNT/A, BoNT/B, BoNT/E, BoNT/F, and BoNT/G, ganglioside binding occurs through a conserved ganglioside binding pocket within the 25 kDa H_{CC} domain (C-terminal HC), whereas BoNT serotypes C and D display two different ganglioside binding sites within their H_{CC} domain (Rummel 2013; Strotmeier et al. 2010).

Following binding to the gangliosides, the membrane-bound ganglioside-toxin complex moves to reach the toxin-specific receptor. Synaptotagmin, a synaptic vesicle protein, has been identified as the receptor for botulinum neurotoxin types B and G (Nishiki et al. 1996; Rummel et al. 2007). BoNT/B and BoNT/G bind via their H_{CC} domain to the intraluminal domains of synaptotagmins I and II when they

a



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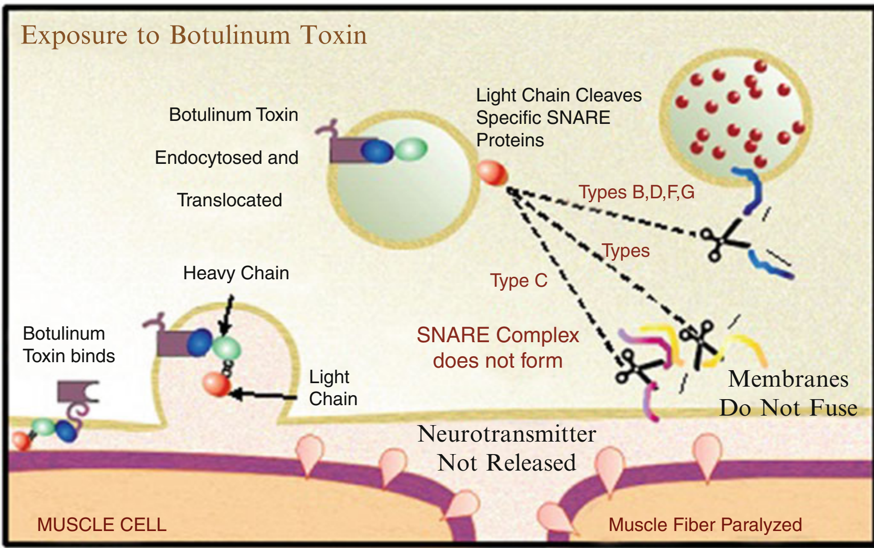


Fig. 2 Schematic model of mode of action of botulinum neurotoxins. (**a**) Synaptic vesicles containing neurotransmitters dock and fuse with the plasma membrane through interaction of the SNARE proteins (synaptobrevin, SNAP-25, and syntaxin). (**b**) Botulinum neurotoxin binds to the presynaptic membrane through gangliosides and a protein receptor followed by internalization into the endosomes via endocytosis. Following this the light chain is translocated across the membrane into the cytosol where it acts as a specific endopeptidase against either of the SNARE proteins. BoNTs cleave their substrates before the formation of SNARE complex (From Kukreja and Singh 2009)

are exposed to the neuronal cell surface during exocytosis. This facilitates the entry of the toxin into the cell during membrane retrieval (Rummel et al. 2007; Willjes et al. 2013). More recently SV2, a synaptic vesicle glycoprotein was identified as a receptor for BoNT/A and BoNT/E (Dong et al. 2006; Mahrhold et al. 2006, 2013). BoNT/D, BoNT/F, and TeNT also employ SV2 for binding and uptake (Rummel et al. 2009; Rummel 2013).

Internalization and Translocation into Neuronal Cytosol

The binding of BoNTs to cell surface receptors is followed by its internalization into cellular compartments by receptor-mediated endocytosis (Schiavo et al. 2000). After the incorporation of BoNTs within the early endosomes, the acidic environment of the endocytotic vesicles is believed to induce a conformational change in the neurotoxin structure and formation of a membrane spanning pore. The membrane interaction and pore formation is thought to facilitate the passage of the catalytic domain across the membrane into the neuronal cytosol (Montecucco and Schiavo 1995; Matteoli et al. 1996).

Several models have been proposed to explain the mechanism of BoNTs translocation across the endosomal membrane. One of the hypothesis suggests that the LC unfolds at low pH and permeates through the narrow transmembrane "tunnel" formed by the HC (Hoch et al. 1985). An alternative hypothesis indicates conformational change occurs in both the light and heavy chains at low pH thus exposing their hydrophobic patches. The toxin HC then forms a transmembrane hydrophilic "cleft" that nests the passage of the partially unfolded LC with its hydrophobic segments facing the lipids (Lebeda and Singh 1999). It has recently been shown that under low-pH conditions, BoNT/A LC undergoes unique structural changes adopting a flexible molten globular conformation by exposing substantial number of hydrophobic groups, thereby facilitating its passage through the narrow membrane channel formed by the BoNT HC (Cai et al. 2006).

A transmembrane chaperone/channel activity of the BoNT HC has been suggested wherein the HC channel ensures a translocation-competent environment for the unfolded LC to reach the cytosol. The light chain is released into the cytosol upon reduction of the disulfide bond (Koriazova and Montal 2003; Fischer and Montal 2007a, b; Montal 2010). The translocation domain belt/loop that wraps around the catalytic domain and partially occludes the active site in the unreduced holotoxin has also been observed to act as a chaperone and as a pseudo-substrate inhibitor of the LC during its translocation across the endosomal membrane (Brunger et al. 2007). More recently, it was shown that although the receptor-binding domain of the HC does not play any direct role in the channel activity for LC translocation, it dictates the pH threshold for channel insertion into the membrane (Fischer and Montal 2013). The above findings indicate that in addition to their individual function, each domain of botulinum neurotoxin functions as a chaperone for others enabling productive intoxication (Fischer and Montal 2013).

Intracellular Zinc Endopeptidase Activity

After internalization into the neuronal cytosol, BoNTs exert their toxic effect by virtue of the metalloprotease activity of the LC which specifically cleaves one of three soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that are integral to vesicular trafficking and neurotransmitter release (Singh 2006). BoNT LCs are a unique group of zinc-dependent metalloproteases that contain the common His-Glu-Xaa-Xaa-His zinc-binding motif (Schiavo et al. 1992). They recognize specific structural motifs on the SNARE proteins comprised of a vesicle-associated membrane protein (VAMP/synaptobrevin), syntaxin, and a 25 kDa synaptosomal-associated protein (SNAP-25). SNARE proteins are key components in the fusion of synaptic vesicles with the plasma membrane and reside on transport vesicles or on the target membranes (Schiavo et al. 2000). When synaptic vesicles come close to the plasma membrane, the SNARE proteins form a multi-protein 20S complex, together with NSF that possesses ATPase activity leading to the fusion of vesicles with the plasma membrane (Sutton et al. 1998; Graham et al. 2001). When these proteins are assembled in the tight complex, the substrates are resistant to in vitro proteolysis by BoNTs (Pellegrini et al. 1995), whereas free proteins are specifically and efficiently cleaved by BoNTs. The proteolysis of one SNARE protein results in a nonfunctional complex wherein the coupling between Ca^{2+} influx and fusion is disrupted (Humeau et al. 2000).

The specific SNARE protein targeted and the site of hydrolytic cleavage vary among the seven BoNT serotypes. BoNT serotypes A and E specifically cleave SNAP-25 at a unique peptide bond. BoNT serotypes B, D, F, and G hydrolyze VAMP/synaptobrevin, at different single peptide bonds, and BoNT/C cleaves both syntaxin and SNAP-25 (Singh 2006) (Fig. 2). These metalloproteases are unique in that they possess exquisite substrate specificity, requiring substrates with a minimum of ~40 residues for efficient cleavage. The three SNARE proteins, VAMP, SNAP-25, and syntaxin, possess a distinct three-dimensional motif (SNARE motif) that is required for specific proteolysis by the neurotoxins (Montecucco and Schiavo 1995). Botulinum neurotoxins are known to recognize the tertiary rather than the primary structure of their proteolytic substrates at the level of the nine-residue long SNARE motif (Rossetto et al. 1994). BoNTs differ with respect to the specific interaction with the SNARE motif (Rossetto et al. 2001a).

The catalytic domain of BoNT/A consists of a mixture of α-helices, β-sheets, and strands with a zinc metalloprotease active site bound deep inside a large cavity. The active site of clostridial neurotoxins has been extensively studied using site-directed mutagenesis and x-ray crystallography (Lacy et al. 1998; Hanson and Stevens 2000; Swaminathan and Eswaramoorthy 2000; Agarwal et al. 2004, 2005; Li et al. 2000; Rigoni et al. 2001; Rossetto et al. 2001b; Binz et al. 2002; Breidenbach and Brunger 2004). Superimposition of the X-ray crystal structures of BoNT/A, BoNT/B, and BoNT/E light LCs reveals virtually identical structures of their active sites (Breidenbach and Brunger 2004). Despite this fact, the BoNT serotypes exhibit unique substrate selectivity and cleavage site specificity suggesting that substrate recognition is not solely dictated by the enzyme's catalytic cleft. The active site of

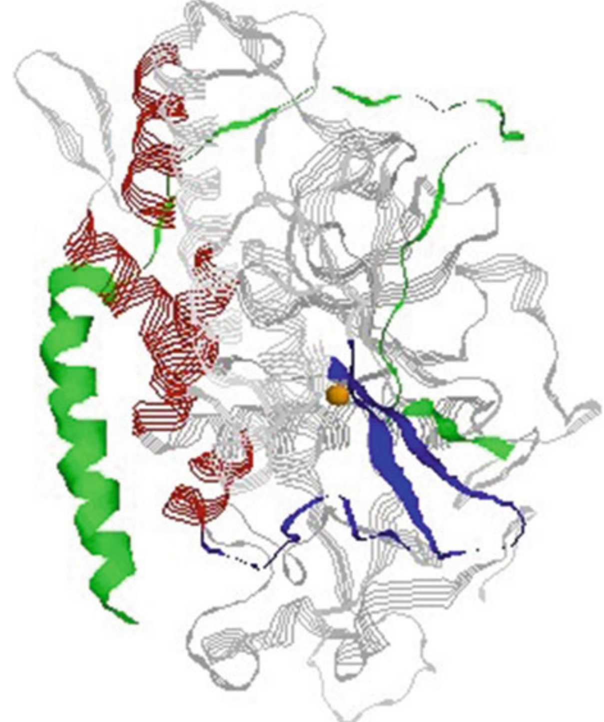


Fig. 3 Schematic diagram of BoNT/A LC (*gray*) co-complexed with SNAP-25 (*green*), displaying multiple sites of enzyme-substrate interaction remote from the active site (*yellow sphere*). A distal α-helical domain of SNAP-25 closely interacts with the α-exosites (*red*), while the β-sheet segment of the C-terminal of SNAP-25 lies in close proximity to the active site and the β-exosites (*blue*)

BoNT/A LC is characterized by HEXXH zinc-binding motif that coordinates the catalytic zinc ion through two His residues, a Glu 224 residue ligating the zinc via a water molecule and Glu 262 that acts as the fourth ligand (Vallee and Auld 1990; Kukreja et al. 2007). Four flexible loops, namely, 50/60 loop (residues 54–68), 170 loop (residues 154–174), 250 loop (residues 242–259), and 370 loop (residues 359–370), form the rim of the active-site cleft and possibly participate in substrate binding (Breidenbach and Brunger 2004; Silvaggi et al. 2007). Structural elucidation of the LC in the presence of substrate has been instrumental in providing evidence for the presence of an array of substrate recognition and binding sites known as exosites that are remote from the active site (Breidenbach and Brunger 2004) (Fig. 3). A model for substrate recognition has been proposed wherein substrate binding is initiated by the interaction of the amino-terminal helical region of SNAP-25 with the enzyme along a hydrophobic patch formed at the interface of four α-helices of the LC referred to α-exosite. The α-exosite is comprised of four α-helices formed by residues 102–113 (α-helix 1), 310–321 (α-helix 2), 335–348 (α-helix 3), and 351–358 (α-helix 4). This is followed by binding at the β-exosite which is comprised of the 250 loop (residues 242–259) and 370 loop (residues 359–370) of BoNT/A LC. This interaction induces conformational changes in the active-site pocket of the enzyme via the 370 loop facilitating efficient enzyme cleavage (Breidenbach and Brunger 2004) (Fig. 3). The multisite binding strategy used by BoNT/A accounts for the extreme selectivity of this enzyme (Breidenbach and Brunger 2005).

Clinical Manifestation of Botulism

Botulism is one of the most lethal paralyzing diseases to afflict humans. The clinical spectrum of botulism continues to expand and is today divided into six clinical categories:

(i) Foodborne botulism is the most common form resulting from ingestion of inadequately processed food that is contaminated with BoNTs (Cherington 1998). Classical foodborne botulism in humans is caused mainly by *C. botulinum* types A, B, and E, although type F has been reported the causative type in at least two outbreaks of food poisoning (Green et al. 1983). The most important vehicles for human botulism are canned foods, meat products, and seafood that are contaminated with the bacterium.
(ii) Wound botulism arises as a consequence of toxin produced in wounds contaminated with the clostridial bacterium. Wound botulism is rare in humans but has increased significantly in recent years among drug users (Cooper et al. 2005).
(iii) Infant botulism is caused by the ingestion of botulinum spores that germinate in the infant's gastrointestinal tract and release toxin. Since the gut microflora in babies are poorly developed, they are more susceptible to botulinum colonization in the intestine (Caya et al. 2004).
(iv) Hidden botulism is an adult variation of infant botulism, which, even though rare, could affect individuals with compromised intestinal flora resulting from abdominal surgery, gastrointestinal wounds, etc. (Freedman et al. 1986).
(v) Inadvertent botulism which is an unintended consequence of treatment with botulinum toxin A or accidental exposure in laboratory workers (Caya et al. 2004).
(vi) Intentional botulism which could result from widespread dispersal of the toxin through contamination of food or water supply or through aerosolization by terrorist organizations and has the potential to cause hundreds of thousands of casualties (Hatheway 1993; Wein and Liu 2005).

Botulism in humans generally manifests as a rapidly progressive symmetrical neuromuscular paralysis. The clinical symptoms of botulism occur fairly quickly between 18 and 48 h postexposure and include dry mouth, double vision, and progressive difficulty in speaking, swallowing, and breathing. Abdominal distension and constipation may also develop. As the disease progresses, intoxication produces descending flaccid paralysis of the diaphragm leading to respiratory failure which may require prolonged mechanical ventilation for several weeks to months (Hughes et al. 1981; Kongsaengdao et al. 2006). Ingested botulinum toxin can paralyze all muscles of the body, and recovery requires re-nervation by new nerve terminal axons and end plates. The rapidity of the onset of symptoms, duration, and the severity of botulism differ based on the specific serotype associated with the exposure (with type A known to cause more severe and longer-lasting symptoms compared to other serotypes) and the level of intoxication (Foran et al. 2003).

Because of their extreme toxicity, ease of production, and robust stability under adverse environmental conditions, BoNTs pose a significant threat as a bioterrorism

weapon. BoNT is the only toxin group listed among the six most dangerous biothreat agents and is a category A biodefense pathogen (Cai and Singh 2007). Although the probability of an incident involving a biological agent is relatively low, the ability of minute amounts of these agents to cause great morbidity and mortality necessitates these biological agents to be classified as weapons of mass destruction (Ramasamy et al. 2010).

Clinical Applications of Botulinum Neurotoxins

Due to their extreme neurospecificity, BoNTs are widely used as therapeutic agents to treat a myriad of neurological disorders, as well as for cosmetic purposes to remove facial wrinkles and frown lines (Rohrich et al. 2003). BoNT/A was approved by the US FDA as early as 1989 for the treatment of strabismus and blepharospasm and then for treating cervical dystonia, axillary hyperhidrosis, and several others. The efficacy of BoNTs in the treatment of pain syndromes, including migraine and myofiscal pain, has been well demonstrated. The high efficacy of BoNT/A coupled with a good safety profile has prompted its empirical use in a variety of ophthalmological, urological, dermatological, and pain disorders (Chen 2012). Incredibly the list of conditions treated with botulinum toxin is expanding at a brisk rate. There are currently three preparations of BoNT/A that are approved by the FDA, namely, BOTOX (onabotulinumtoxinA; Allergan, USA), Dysport (abobotulinumtoxinA; Ipsen UK), and Xeomin (incobotulinumtoxinA; Merz, Germany). Botulinum neurotoxin serotype B (MYOBLOC, Solstice Neurosciences, USA) was also approved by FDA in year 2000 (Chen 2012). The remarkable therapeutic utility of botulinum toxin lies in its ability to specifically and potently inhibit involuntary muscle activity for an extended duration.

Although the effects of BoNTs can be lethal, local applications at extremely low doses (200 million times less than the lethal dose) have proven to be safe and effective in clinical applications. Systemic side effects are rare when BoNT remains localized at the site of action (Truong and Jost 2006). However, it is likely that an accidental overdose, misuse, and/or adverse side effects (Cote et al. 2005) may result in systemic toxin exposure providing further impetus for development of effective countermeasures.

Opportunities for Therapeutics Development That Emerge from the Multiple Stages of BoNT Intoxication

BoNT remains a topic of relevant human health concern due to its expanding use in clinical medicine and scientific research, as well as the continual threat of its use as a bioweapon. Even though rarely encountered, botulism is a life-threatening disease, and the treatment requires intensive medical resources needed for supportive care, and in case of a bioterrorism event, it may quickly overwhelm the local health-care systems. Realizing this, in the recent years widespread efforts have been directed to develop improved detection technologies and effective countermeasures against botulism.

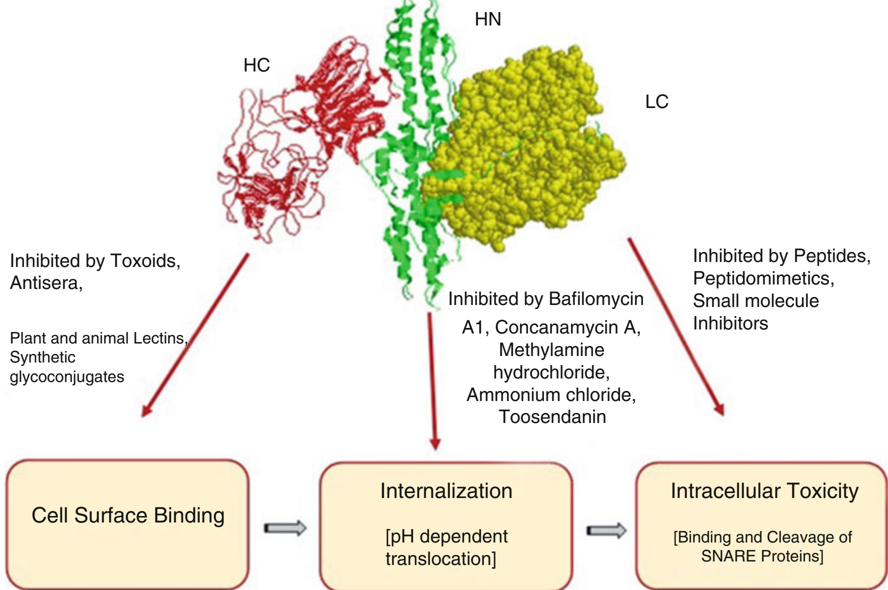


Fig. 4 Schematic model of BoNT domains and their role in the three main steps of BoNT intoxication at the neuromuscular junction (binding, internalization, and intracellular poisoning). HC (*red*) is the heavy chain receptor-binding domain, HN (*green*) is the heavy chain translocation domain, and LC (*yellow*) is the light chain catalytic domain. Inhibitors or inhibitor classes that target these specific stages of BoNT action are indicated in the diagram

Like other infectious disorders, the detailed mechanism of BoNT pathogenicity and its structure-function relationship provide valuable targets for development of antidotes against botulism. As previously described, the BoNT molecule is divided in clear functional domains. The three major activities of BoNTs that lead to blockage of neurotransmitter release, namely, cell surface binding and internalization, protein translocation, and enzymatic cleavage of the intracellular substrate, are all provided by independent domains: the HC, HN, and LC, respectively. Thus, each discrete stage of the BoNT mechanism of action provides an opportunity for pharmacological intervention, and the inhibition of BoNT intoxication could be achieved by targeting one or more of these specific stages of BoNT action (Fig. 4). Current progress in the design of therapeutics targeting these stages is described below.

Inhibition of Toxin-Receptor Binding

Active and Passive Immunization by Vaccines

Vaccination is an effective strategy for providing specific protection against exotoxins like botulinum toxin by eliciting neutralizing antibodies that prevent binding of the toxin to the specific receptor. Some of the earliest efforts for vaccine

development were initiated during World War II due to the concerns about the toxin being potentially employed as a biological weapon. The initial development of formalin-inactivated diphtheria and tetanus toxoid vaccines had prompted efforts toward the development of botulinum toxin vaccine by treating crude *C. botulinum* extract with formalin. This included an investigational crude alum-precipitated bivalent AB botulinum toxoid that was developed by the Department of Defense (DoD) and administered to at-risk individuals during the US biological warfare program in 1946 (Nigg and Hottle 1947). These developments led to the formulation of a pentavalent (ABCDE) botulinum toxoid by Parke-Davis in 1957 and thereafter by the Michigan Department of Public Health. CDC and DoD continued to distribute the pentavalent botulinum toxoid vaccine to immunize thousands of at-risk laboratory and military personnel for over half a century, as a preventative measure against botulinum. However, its use was discontinued in 2011 due to several shortcomings including declining immunogenicity and potency and an increased occurrence of adverse reactions upon its continual use (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6042a3.htm). In the USA, there is currently no FDA-licensed prophylactic treatment against botulism.

Studies in the late 1980s that demonstrated the production of neutralizing antibodies from the recombinant fragments of tetanus toxin have guided the development of recombinant BoNT (HC) vaccines. Recombinant BoNT HC has been expressed in *Escherichia coli* and *Pichia pastoris* and evaluated for their protection against BoNT-induced death in animals (Clayton et al. 1995). Recombinant HC was found to be highly immunogenic and produced high levels of effective antibodies against the toxin that could be used as co-treatment for botulism (Webb et al. 2007; Yu et al. 2009). Recently the potential power of recombinant vaccines was also investigated in an aerosolized toxin exposure model which is of great significance since in the event of a bioterrorist attack, toxin is most likely be deployed in this form. This study demonstrated that recombinant BoNT/B HC can provide 50 times more protection than the LCt_{50} value of BoNT/B against an aerosol exposure in rhesus monkeys (Boles et al. 2006).

Recent research efforts have focused on the development of more safe, cost-effective, and widely accessible vaccines. The potential use of a non-catalytic recombinant form of botulinum toxin was investigated wherein two single point mutations on the light chain domain rendered the toxin inactive and unable to cleave its substrate, SNAP-25. Mice immunized with BoNT/A^{RYM} survived subsequent challenges of 10,000 times that of BoNT/A LD_{50}, thus making this approach viable in the production of future vaccines (Pier et al. 2008; Ramasamy et al. 2010).

Several DNA-based vaccines have also been investigated due to their ease of production, purity, and storage. Plasmid DNA replicon vectors derived from alphaviruses were used to boost immune responses (Yu et al. 2007). The plasmid DNA replicon vaccine (pSCARSHc) encoding the BoNT/A HC provided better protection against BoNT/A in mice compared to a conventional plasmid DNA vaccine (pcDNASHc) encoding the same antigen (Ramasamy et al. 2010). In another study, an adenovirus-vectored recombinant vaccine based on BoNT/C HC elicited a robust immune response against BoNT/C over a

prolonged time period (Zeng et al. 2007) making them potentially promising vaccine candidates for botulism.

A bivalent HC subunit vaccine produced in *P. pastoris* (rBV A/B) provided short- and long-term immunity against neurotoxin challenges in mice and nonhuman primates (Hart et al. 2012). DoD provided additional funding to DynPort Vaccine Company for continued development of the recombinant bivalent botulinum vaccine for protection against BoNT/A and BoNT/B, since these two serotypes are known to be responsible for approximately 85 % of all cases of botulism. The safety and efficacy of this vaccine has been demonstrated in phase I and phase II clinical trials and further studies are being carried out to seek FDA licensure (Rusnak and Smith 2009; http://www.csc.com/dvc/press_releases/77621-csc_s_dynport_vaccine_company_releases_results_for_phase_2_botulinum_vaccine_clinical_trial).

In March 2013, the US FDA approved the heptavalent botulism antitoxin (HBAT), made by Cangene Corporation (Canada), as the first product to treat all serotypes of botulism. HBAT contains equine-derived antibodies against all known BoNT serotypes (A–G) and is the only botulinum antitoxin available in the USA for naturally occurring non-infant botulism (www.hhs.gov/news/press/2013pres/03/20130322a.html). It is indicated for sporadic cases of life-threatening botulism and is also stockpiled for the eventuality of botulinum toxins being used in a bioterrorist attack. This antitoxin replaced the previously used equine bivalent botulinum antitoxin AB and the investigational monovalent equine antitoxin E (BAT-AB and BAT-E, Sanofi Pasteur).

For the treatment of infant botulism, the US FDA has approved the use of human botulism immune globulin intravenous (BIG-IV/BabyBIG), which is available through the California Department of Health Services. BabyBIG is derived from pooled plasma from adults vaccinated with the pentavalent botulinum toxoid who developed high titers of neutralizing antibodies against BoNT/A and BoNT/B. When administered promptly, BabyBIG is safe and effective in minimizing the severity of illness (Arnon et al. 2006; Dembek et al. 2007). Also, in contrast to the adult antitoxin, BabyBIG does not pose a risk for anaphylaxis or possible hypersensitivity to equine antigens (Dembek et al. 2007).

In general, antitoxins can neutralize toxin molecules that are not yet bound to the nerve endings and clear them from circulation, thus limiting nerve damage and progression of the disease (Webb et al. 2012). Early treatment with antitoxins after BoNT exposure has reduced neurological manifestations and has been most effective if administered within 24 h of the onset of botulism (Willis et al. 2008). Because of the rapid onset of symptoms following BoNT intoxication, prophylactic vaccines are subject to several shortcomings including the limited availability for a large number of intoxicated patients in the event of a bioterrorist attack. Moreover, polyclonal antibodies consist of a multitude of diverse antibodies that bind to multiple epitopes, and there are often many side effects associated with administration of equine antibodies such as serum sickness, hypersensitivity reactions, anaphylactic shock, etc. (Willis et al. 2008). Other challenges associated with polyclonal preparations include batch-to-batch variations in preparation and potential risk of transmission of infectious diseases, thus

making antitoxins less effective as prophylaxis treatment (Willis et al. 2008). To balance between the need of scarce equine antitoxin with its potential side effects and the advantages of early treatment after an intentional or unintentional outbreak, it has been suggested to closely observe exposed patients and administer antitoxin when early signs appear.

Neutralizing monoclonal antibodies (mAbs) may prove to be valuable therapeutic agents against botulinum intoxication and viable substitutes for polyclonal antisera. This may be due to the fact that they are relatively easy to produce, have batch consistency, and do not carry any infectious risks, thus providing newer avenues to treat botulism with lesser side effects (Cai and Singh 2007). The fact that mAbs recognize only a single epitope means that they have limited neutralization capability against pathogens that exhibit antigenic variation. So far, no single mAb has significantly neutralized BoNTs (Nowakowski et al. 2002; Marks 2004). However, higher neutralizing effect was observed when three monoclonal antibodies were administered as a cocktail suggesting that effective antibody therapy against BoNT intoxication may require synergistic effect of several antibodies working together (Nowakowski et al. 2002; Meng et al. 2012). In partnership with National Institute of Allergy and Infectious Diseases (NIAID), recombinant monoclonal antibodies that effectively protect against BoNT/A, BoNT/B, and BoNT/E serotypes are currently being produced and tested for FDA licensure by XOMA Corporation, CA. Antibodies that protect against the other toxin serotypes (i.e., C, D, F, and G) are also in development.

An important limitation of all above indicated antitoxin and neutralizing antibody treatments is that they must be administered before the penetration of the toxin into the neuronal cytosol after which they are no longer effective. Hence, the therapeutic window for administering antitoxins is very limited (Marks 2004). Moreover, the flaccid muscle paralysis caused by BoNTs can last for several months, with patients requiring expensive, long-term respiratory care (Arnon et al. 2001; Wein and Liu 2005). Among the BoNT serotypes, BoNT/A persists the longest within neurons and requires the longest time for recovery of neuromuscular function (Davletov et al. 2005). This long duration of activity has made BoNT/A the serotype of choice for the treatment of several medical disorders as well as in cosmetic applications (Chen 2012). Consequently, the clinical value of BoNT/A has made mass vaccination a less desirable option for preventing botulism, as the immunity from botulism would neutralize the potential therapeutic benefits of the enzyme. However, the increasing use of BoNT/A in clinical applications also carries with it a heightened risk of accidental overdose. Clearly, there is a pressing need for new, and more effective therapeutic antidotes to counter BoNT intoxication, which unlike anti-BoNT antibodies will possess the ability to counter BoNT/A activity within the neuronal cytosol post-intoxication.

Small-Molecule Inhibitors of Toxin-Receptor Binding

The heavy chain in BoNTs mediates the binding of the toxin with the ganglioside and glycoprotein receptor at the nerve terminal (Nishiki et al. 1994). Thus,

inhibition of BoNT intoxication could be achieved by a small molecule that interferes with the ability of BoNT to interact with cellular receptors or a receptor antagonist that inhibits BoNT binding to the cellular receptor. In the former approach, polysialated gangliosides such as G_{T1b} have been shown to be potential receptors for BoNT/A (Simpson and Rapport 1971). Free G_{T1b} was shown to block binding of BoNT/A to synaptosomes (Kitamura et al. 1980). More recently, synthetic glycoconjugates based on G_{T1b} have prevented SNAP-25 cleavage in spinal cord cells of rat embryos (Kale et al. 2007). In the second approach, lectins of both plant and animal origin, *Triticum vulgaris* and *Limax flavus*, respectively, competed with BoNT for cellular binding (Bakry et al. 1991).

Inhibition of pH-Dependent BoNT Translocation

BoNT-induced muscle paralysis involves encapsulation of the holotoxin into endosomes and subsequent acidification which facilitates translocation of the BoNT LC into the neuronal cytosol where it acts as a Zn^{2+}-dependent endoprotease (Singh 2000). Exposure of the toxin to ammonium chloride and methylamine hydrochloride exhibited concentration and time-dependent antagonism against BoNT-induced paralysis by neutralizing the endosomal pH (Simpson 1983). However, the amines were found to act solely by inhibiting endosome acidification thus antagonizing the internalization of the toxin. They neither exhibited toxin inactivation nor affected the tissue function irreversibly.

Acidification of endosomes requires a cellular ATPase that pumps protons from the cytoplasm into the vesicle lumen. Bafilomycin A1, an ATPase inhibitor that prevents endosomal acidification and thus BoNT translocation, is a universal antagonist of BoNTs A–G as well as tetanus toxin (Simpson et al. 1994). An additional ATPase inhibitor, concanamycin A, has also been recently shown to prevent cleavage of SNAP-25 in pretreated cultured neurons by inhibiting endosomal acidification (Keller et al. 2004). Several aminoquinoline analogs have also been tested either alone or in a combinatorial approach and have been shown to suppress BoNT-induced neuromuscular blockage by inhibiting endosome acidification (Adler et al. 1997).

In the early 1980s, studies on the triterpenoid toosendanin, a traditional Chinese medicine, demonstrated its anti-botulinum properties in animals as it was shown to protect monkeys from BoNT/A-, BoNT/B-, and BoNT/E-induced death in a dose-dependent manner (Li et al. 1982; Zou et al. 1985; Shi and Li 2007). Single-molecule channel experiments using toosendanin and its tetrahydrofuran analog in mouse bioassays and rat spinal cord cell assays showed that toosendanin exhibits an unprecedented bimodal action within the protein conduction channel acting as cargo-dependent inhibitor of LC translocation and as a cargo-free activator of the HC channel (Fischer et al. 2009). In order to elucidate the mechanistic nature of the anti-botulinum properties of toosendanin, several of its analogs have since been prepared and studied including a function-oriented synthesis strategy in order to determine the structural features that are important for the anti-BoNT activity of this compound (Nakai et al. 2009, 2010).

All the above studies argue for further development of pharmacological countermeasures that target the early phases of BoNT intoxication mechanism such as binding, endocytosis, and translocation. However, due to the short window of time available for effective intervention, this strategy may not prove to be useful, since it was observed that ammonium chloride, methylamine hydrochloride, and concanamycin A could provide protection only when added either prior to or within 10–20 min after toxin challenge (Simpson 1983; Keller et al. 2004). In addition, the potential side effects of these agents in interfering with the regular cellular endocytosis also need to be considered. Thus, targeting the early steps of BoNT intoxication may not be advantageous over existing antitoxin treatments.

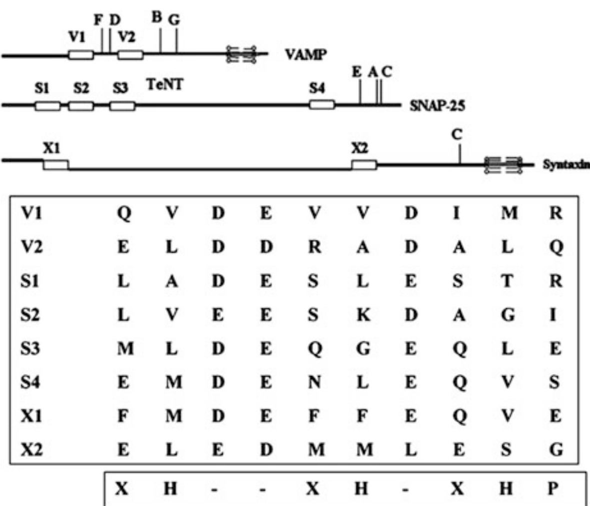
Inhibition of BoNT Metalloprotease Activity

BoNTs inhibit neuronal exocytosis by specifically cleaving SNARE proteins of the exocytosis machinery; the assembly of which plays a critical role in membrane fusion and neurotransmitter release (Singh 2006). Thus, targeting BoNT LC zinc metalloprotease activity provides an additional potential mode of BoNT inactivation. Different types of BoNT metalloprotease inhibitors have been discovered including peptides, peptidomimetics, and small molecules that inhibit BoNT LC activity.

Peptide-Based Inhibitors

Based on the available information on BoNT substrates, several small peptides have been developed as competitive inhibitors for the BoNT endopeptidase activity (Schmidt et al. 1998). Based on the cleavage site sequence in SNAP-25 (EAN**QR**AT, where Q and R is the cleavage site for BoNT/A), short peptides have been designed to achieve high inhibition effect on BoNT/A endopeptidase activity (Schmidt et al. 2001). Since sulfhydryl groups readily complex zinc, it was assumed that substitution of key amino acid residues with cysteine would inhibit BoNT enzyme activity. Short peptides containing a common sequence of (C) RATKML have also been identified as the effective inhibitors of BoNT/A. Further modification of this common sequence by replacing cysteine with 2-mercapto-3-phenylpropionyl (mpp) has yielded a peptide with a K_i value of 330 ± 50 nM (Schmidt and Stafford 2002).

The three SNAREs, VAMP, SNAP-25, and syntaxin, possess a distinct three-dimensional nine-residue long motif (SNARE motif) that is required for specific proteolysis by the neurotoxins (Rossetto et al. 1995). There are two copies of the motif in VAMP (V1 and V2), two copies in syntaxin (X1 and X2), and four copies in SNAP-25 (S1-S4) (Fig. 5). BoNTs differ with respect to their specific interaction with the SNARE motif (Rossetto et al. 2001a). Targeting the SNARE motifs provides another alternative to design inhibitors against BoNT activity. To investigate the potential role of the conserved motifs in BoNT recognition, a set of peptides corresponding to the conserved segments in VAMP, SNAP-25, and syntaxin were synthesized and tested as inhibitors of neurotoxin activity in vivo and in vitro and as immunogens (Rossetto et al. 1994). It was observed that V2, a



V1	Q	V	D	E	V	V	D	I	M	R
V2	E	L	D	D	R	A	D	A	L	Q
S1	L	A	D	E	S	L	E	S	T	R
S2	L	V	E	E	S	K	D	A	G	I
S3	M	L	D	E	Q	G	E	Q	L	E
S4	E	M	D	E	N	L	E	Q	V	S
X1	F	M	D	E	F	F	E	Q	V	E
X2	E	L	E	D	M	M	L	E	S	G
	X	H	-	-	X	H	-	X	H	P

Fig. 5 Conserved segments in the SNARE motif of VAMP, SNAP-25, and syntaxin. The SNARE motif consists of nine residues which is common to all three substrates: hydrophobic residue (*H*), Asp or Glu residue (-), polar residue (*P*), and any residue (*X*) (Modified from Rossetto et al. 1995)

synthetic peptide based on VAMP, and S1, a synthetic peptide based on SNAP-25, inhibited cleavage of the three SNARE substrates and were found to prevent neurotransmitter blockage in *Aplysia californica* neurons (Rossetto et al. 1994). While therapeutic agents targeting the active site will have to be tailored to individual variants of BoNTs, agents based on SNARE motif have the potential to treat poisoning by all forms of BoNT.

Hinge peptide mini libraries containing the acidic amino acids Asp and Glu (conserved in the SNARE motif), the scissile bond amino acids Gln and Arg in BoNT/A, and the zinc chelators His and Cys have been designed. Each library was based on the general formula acetyl-X_1-X_2-linker-X_3-X_4-NH_2, where Xn is one of the six amino acids residues, and the linker was 4-aminobutyric acid (Hayden et al. 2000). The linker was chosen to enhance BoNT recognition by altering the conformation of the amino acid backbone (Willis et al. 2008). These libraries exhibited pronounced inhibition of BoNT/A protease activity (Hayden et al. 2000). Deconvolution of these libraries by employing positional scanning synthetic combinatorial library (PS-SCL) technology was accomplished by fixing one position of the amino acid residue at a time beginning at the C-terminus. Higher inhibitory potencies were observed in the secondary library subsets when the two amino acid residues were fixed on the opposite sides of the hinge. This probably results from the increased flexibility of the central hinge moiety which creates a greater opportunity for side-chain interactions. The library subsets uncovered in this study may provide new leads in to the design of BoNT inhibitors (Moore et al. 2006).

A recent study demonstrated the rescue of botulinum-poisoned mouse spinal cord nerve cells by mastoparan-7, a peptide constituent of bee venom that was delivered through a drug delivery vehicle (DDV) constructed from the nontoxic fragment of botulinum neurotoxin itself. It was proposed that mastoparan-7 induces a physiological host response that is unrelated to SNAP-25 but linked to the phospholipase signal transduction pathway, thus providing new avenues to examine

the mechanism of exocytosis and also to examine alternative cellular mechanisms of botulinum neurotoxin action (Zhang et al. 2009a, b; Singh et al. 2010).

Peptide-based inhibitors in general suffer from two major limitations of poor bioavailability and metabolic instability, thus limiting their ability to reach the BoNT endopeptidase within neurons. Molecular docking and modeling studies between lead peptides and BoNT, based on their crystal structure, can lead to identification of pharmacophoric groups for the design of effective peptidomimetics as inhibitors against BoNT intoxication.

Inhibition of the long-lasting endopeptidase activity of BoNT resulting in the proteolysis of the SNARE proteins involved in exocytosis has been the major focus of research efforts for designing small-molecule antidotes against botulism, with the potential to reverse the paralysis syndrome of botulism. Small-molecule non-peptidic inhibitors (SMNPIs) have the advantage of being delivered into the intoxicated cells post-toxin exposure. Compared to the antibody-based therapy, these small-molecule inhibitors have better bioavailability, chemical stability, and better cell and tissue permeation. Therefore, a small-molecule inhibitor has the potential to be an effective oral therapy against botulism.

Small-Molecule Inhibitors of BoNTs

Several small-molecule inhibitors of BoNT LCs have been identified so far by screening libraries of either drug-like small molecules that are available commercially or those that are stored in national repositories. They have also been identified by creating a virtual library of compounds derived by an in silico chemical transformation of commercially available set of compounds. Several hydroxamate-based compounds and mercaptoacetamides have been identified as active-site inhibitors of BoNT/A LC since they exhibit competitive kinetics versus the SNAP-25 substrate (Boldt et al. 2006; Silvaggi et al. 2007; Stowe et al. 2010; Moe et al. 2009; O'Malley et al. 2013).

X-ray crystal structure studies of small-molecule inhibitors of BoNT/A LC including 2,4-dichlorocinnamic hydroxamate, a potent in vivo inhibitor, were carried out to assess the role of conformational flexibility of the active site of BoNT LC (Silvaggi et al. 2007). As shown in Fig. 6, the inhibitory compound, 2,4-dichlorocinnamic hydroxamate, binds with the cinnamyl side chain oriented toward the 370 loop that constitutes a part of the β-exosite and forms a rigid wall at one end of the active-site cleft. The hydroxamate moiety displaced the zinc-ligating water molecule observed in the native structure and the hydroxyl oxygen coordinated the catalytic Zn^{2+} ion. The active site showed remarkable flexibility conforming closely to the contours of the inhibitor, thus providing leads to the design and optimization of more effective small-molecule inhibitors of BoNTs (Silvaggi et al. 2007).

Development of a variety of high-throughput assays for screening a large library of diversified small-molecule (non-peptidic) compounds made it feasible to identify these inhibitors against BoNT protease activity. After screening a number of compounds from the libraries, a common pharmacophore was identified which provides scaffolds for designing potent inhibitors against BoNTs (Burnett et al. 2003; Cai and Singh 2007). Optimization studies utilized molecular dynamics simulations of a complex formed between zinc endopeptidase and the inhibitor, to

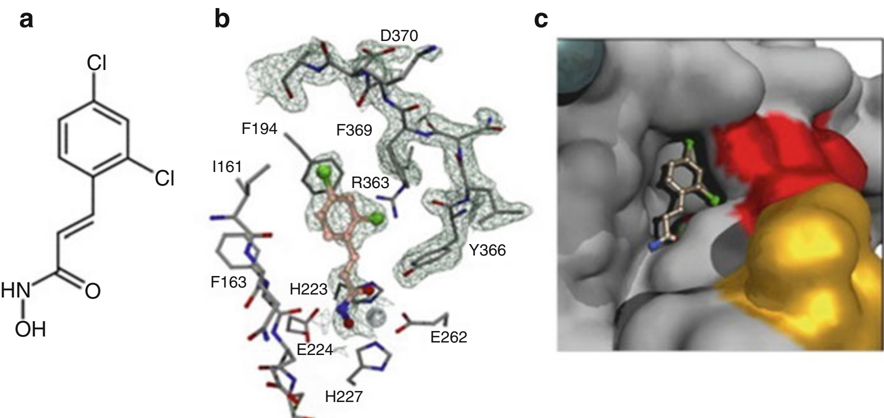
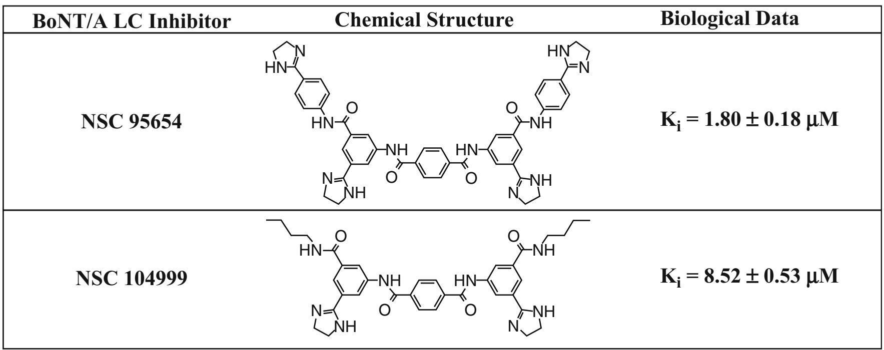


Fig. 6 (**a**) Chemical structure of 2,4-dichlorocinnamic hydroxamate. (**b**) Stereo representation of BoNT/A LC (1–424) bound to 2,4-dichlorocinnamic hydroxamate. Active-site residues are represented as cylinders with *gray* carbon atoms. The inhibitor is shown in ball-and-stick representation with brass carbons. The catalytic Zn^{2+} ion is denoted by a metallic sphere. (**c**) Conformational changes in the active site of BoNT/A LC seen upon binding to the inhibitor. The 370 and 250 loops are colored *red* and *orange*, respectively (From Silvaggi et al. 2007)

identify novel BoNT inhibitors based on the crystal structure of BoNT/A light chain (Roe and Ypang 1999; Tang et al. 2007). The crystal structures of BoNT light chains have been established (Segelke et al. 2004; Breidenbach and Brunger 2004); however, they do not adequately describe the dynamic properties of the bioactive form of the enzyme in solution where it represents a manifold of conformational substrates. The active site of BoNT/A LC exists in a dynamically flexible conformation that is likely to facilitate specific interactions with its substrate, SNAP-25, for its optimum and selective enzymatic activity (Kukreja and Singh 2005). This concern was highlighted when the potent LCA inhibitor mpp-RATKLM (Schmidt and Stafford 2002) failed to generate satisfactory binding conformations when docked into the enzyme structure (Burnett et al. 2007a). The pseudo-peptide mpp-RATKML is a potent (K_i = 330 nm), specific, and competitive inhibitor of the BoNT/A LC. Due to its size and the extensive amount of empirical structure-activity data available for this compound, it was an ideal candidate for molecular docking studies. A molecular dynamics study was thereafter initiated wherein favorable surface conformations between mpp-RATKML and BoNT/A LC were used. Several key requirements for potent small molecules were postulated from this study to identify key intermolecular contacts and map available steric volume within the BoNT/A LC substrate cleft (Burnett et al. 2007a).

Several non-Zn^{2+}-chelating small-molecule (non-peptidic) inhibitors of BoNT/A LC have since been designed and continually integrated into the pharmacophore-based design to guide the rational design of more potent SMNPI derivatives (Hermone et al. 2008; Solaja et al. 2008; Nuss et al. 2010). Recently two SMNPIs, NSC 95654 and NSC 104999 (Fig. 7), were shown to prevent BoNT/A-induced cleavage of



BoNT/A LC Inhibitor	Chemical Structure	Biological Data
NSC 95654		$K_i = 1.80 \pm 0.18\ \mu M$
NSC 104999		$K_i = 8.52 \pm 0.53\ \mu M$

Fig. 7 Chemical structures and Ki values of NSC 95654 and NSC 104999 that inhibited BoNT/A – induced cleavage post-intoxication within the neuronal cytosol in embryonic chick motor neurons (From Ruthel et al. 2011)

SNAP-25 post-intoxication in embryonic chick motor neurons rendering them as promising chemotypes for further therapeutic development (Ruthel et al. 2011).

A series of benzylidene cyclopentenedione-based inhibitors have been reported that inhibit BoNT/A LC metalloprotease by putatively forming a covalent bond with the enzyme (Capkova et al. 2009; Li et al. 2010). Several 4-amino-7-chloroquinoline-based antimalarial compounds have been identified as some of the most potent non-Zn^{2+}-chelating small molecule inhibitors of BoNT/A (Burnett et al. 2007b; Solaja et al. 2008). Recently, more BoNT/A LC inhibitors that exhibited significant antiprotozoal activity were synthesized, and optimization studies were designed to improve their ligand efficiency and inhibitory potency (Opsenica et al. 2013).

Several quinolinol derivatives have been reported to inhibit BoNT/A in cell- and tissue-based assays (Roxas-Duncan et al. 2009; Lai et al. 2009). A recent study demonstrated the efficacy of several hydroxyquinolinol derivatives that were selected by in silico screening of ChemBridge and NCI database libraries in protecting mice against pre- and post-BoNT/A challenge by blocking the active-site zinc atom (Singh et al. 2012).

A natural product from *Echinacea*, D-chicoric acid, has been identified to inhibit BoNT/A LC zinc metalloprotease activity by binding to an exosite and displaying noncompetitive partial inhibition of the LC with a sub-micromolar inhibition constant (Silhar et al. 2010). L-chicoric acid was found to inhibit the substrate binding to BoNT/A as well as BoNT/B LC via disruption of the SNARE-binding motif to the protease, thus implicating an exosite mechanism of action activity (Salzameda et al. 2011).

Many of the studies carried out for identification of small-molecule inhibitors for BoNT LCs have employed a more stable and truncated form (with comparable catalytic properties to the full-length enzyme) of LCs. In a recent study on the kinetics of the active sites and exosites of these two forms of the enzyme using molecular dynamics simulations, considerable flexibility in the C-terminus of the full-length BoNT LC/A was observed. This flexibility was found to be responsible

for the potency shifts of active-site proximally binding inhibitors whereas the distal binding exosite inhibitors remained equipotent (Silhar et al. 2013).

Recently, RNA aptamers were employed to effectively inhibit BoNT/A LC endopeptidase activity in a noncompetitive manner (Chang et al. 2010). Aptamers are single-stranded oligonucleotides (either DNA or RNA) that form unique three-dimensional structures which provide the basis for high binding specificity and affinity toward their targets (Cai and Singh 2007). In addition, aptamers exhibit low toxicity, non-immunogenicity, better tissue penetration (due to their smaller size), favorable pharmacokinetics, and established quality control rendering aptamers an attractive candidate for therapeutic countermeasures against the deadly BoNT (Cai and Singh 2007).

Clinical Diagnosis of Botulism

Although uncommon, botulism remains a subject of considerable concern because of its high mortality if not treated with urgency. As highlighted above, BoNT intoxication progresses fairly rapidly leading to descending flaccid paralysis which can be fatal. Due to the severity of the disease and the level of care involved, rapid detection of toxin in patients at the earliest stages of the disease is necessary for proper treatment.

Preliminary diagnosis of botulism is based on clinical suspicions which may be nonspecific and difficult to associate with botulinum intoxication and could easily be confused with symptoms of some other neuromuscular disorders. Drug and alcohol abuse may also prolong the time for diagnosis to be made (Lindstrom and Korkeala 2006). Definitive diagnosis requires identification of toxin in serum, vomitus, gastric aspirate, and stool. Due to the extremely low lethal doses of BoNTs, it is difficult to induce host responses detectable for diagnostics. Laboratory diagnostics need to focus on the rapid and sensitive detection of the toxin in clinical/biological samples.

Significant efforts have been made in the last several years to develop improved technologies for detection of BoNTs which can evaluate either overall activity of BoNT, including the three functional activities (binding, translocation, and enzyme), or assays that can detect the toxin based on its enzyme activity (reviewed in Cai et al. 2007; Capek and Dickerson 2010; Singh et al. 2013). Some of the detection methods using in vivo animal testing or in vitro technologies are described below.

Mouse Lethality Assay (MLA)

The current standard confirmatory test to detect the toxin is the mouse bioassay also known as mouse lethality assay (MLA) and is considered to be the "gold standard" for detecting all seven BoNT serotypes (Solomon and Lilly 2001). This assay involves intraperitoneal injection of mice with dilutions of the toxin and

determining the dilution at which 50 % of the mice die. The toxin type is determined by neutralization of the toxins with specific antitoxins. Alternatively the sample could be treated with antitoxins before administration to mice. While the method is very specific, any of the seven serotypes of BoNT can be identified. The mouse lethality assay is very sensitive, with one intraperitoneal mouse 50 % lethal dose (LD_{50}) corresponding to ~10 pg, a concentration that corresponds to one minimum lethal dose (MLD) which is the minimum amount of toxin (or the highest dilution of sample) that kills all the inoculated mice (Ferreira 2001). In addition to food and microbiological samples, the assay has been successfully used with fecal, wound, gastric, and serum samples. However, several drawbacks are associated with this assay which includes the laborious and time-consuming nature of the test as it can take up to 4 days or even longer if toxin serotyping is performed. The test requires the use of expensive specialized animal facilities staffed with highly trained personnel. In addition there also exists an ethical dilemma due to the use of laboratory animals in the assay. The assay cannot be automated and is not suitable for routine toxin quantification of samples. Also, more importantly, the assay would not be able to meet the testing capacity required in the event of a real biodefense scenario.

Several variations of the mouse bioassay have been developed that are more cost-effective, require fewer animals, and do not use death as the end point. However, none of them are treated as the standard for confirmation of botulism. A neuromuscular bioassay that makes use of intact hemidiaphragm preparations with attached phrenic nerves from mouse or rat have been evaluated (Sheridan et al. 1999; Rasetti-Escargueil et al. 2009). A recently developed in vivo method that uses mouse toe-spread reflex model was tested to detect BoNTs spiked into buffer, serum, and milk samples (Wilder-Kofie et al. 2011). Although these assays are more sensitive and faster and provide a more humane alternative to the current mouse bioassay, there are certain limitations associated with these. As with all in vivo methods, they require the use of expensive animal facilities and sophisticated equipment and training. Moreover, these assays are not amenable for use with complex matrices (Grate et al. 2010).

In order to combat these shortcomings of the animal-based assays, significant advancements have been made in developing alternate in vitro assays for BoNT detection. Some of the most commonly used ones are described below.

Immunological Assays for BoNT Detection

A wealth of immunoassays for the detection of botulinum neurotoxins have been reported. Enzyme-linked immunosorbent assay (ELISA), which measures the antigen-antibody interaction, has been used for over two decades for BoNT detection (Cai et al. 2007; Sharma and Whiting 2005; Stanker et al. 2008; Scotcher et al. 2010). ELISAs have been extensively tested with purified botulinum toxin, *C. botulinum* cultures, and foods contaminated with botulinum (Lindstrom and Korkeala 2006). The ELISA-based methods have also successfully been used to

characterize the toxin in its progenitor form (complexed with neurotoxin-associated proteins), which is the virulent factor for foodborne botulism (Sharma et al. 2006). Several modifications have been made in the ELISA-based method that enhance the sensitivity of BoNT detection.

An enzyme-linked coagulation assay (ELCA), developed as an amplification system for ELISA, was found to be equally sensitive as the mouse bioassay and performed well with clinical complex matrices like blood and feces. This sensitive method relied on a sophisticated amplification system utilizing a snake venom coagulation factor but was limited by its complexity, reagent expense, and longer testing times (Doellgast et al. 1993). Recently, another amplified ELISA assay known as the digoxigenin-ELISA (DIG-ELISA) was developed by US FDA and CDC for detection of BoNT/A, BoNT/B, BoNT/E, and BoNT/F in food, serum, and environmental samples. This assay makes use of BoNT serotype-specific polyclonal antibodies to capture the toxin and DIG-labeled serotype-specific polyclonal antibodies as secondary antibodies. The secondary antibodies are then detected by anti-DIG-labeled tertiary antibodies conjugated to horseradish peroxidase. This assay was found to be highly sensitive and could be completed within 6 h (Sharma et al. 2006).

Peptide-based capture agents have also been developed for detection of BoNTs by ELISA. A BoNT/A-specific 11-mer cyclic peptide was identified using phage display technologies. This peptide displayed strong binding affinity to BoNT/A and a detection limit of 1 pg/mL was achieved by using a highly sensitive chemiluminescent substrate (Ma et al. 2006).

A sensitive electrochemiluminescence (ECL) immunoassay platform has been used for detection of BoNTs in food matrices (Cheng et al. 2009; Cheng and Stanker 2013). This study used a similar immunoassay format to ELISA, except the output signal was not generated by the enzymatic hydrolysis of the luminescent substrate. Instead the luminescent signal was produced from ruthenium-labeled (SULFO-TAG™) reporter antibodies by application of an electric potential. More recently, an image-based 96-well Meso Scale Discovery (MSD) electrochemiluminescent (ECL) assay platform was also used for rapid detection of BoNTs in dairy milk products (Sachdeva et al. 2013).

Lateral flow tests or lateral flow assays (LFA) are handheld or dipstick assays based on capture agent-analyst reaction that are carried out using a nitrocellulose strip. The BoNT-containing liquid sample applied to the test well rehydrates the antitoxin antibody that is labeled with gold nanoparticles. The toxin-antibody complex then migrates along the nitrocellulose membrane placed between two plastic strips. In the sample window, the BoNTs bind with the antitoxin antibody-gold particle conjugate and change color by forming a red line in the sample window, the intensity of which is directly proportional to the amount of BoNT present in the sample. The sensitivity of LFAs usually is lower compared to ELISA assays but is found to be easy to use, takes less than 15 min to perform, and does not require any sophisticated equipment (Sharma et al. 2005; Cai et al. 2007). An improved LFA was developed for BoNT detection using ganglioside-liposomes as the capture agent in the sample zone to enrich BoNT from the samples

(Ahn-Yoon et al. 2004). The sensitivity of this test was reported at 15 pg/mL for pure toxin but was not tested for BoNT in food matrices. To date several LFAs have been validated to detect BoNT in a wide variety of food matrices (e.g., Sharma et al. 2005; Gessler et al. 2007; Chiao et al. 2008).

Several other detection techniques have been efficiently used in BoNT detection, including flow cytometry where toxin detection and quantification in food matrices is carried out in a multiplexed format using highly sensitive fluorescence immunoassays (Pauly et al. 2009; Ozanich et al. 2009). Immuno-PCR method which uses an antibody that is conjugated to a reporter DNA molecule has also been used for detection of BoNTs. Amplification of the DNA fragment that is conjugated to the toxin-specific detection antibody is then carried out by conventional or real-time PCR. BoNT/A could be detected in concentrations as low as 1 pg/mL using this technique (Wu et al. 2001; Chao et al. 2004).

Endopeptidase Activity Assays

BoNTs exhibit endopeptidase activity against proteins of the neuroexocytosis machinery. This opens new avenues for BoNT detection wherein the measured analyte is the cleaved product generated by BoNT enzyme action and is directly proportional to the toxin concentration in the sample. Fluorescence, or fluorescence resonance energy transfer (FRET), and Endopep-MS are some of the most relevant methods developed for detection of BoNTs by virtue of their endopeptidase activity.

Recently a FRET-based assay developed for BoNTs detection in neuronal cells employed recombinant VAMP or SNAP-25 fragments fused with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) at each end (Dong et al. 2004). When the substrate was intact, FRET between the CFP and YFP pair was observed. Upon cleavage, FRET between the CFP and YFP was reduced or abolished allowing for direct quantification of toxin concentration. The detection limits observed for these FRET-based assays were up to 2 ng/mL for BoNT/A and BoNT/E and up to 30 ng/mL for BoNT/B and BoNT/F (Parpura and Chapman 2005).

An assay with a large immunosorbent surface area (ALISSA) was established based on the enzymatic activity of BoNTs. BoNT/A was immobilized on a bead-based immunoaffinity matrix cleavage and its enzymatic activity was measured by the cleavage of a fluorescent substrate (SNAP-tide). This assay was four to five orders of magnitudes more sensitive and considerably faster than the traditional MLA assay and could detect toxin in attomolar concentrations in complex biological matrices like serum, milk, and carrot juice and in the diluent fluid used in the MLA (Bagramyan et al. 2008).

Mass spectroscopy also provides for a very sensitive detection tool for the endopeptidase activity of BoNTs. Endopep-MS has been effectively used not only for the quantitative detection of BoNTs in sample matrices but also to differentiate between BoNT serotypes by the use of high-affinity serotype-specific monoclonal antibodies (Kalb et al. 2009, 2010). This is of particular importance as it would aid in choosing the right antitoxin treatment in the clinic.

Cell-Based Assays

Cell-based assays that evaluate the overall activity of BoNTs including receptor binding, translocation, and enzymatic activity are promising alternatives to the mouse bioassay. Numerous cell-based assays have been developed for use with BoNTs that employ different neuronal and nonneuron-derived cell lines. Some of these include chick embryo neuronal cells (Stahl et al. 2007), mouse and rat spinal cord cells (Hall et al. 2004; Pellett et al. 2007), PC12 cells (Dong et al. 2004), neuroblastoma cells N2A, and BE(2)-M17 cells (Eubanks et al. 2007; Hale et al. 2011). Intracellular BoNT activity can be detected by monitoring the immunoblot detection of the BoNT-induced cleavage of the substrate or by a specific FRET-based sensor (Dong et al. 2004).

Continued efforts to develop more robust cell-based assays are underway. Recently the US FDA approved an in vitro cell-based assay for the evaluation of the potency of onabotulinumtoxinA/BOTOX manufactured by Allergan Inc., for use as a possible replacement of the mouse bioassay. Although cell-based assays may be viable substitutes for the mouse assay, they do have certain disadvantages including extensive maintenance of cell lines and the need for tissue/cell culture facilities. Moreover, the assay must be effectively applied to testing of food and clinical samples.

Conclusion and Future Directions

From a deadly food poison to frontline medicine, the discovery, understanding, and utilization of botulinum neurotoxins have been an intriguing story. Botulinum neurotoxins belong to a new class of metalloproteases endowed with unique features of substrate specificity, cleavage site selectivity, and mode of activation. Botulinum toxin has been of concern as a biowarfare weapon since World War II and remains to be a potential bioterrorist threat to the public. In addition, extensive clinical use of its highly diluted form to treat a wide variety of disorders and pain and its use in cosmetic applications add to the concern of a systemic toxin exposure in case of an accidental overdose or side effects.

Advancements in understanding the toxin's structure and function have been valuable in deciphering the mechanisms of membrane exocytosis and have led to widespread efforts in the design and development of therapeutics to combat the deadly botulism. These advances have also provided us with molecular clues for further discoveries following the neurotoxin's use as tools in neurobiology and cellular biology.

Currently there are no approved pharmacological treatments for BoNT intoxication. Antibody-based antitoxins are the only treatment available, but they only remove toxins circulating in the system and not the toxin already inside the neuronal cells. Tremendous strides have been made in the design of more sophisticated toxoids and recombinant heavy chain fragment vaccine candidates. These include improvements in their manufacturing processes and costs, routes of administration, increased potency, and immunogenicity. Their therapeutic utility however

remains unconvincing and limited due to reluctance of the population for general immunization owing to the therapeutic and cosmetic use of botulinum neurotoxins themselves. Furthermore, their limited window of efficacy, short serum half-life, and several safety concerns also need attention. With the potential of bivalent Hc vaccine rBV A/B being licensed by the FDA, future vaccine development should focus on developing recombinant Hc vaccines for other serotypes such as C, E, and F, potentially as a trivalent vaccine, or a pentavalent vaccine for all botulism causing serotypes.

Since the light chain of BoNTs is the catalytic moiety that fully accounts for BoNT toxicity, inhibition of the catalytic protease with small-molecule inhibitors has provided an attractive and promising approach to counter the effects of botulism poisoning. Due to the major limitation of peptide-based inhibitors of their inability to reach the endopeptidase within neurons, the design of small-molecule non-peptidic inhibitors, among others, has offered an excellent opportunity for the development of post-botulinum exposure therapeutics. Despite the plethora of new data that have emerged in the last few years, there have been several discrepancies in the effectiveness of these inhibitors developed so far against botulism under in vitro, ex vivo, and in vivo conditions, perhaps arising from the structural differences that might be encountered under these conditions. Very few of these small-molecule antagonists have been assessed in animal models and none have advanced to preclinical development so far. These difficulties could be attributed to the unusually large enzyme-substrate interface that would require a small molecule with a very high binding affinity to block substrate binding. Also BoNT domains are known to exhibit considerable conformational flexibility thus making the design of effective inhibitors complicated. Hence, there is a crucial need to further refine the design strategy in order to identify new candidates or to develop analogs of the lead molecules with remarkable potency, safety, and efficacy that also demonstrate suitable absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles. Techniques such as high-throughput screening and combinatorial chemistry provide powerful tools for the discovery of next-generation botulism countermeasures. In addition, combining other random screening techniques (such as phage display, small-molecule library screening, SELEX), and rational design, will greatly facilitate the drug discovery process for identification of potential therapeutics with improved potency and bioavailability.

Increased research efforts are also needed toward the establishment and validation of effective models such as animal bioassays (e.g., in primates) or cell-based assays to test the in vivo efficacy of therapeutics against BoNTs, since designing a human clinical trial for testing of potential drugs against botulism is difficult.

Since BoNTs pose a threat of being employed as biowarfare agents, their rapid, sensitive, and serotype-specific detection in clinical samples is an essential component in the diagnosis and treatment of the deadly botulism disease yet remains to be achieved. FDA currently recommends the standard mouse bioassay to determine the potency of BoNTs in clinical and food samples. However, these animal-based assays come with several disadvantages as mentioned earlier, and in order to overcome these shortcomings, significant progress has been made in designing

sensitive in vitro diagnostics to detect active as well as inactive forms of the neurotoxin. A preferred detection platform would incorporate assays that are simple and quick, offer multiplexing capability, and are easy to automate with high accuracy, good precision, and sensitivity comparable to the traditional mouse bioassay. The data generated by any of these also need to be further validated and compared directly to the mouse bioassay.

In addition to currently developed technologies, many new technologies developed for proteomics research can be applied to the laboratory diagnostics of botulism. To this end, technologies such as protein microarrays with multichannel microfluidic devices can provide a platform for multiplex and high-throughput large-scale screening for BoNT detection. Aptamers which are small nucleic acid (DNA or RNA) molecules selected for a particular function have the potential to be used as capture agents for sensitive real-time detection of BoNTs. They act in the same way as antibodies but carry the advantage of consistent production through chemical synthesis, good stability, and high binding specificity to their targets. Aptamers thus provide a promising platform for rapid diagnosis for many biodefense agents, from protein toxins to small molecules.

The unique mode of action of BoNTs on the host system is likely to generate a specific response from the host upon infection with toxins. These responses in the GI tract and in neuronal cells were recently studied, and these could potentially provide for a valid measure for the clinical diagnosis of BoNTs. The challenge, however, is that due to extremely low doses of BoNT that induce toxicity, there may not be detectable responses before the symptoms fully develop. With the advancement in technologies and highly sensitive instruments in the area of proteomics research, the possibility of using the host response for the development of biomarkers for botulism diagnosis can be explored.

Neuronal cell-based assays that can evaluate the overall activity of BoNTs could provide an alternate approach to the mouse bioassay, since they eliminate the use of large number of animals for toxin diagnostics. These however are accompanied by certain disadvantages such as longer testing time and extensive high-cost maintenance of cells lines. Recently FDA has approved a cell-based assay for the evaluation of potency of onabotulinumtoxinA manufactured by Allergan.

With the array of technologies being developed for detection and identification, it is quite feasible in the near future to develop fast, sensitive, and robust system of diagnostics for patients exposed to BoNTs. Therapeutics against botulism has remained out of reach so far and requires sustained efforts by scientists and funding agencies to develop effective pharmaceutical(s).

Cross-References

▶ Antidotes to Botulinum Neurotoxin
▶ Botulinum Toxin: Present Knowledge and Threats
▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures

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Aptamers as New Agents Against Biotoxins 22

Shuowei Cai and Pavithra Janardhanan

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Abstract

Biological toxins can be used by hostile states or terrorists as biological weapons and pose a great threat to public health and homeland security. Yet there are no effective countermeasures for most biological toxins. Aptamers are short single-stranded DNA or RNA oligonucleotides with high affinity and specificity to their targets. They are selected through the entire in vitro process and have many advantages over antibodies, leading them to have great potential for many therapeutic and diagnostic applications. In this chapter, the potential of aptamers as novel countermeasures and detection platform against biological toxins has

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been reviewed, along with the discussion of four biological toxins: ricin, botulinum neurotoxins (BoNTs), staphylococcal enterotoxin B (SEB), and epsilon toxin of *Clostridium perfringens*, which are among the highest level of biothreat agents. This approach opens new avenues to combat biothreat and bioterrorism against public health and national security.

Introduction

Biological toxins are produced by living organisms and include both small molecules and protein toxins. These are invisible to the human eye when dispersed in aerosol form, odorless and tasteless, and more potent than most lethal chemical agents. Generally, there is lack of medical countermeasures against many of the biological toxins. These toxins can be produced from widely available pathogens which can be grown for legitimate biomedical research or obtained from soil or infected animals or humans. Many of them can be used by hostile states or terrorists as biological weapons and therefore, pose great threat to public health. The US government has long realized those potential biothreats and has prepared to address those challenges. Centers for Disease Control and Prevention (CDC) has listed over 80 biological agents as biothreat agents (including biological toxins, bacteria, viruses, and fungi) and separated them into three categories, based on how easily they can be spread and the severity of illness or death they cause (http://www.bt.cdc.gov/agent/agentlist-category.asp). Category A agents are the highest priority agents, because they can be easily disseminated or transmitted from person to person, result in high mortality rates and have the potential for major public health impact, might cause public panic and social disruption, and require special efforts for public health preparedness (http://www.bt.cdc.gov/agent/agentlist-category.asp). Category B agents are the second highest priority agents and referred to those that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates, and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance (http://www.bt.cdc.gov/agent/agentlist-category.asp). Category C agents are the third highest priority agents that are considered emerging threats for disease (http://www.bt.cdc.gov/agent/agentlist-category.asp). In conjunction with the Department of Homeland Security and CDC, the National Institute of Allergy and Infectious Diseases (NIAID) has also developed a list of priority pathogens which provides guidelines for responding to emerging pathogen threats in the United States (http://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx). To further safeguard the agents that could pose severe threats to public health, plant and animal health, or animal or plant products, the US government has established the Federal Select Agent Program, which is jointly administered by the Centers for Disease Control and Prevention/Division of Select Agents and Toxins and the Animal and Plant Health Inspection Services/Agricultural Select Agent Program (http://www.selectagents.gov), regulating currently 66 agents and toxins. Under the Select Agent Program, 13 agents and toxins have been designated as Tier 1 agents because these biological

agents and toxins present the greatest risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence and pose a severe threat to public health and safety and thus are subjected to more rigid regulation on security and biosafety.

High-molecular-weight protein toxins, such as ricin, botulinum neurotoxins (BoNTs), staphylococcal enterotoxin B (SEB), and epsilon toxin of *Clostridium perfringens*, are regarded as potential biological warfare agents and could be used for small- or large-scale attacks on the basis of their availability, ease of production, high toxicity, and lack of medical countermeasures. They are on both CDC list and NIAID priority list of pathogens and are the only biological toxins on the Category A and B list (BoNTs on category A list, while ricin, SEB, and epsilon toxin of *Clostridium perfringens* on category B list). Furthermore, BoNTs, SEB, and ricin belong to select agents that are regulated by Federal Select Agent Program, and BoNTs belong to Tier 1 select agent (http://www.selectagents.gov). Toxins are of particular concern to homeland defense because they can be used as biological weapons by terrorists and can affect the public health in a relatively short time as no organism growth time is required (Arnon et al. 2001; Greenfield et al. 2002; Rosenbloom et al. 2002; Wein and Liu 2005). In this chapter, these four biothreat toxins are focused, and a group of new biological molecules, aptamers, is discussed as potential therapeutics and novel detection platform to counter the threat of these biothreat toxins.

Aptamers

Aptamers are unique, single-stranded oligonucleotides (DNA or RNA) that bind to their targets with very high affinity and specificity. Technically, aptamers could be isolated against any protein known to mankind (Tuerk and Gold 1990; Nimjee et al. 2005). In solution, aptamers form intramolecular interactions that fold the molecule into a unique three-dimensional structure, providing the basis for high affinity and specificity towards their targets. Thanks to specific and tight interactions, aptamers serve as valuable tools to modulate or block functions of proteins. The screening process for aptamers is popularly termed as SELEX (systematic evolution of ligands through exponential enrichment) (Tuerk and Gold 1990). The process of SELEX starts with generation of a library of randomized DNA or RNA sequences (Fig. 1). Usually this library contains 10^{14}–10^{15} different DNA or RNA species that fold into different structures depending on their particular sequence. The library is incubated with the target protein of interest, and those DNAs or RNAs present in the library that bind to the protein are separated from those that do not. The retained DNAs or RNAs are then amplified by PCR (in case of RNAs, by reverse transcription/PCR and in vitro transcription) to generate a pool of DNAs or RNAs that have been enriched for those that bind to the target of interest. This selection and amplification process is repeated (usually 4–20 rounds) until the winning DNA or RNA ligands with the highest affinity for the target protein are isolated. The winning ligands are defined as aptamers that are then cloned and sequenced.

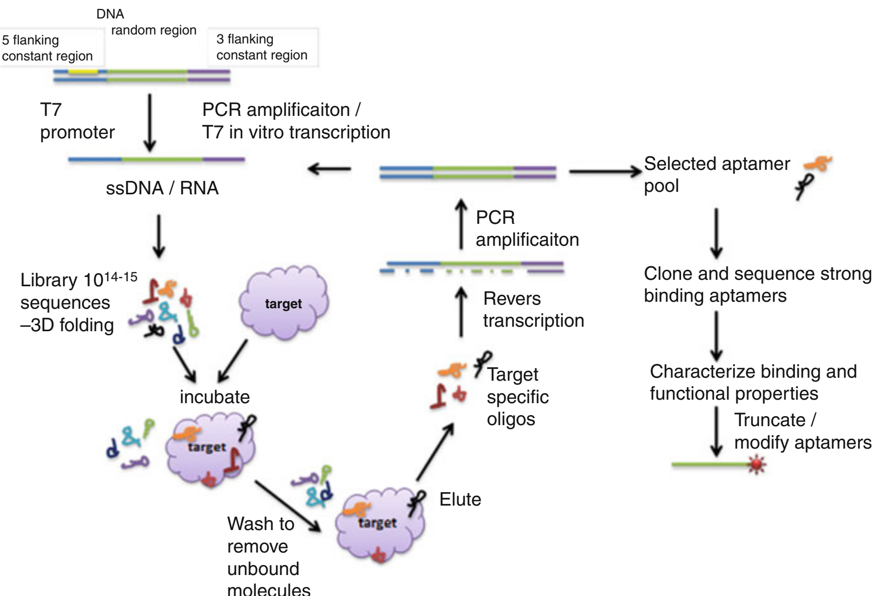

Fig. 1 Scheme of SELEX. A library of DNA or RNA molecules is incubated with the target of interest, and the bound oligos are separated from the unbound. The bound sequences will be amplified by PCR (RT/PCR in case of an RNA library). The selected pool then enters a new round of selection. After a few rounds of selection, the enriched aptamers sequences with a strong binding affinity for their target are cloned, sequenced, and characterized

Several properties of aptamers make them as attractive therapeutic and diagnostic agents that rival and in some cases, surpass antibodies. Those are listed in Table 1. Binding of an aptamer is a highly specific interaction, with the ability to discriminate targets on the basis of subtle structural differences (Jenison et al. 1994). The properties of aptamers make them ideal for both therapeutic and diagnostic applications.

Therapeutic Applications of Aptamers

Aptamers rival antibodies in terms of affinity and specificity for targets (Table 1) and are considered very promising for in vivo therapy. Aptamers are widely applied towards a variety of targets, from small molecules to proteins, and even for whole organisms. Most in vivo targets for aptamer-based therapy are at extracellular levels, which are easier to access and represent less stringent pharmacological challenge. Aptamers have been used for several areas as investigational therapies. They have been used to target coagulation factors as anticoagulants (Nimjee et al. 2005); target the vascular endothelial growth factor (VEGF) for an array of diseases, such as cancers, retinopathies, and age-related macular degeneration (www.Macugen.com); target virus proteins for HIV, influenza, and hepatitis C

Table 1 Aptamers versus antibodies (Cai and Singh 2007)

Aptamers	Antibodies
Binding affinity high (in low picomolar to low nanomolar, in terms of dissociation constant, K_d)	Binding affinity high (in low picomolar to low nanomolar, in terms of dissociation constant, K_d)
Entire selection is an in vitro chemical process and therefore can target any proteins, small molecules, and whole organisms	Selection needs a biological system and is therefore difficult to raise antibodies against toxins or non-immunogenic targets
Iterative rounds against the targets limits screening processes	Screening monoclonal antibodies is time-consuming and expensive
Uniform activity from batch to batch	Activity of antibodies vary from batch to batch
Wide variety of chemical modifications to molecule for diverse functions	Limited modifications of molecules
Return to original conformation after temperature insult or other chemical denaturation process	Temperature and environment sensitive and undergo irreversible denaturation
Unlimited shelf life	Limited shelf life
No reported immunogenicity	Significant immunogenicity
Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse the effect of antibodies
Can select for ligands under different conditions for in vitro diagnostics	Limited to physiological conditions for optimizing antibodies for diagnostics
Easy to label reporter molecules (such as fluorescence dye) to the aptamers at precise location identified by user	Difficult to label the reporter molecules at precise location

(Romero-Lopez et al. 2005; Khati et al. 2003; Jeon et al. 2004); target antibodies and cytokines for autoimmune and inflammation diseases (Hwang et al. 2003); target neuropathological targets for neurodegeneration diseases, such as Alzheimer disease and transmissible spongiform encephalopathies (TSEs) (Ylera et al. 2002); and target membrane biomarkers for cancer (Scaggiante et al. 2013). The first aptamer-based drug, Macugen®, has been approved by FDA for age-related macular degeneration (www.Macugen.com).

Compared to antibodies, aptamers present several advantages for in vivo therapeutic applications. There are no reports of aptamer-induced immune response, which is one of the major limitations of antibody-based therapy. Due to their small size (about 10 kDa, compared to 150 kDa antibodies), aptamers are likely to produce better tissue penetration. Also, aptamers are chemically synthesized at relatively low cost and have better batch-to-batch reproducibility. Additionally, aptamers can be modified to adjust the pharmacokinetic profile. Further, to reverse the effect produced by aptamers, antisense molecules against aptamers could be used.

Unmodified aptamers have a short half-life in the blood circulation due to the rapid nuclease degradation and the fast clearance rate. This is an advantage for some therapeutic areas, such as thrombin aptamer for anticoagulation therapy, but is likely to be a problem for the majority of other in vivo applications. Chemical

Fig. 2 Possible modifications on an oligonucleotide strand to generate modified oligonucleotide libraries for the SELEX process. Big N represents the base (purines or pyrimidines). Modification 2′ position of the sugar confers nuclease stability. Modifications at C-5 position of the pyridines could be used to generate covalent links with targets. I and Br represent iodine and bromide, respectively. F represents fluorine

modifications have been developed to improve the nuclease resistance of oligonucleotides (Fig. 2). When conjugated to high-molecular-weight polyethylene glycol (PEG), the half-life of aptamers is much longer (9–12 h) (Tucker et al. 1999). Aptamers also can be formulated into a long-lasting formulation by emulsion with the biodegradable polymer microsphere, such as poly-lactide-glycolide (PLG), which can achieve a half-life of months. The tunability of the pharmacokinetics makes aptamers as good candidates for many therapeutic areas. While aptamers are easier to access the extracellular targets, when combined with the appropriate delivery system, they can also target the intracellular targets. Extensive work is underway and numerous approaches are under development to achieve this formidable task.

Potential of Aptamers for Rapid Detection and Diagnostics

Aptamers are not only great candidates for therapeutics but also have immense potential for rapid detection and diagnostics to counter biothreats. To this end, aptamers can be used for both heterogeneous assays and homogeneous assays. For heterogeneous assay format (Fig. 3), aptamers can be immobilized on a solid surface in an array format or in a biosensor format. The stability and reversibility under changing experimental conditions make aptamers superior to antibodies (Jayasena 1999).

A more attractive assay format is a real-time detection format, which can be designed as both homogeneous and heterogeneous assay systems. Fluorescence anisotropy can be used for readout as illustrated in Fig. 4. Unlike antibodies, the aptamers are small (about one-tenth of antibodies). Upon binding to its target (such as proteins), the tumbling rate will be significantly changed, and therefore,

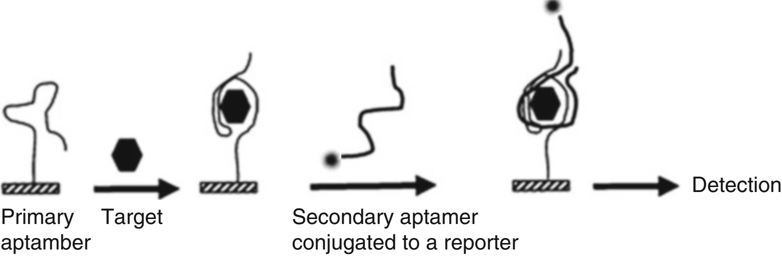

Fig. 3 Aptamer-based assay in heterogeneous format, using a secondary aptamer as reporter molecule (Cai et al. 2007)

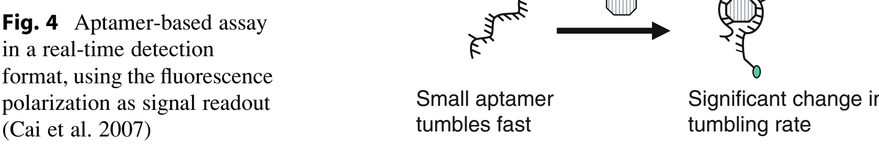

Fig. 4 Aptamer-based assay in a real-time detection format, using the fluorescence polarization as signal readout (Cai et al. 2007)

fluorescence anisotropy will be changed. This type of assay can be used not only in homogeneous solution assay but also in heterogeneous assay, such as sensor-based assay, by immobilizing the fluorophore-labeled aptamer on the sensor. This will significantly increase the testing speed.

Molecular beacon, a novel class of fluorophore being used for detection of the nucleic acid sequences of specific pathogen, can be used for real-time detection of protein, when using the aptamer as the communicator (Tan et al. 2004). Molecular beacons are simple hairpin loops of nucleic acids, with a fluorophore attached to one terminus and a quencher attached to the other. This mode of attachment brings the fluorophore close to the quencher when the molecular beacon is folded into hairpins, and the fluorophore is quenched. The nucleic acid sequences in the loop of a molecular beacon are designed to be complementary to the target of interest. The nucleic acid sequences in the loop of the molecular beacon interact with the target sequence to form an intermolecular hybrid, during which, the step of the beacon unfolds, moving the fluorophore away from the quencher. The end result is the emission of fluorescence. Aptamers can communicate with molecular beacons for a real-time detection of proteins.

Current Development of Aptamers as Novel Detection Platforms and Countermeasures for Biothreat Toxins

Due to the unique in vitro selection process for identification of aptamers, the selection time is much shorter than raising antibodies or identification of small molecule leads; thus, development time for aptamer-based therapeutics could be much shorter than development of traditional therapeutics (small molecules and

antibodies). This is specifically important in winning any possible war against bioterrorism, especially for the emerging threats that currently do not have countermeasures. Aptamers have also been proven as a rapid diagnostic and detection tools for many biodefense agents, from protein toxins to small molecules. Here, the applications and potentials of aptamers for four biothreat agents, ricin, botulinum neurotoxin, staphylococcal enterotoxin B, and epsilon toxin of *Clostridium perfringens*, the only four biological toxins on the list of biodefense priority agents, will be focused.

Ricin

Ricin is a highly toxic plant protein found in the seeds of castor bean. It is a glycoprotein that inhibits protein synthesis by depurinating the 28S ribosomal RNA. While the estimated oral LD_{50} of ricin in human is about 1–20 mg/kg due to their poor absorbance in the GI tract, the lethal dose by inhalation or injection is about 3–5 μg/kg (Shea and Gottron 2010). This is marginal compared to BoNT and SEB. However, ricin can be prepared in large quantities without great technological need. Each year, one million tons of castor beads are processed in the production of castor oil worldwide; the waste mash contains 5 % ricin by weight (Shea and Gottron 2010). Ricin is stable under ambient conditions; combined with its worldwide availability in bulk quantities, it has potential for weaponization and has been used by domestic terrorists (Shea and Gottron 2010). More recently, incidents involved the letters containing ricin sent to Mississippi Republican Senator Roger Wicker, New York City Mayor Michael Bloomberg, and US President Barack Obama in April and June of 2013 (Associated Press 2013).

Ricin is a globular, glycosylated heterodimer of approximately 64 kDa, consisting of A and B chains, with similar molecular weight for each chain, linked through disulfide bond. It is classified as type II ribosome-inactivating protein (RIP). Like other A-B-type toxin, two chains of ricin have their distinct function, with ricin toxin B chain (RTB) responsible for receptor binding and delivery of toxin to the targeted cells, while ricin toxin A chain (RTA) responsible for the catalytic activity (Doan 2004).

RTA is categorized as an *N*-glycosidase (Endo et al. 1987), which removes bases from nucleic acids, resulting in catalytic and irreversible inactivation of 28 s rRNA and inhibition of protein synthesis. In rats, RTA specifically and irreversibly hydrolyses the *N*-glycosidic bond of the adenine residue at position 4324 (A4324) within the 28S rRNA but leaves the phosphodiester backbone of the RNA intact (Endo and Tsurugi 1987).

RTB has a binding site for galactose on each end of its barbell-shaped structure (Wales et al. 1991), allowing binding to cell surface membrane sugars (galactose and *N*-acetyl galactosamine). Since most cell surface proteins are glycosylated with sugars, RTB can bind to many sites at the cell surface on the order of 10^6–10^8 ricin molecules per cell surface (Sandvig and van Deurs 2002). Upon binding to the cell surface, ricin is internalized through receptor-mediated endocytosis.

The internalized toxin then must be routed to specific cellular compartment (endoplasmic reticulum) for RTA chain to exert its toxicity. It is thought that internalized ricin has three routes intracellularly: (i) dissociation from binding sites in the endosome and transportation to the lysosome where it is degraded, (ii) remaining attached to their binding sites in the endosome and being recycled to the cell surface by vesicular and tubular structures that bud off the membrane of the endosome, and (iii) retrograde transport to endoplasmic reticulum (ER) through trans-Golgi network (Spooner et al. 2006). It is estimated that only a small amount of ricin reaches the ER, while the rest is routed to degradation and recycling (Spooner et al. 2006).

There is no specific treatment for ricin exposure, only treatment of signs and symptoms, including fluid and electrolyte management (Doan 2004). Candidate vaccines are being developed (Dembek et al. 2011). The most promising progress for vaccine development against ricin has been on genetic engineering to eliminate its catalytic activity and its ability to induce vascular leaking (O'Hara et al. 2012).

The advantages of aptamers make them attractive detection platforms and therapeutic countermeasures for biothreat toxins. Given the strong history and uses as biothreat agents, ricin has become the most studied toxin for aptamer development (Lauridsen and Veedu 2012). The first reported RNA aptamer against the RTA was developed in 2000 by Professor Andrew D. Ellington's lab at University of Texas, Austin (Hesselberth et al. 2000). This 31-nucleotide RNA aptamer against RTA has shown high affinity to RTA and showed protective effects against ricin-induced ribotoxicity in cell-based luciferase translation and cell cytotoxicity assays (Fan et al. 2008). RNA aptamer that inhibits RTA enzymatic activity represents a novel class of nucleic acid inhibitor that has the potential to be developed as a therapeutic agent for the treatment of ricin intoxication.

In addition to their potential as therapeutics against ricin intoxication, aptamers are also applied into rapid detection tool for ricin. Due to their robustness of structural stability and structural reversibility under different conditions, aptamers can be integrated into different detection platforms and be reused for multiple times without loss of function. For example, anti-ricin aptamers have been integrated into nanopore-based sensor to sense ricin at single-molecule resolution (Ding et al. 2009). Antibody-based nanopore platform, on the other hand, due to bulky size of antibody, has been difficult to sense analyte at single-molecule level (Gu et al. 2009). A DNA aptamer against ricin B chain was also identified recently and has been integrated into surface-enhanced Raman scattering (SERS) platform to rapidly detect intact ricin in different food matrices (Lamont et al. 2011).

Botulinum Neurotoxin

BoNTs are produced by anaerobic *Clostridium botulinum* and are the cause of botulism, a life-threatening neuroparalytic disease. There are seven serotypes of BoNT currently known, designated A to G. They are the most toxic substances known to human being and are extremely potent food poisons, with the mouse LD_{50} of 1 ng/kg for type A (Gill 1982; Rosenbloom et al. 2002). All seven toxins cause a

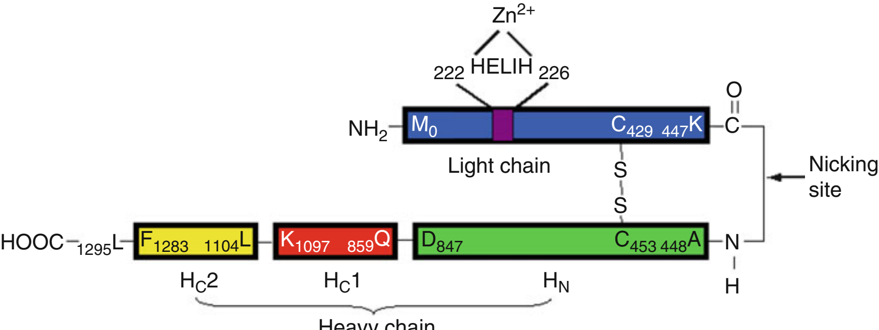

Fig. 5 Schematic structure of BoNT/A represents common features of clostridial toxins

similar clinical disease, botulism, characterized by flaccid paralysis. Classical foodborne botulism is caused by ingestion of food where the bacteria have produced the neurotoxin. Foodborne botulism in humans is caused by *C. botulinum* types A, B, and E and occasionally by type F (Hatheway 1993; CDC 1998; Gupta et al. 2005), whereas in animals, it is always caused by *C. botulinum* types C1 and D (Hatheway 1993).

BoNTs are large proteins with molecular weight of 150 kDa. They are produced by bacteria as a complex, containing the neurotoxin and its associated proteins (Singh 2006). They remain inactive as a single chain after synthesis and are activated by protease nicking to form a dichain molecule (a 50 kDa light chain and a 100 kDa heavy chain) linked through a disulfide bond (Fig. 5) (Singh 2006). The light chains (LCs) of neurotoxins are zinc endopeptidase, which cleave several proteins involved in synaptic vesicle docking and fusion and therefore block the release of acetylcholine (Singh 2006). The heavy chain (HC) plays an accessory role of binding to the target nerve cells (through its C-terminus) and translocating the LC into the cell cytoplasm (through its N-terminus) (Singh 2006).

Human botulism can occur in one of three natural forms: foodborne, wound, and intestinal (infant botulism and adult sporadic botulism). The foodborne botulism occurs by ingestion of preformed toxin from contaminated food (Sobel et al. 2004; Arnon et al. 2001). Wound botulism is rare but increasingly among injecting drug abusers (Centers for Disease Control and Prevention (CDC) 1998). It results from the growth of *C. botulinum* spores in a contaminated wound with in vivo toxin production (MacDonald et al. 1985). Another form of botulism, intestinal botulism (infant botulism and adult infectious botulism), is caused by colonization (infection) of the intestine by spores of *C. botulinum*, with subsequent in vivo toxin production (CDC 1998). It is often infectious to infants as the gut microflora of small babies is poorly developed, and thus *C. botulinum* spores may germinate and form a toxin-producing culture in the intestine. The adult form of infectious botulism is rare and resembles infant botulism in its pathogenesis and clinical status. It results from colonization of the intestinal tract by toxin-producing clostridia (CDC 1998). Individuals with altered intestinal flora due to abdominal

surgery (CDC 1998), prolonged antimicrobial treatment, or gastrointestinal wounds and abscesses are particularly vulnerable (CDC 1998). Inhalational botulism does not occur naturally, but could be used deliberately by terrorists (Arnon et al. 2001). All forms of botulism are results from absorption of botulinum neurotoxin into the circulation from either a mucosal surface (gut, lung) or a wound.

The onset of paralytic manifestations occurs from a few hours to over a week after intoxification, but in majority of cases, paralysis occurs in 12–72 h (CDC 1998). The inhalation of botulinum toxin follows a similar time course based on primate study (Arnon et al. 2001).

Botulism presents with acute, bilateral cranial nerve palsies that are followed by descending flaccid paralysis (CDC 1998; Greenfield et al. 2002). The earliest clinical signs include blurred vision from dilated pupils, dry mouth, dysarthria, and dysphagia (Greenfield et al. 2002). Mentation of patients remains normal. Fever normally is absent. As weakness descends, bulbar manifestations are followed by loss of head control and deterioration in muscle tone. Constipation and other autonomic dysfunction may also develop. The clinical symptoms are similar regardless of botulinum toxin types (CDC 1998). However, the extent and pace of paralysis may vary considerably depending on the different antigenic types (CDC 1998). Individual susceptibility may also play a role in the extent of manifestations. Cerebrospinal fluid, routine blood tests, and brain imaging studies are typically normal (Greenfield et al. 2002).

The high toxicity of BoNT poses great challenge for development of both appropriate treatment and diagnostics of botulism. Currently, there is no efficient method for early diagnosis of botulism (Cai et al. 2007; Singh et al. 2013). The standard mouse bioassay takes 4 days to complete and cannot meet the clinical need for diagnosis. Therefore, the diagnosis of botulism relies on symptoms development (12–72 h after exposure) and is confirmed by mouse bioassay. The symptoms of botulism are often confused with other neuromuscular disorders, such as Guillain-Barré syndrome, myasthenia gravis, or stroke (Greenfield et al. 2002; Rosenbloom et al. 2002), therefore making the early diagnosis of botulism more difficult. Note that an early diagnosis makes a strong foundation for the prophylactic treatment of botulism.

Current therapy for botulism involves the respiratory supportive care and the administration of antitoxin. The only antitoxins available are equine antitoxin (neutralizing antibodies against BoNT/A, BoNT/B, and BoNT/E and an investigational heptavalent (against ABCDEFG) antitoxin) and BabyBIG®, derived from the blood of human donors vaccinated with a pentavalent (ABCDE) toxoid vaccine. The antitoxin has to be administered before toxins reach the nerve cells. The treatment window for using antitoxin is short; once the symptoms are developed, the antitoxin is not effective, since the antitoxin cannot get into the nerve cell to neutralize the toxin. The flaccid muscle paralysis caused by BoNTs lasts for several months (Cherington 2004). Patients who have already developed the symptoms have to be put under respiratory intensive care for several months (Greenfield et al. 2002; Rosenbloom et al. 2002). The estimated cost for each botulism patient under respiratory supportive care could be as high as $350,000 (Wein and Liu 2005).

This puts a large burden on hospitals, both financially and resource-wise. In times of a bioterror attack, there will be a public health crisis due to the lack of effective antidotes against botulism, both for prophylactic and postexposure treatments and reliable diagnostics (Cai and Singh 2007; Singh et al. 2013).

There used to be an investigational pentavalent (ABCDE) toxoid available for vaccination of the high-risk population (such as lab workers working on BoNTs and military personnel to prevent possible attack). However, the mass immunization is not feasible and desirable, because the botulism outbreaks are rare and, more importantly, BoNT is being used as an effective therapeutic agent against numerous neuromuscular disorders and for a wide range of cosmetic applications.

New therapies for botulism under development include antibody-based therapy (Arnon et al. 2001) and small molecule-based inhibitors (Cai and Singh 2007). The antibody-based therapy mainly targets BoNTs at the circulation level; therefore, the administration window is short. A passive immunization for prophylaxis is possible, but it needs to have an exceptional safety profile, since many individuals to be treated do not know their actual exposure status. Small molecule-based inhibitors have better tissue permeation property and when targeting endopeptidase activity of BoNT have the potential to be therapeutics to reduce the severity of botulism symptoms and reverse the paralysis caused by botulism. However, despite substantial efforts in this area in the last decade, no strong inhibitors have been identified, partially due to the unique flexible structure of BoNT (Cai and Singh 2007). Another hurdle for small molecule endopeptidase inhibitors is how to deliver such inhibitors into the cytoplasm of the peripheral cholinergic nerve cells.

As a promising class of biomolecules, aptamers have been employed as novel rapid detection and potential therapeutics for botulinum neurotoxins. Tok and Fisher first reported the selection of high-affinity ssDNA aptamers for BoNT/A HC peptide (a short, 19a.a. peptide of the ganglioside-binding receptor) and BoNT/A toxoid. Using a single microbead SELEX procedure, they obtained aptamers with μM and nM affinities for the HC peptide and toxoid, respectively (Tok and Fischer 2008). Later, in December 2008, Fan et al. reported a rapid screening procedure to select DNA aptamers for botulinum neurotoxin along with other targets using ASExp (aptamer selection express) (Fan et al. 2008). Using a single round of microfluidic SELEX (M-SELEX) procedure, Lou et al. reported DNA aptamer with low nM affinity to the BoNT/A LC (Lou et al. 2009). Subsequently, the utility of these aptamers in the detection of BoNT/A toxoid and LC was demonstrated. An electrochemical sensor monitoring conformational change in aptamer demonstrated the detection of 40 pg/ml BoNT/A toxoid in 24 h (Wei and Ho 2009). Within 2 years, the same group reported a feedback system control scheme to improve the detection time to 5 min (Wei et al. 2011). In 2012, Bruno et al. screened DNA aptamer beacons for the BoNT/A LC and reported a fluorescence FRET assay for detection of 1 ng/ml of the toxin domain in buffer and dilute soil suspensions within 20 min (Bruno et al. 2012). These findings reveal that high-affinity aptamers can be easily screened for the different domains of BoNTs. The selection of aptamer is based on the structural recognition between aptamer and its target; thus, aptamer is highly specific to its target's structure. Recently, an RNA aptamer-specific targeted

to native-folded light chain of BoNT/A has been identified (Chang et al. 2010), and its utility for rapid detection of natively folded toxin has been demonstrated (Janardhanan et al. 2013). The unique structural recognition mechanism of aptamers provides great advantages over traditional immunological-based assay, since it can be used to detect targets in appropriate folding. This is specifically important for biothreat toxins, since they can be used to rapidly detect active toxin, which is the real threat.

Beyond being a ricin inhibitor, aptamers are also explored to be potential inhibitors against BoNT. Recently, three RNA aptamers have been identified, showing strong binding affinity to light chain of BoNT/A, as well as strong inhibition activity against the endopeptidase activity of BoNT/A (Chang et al. 2010). The half maximal inhibitory concentration (IC_{50}) values for those aptamers are in low nanomolar range. Enzyme kinetics studies showed that they exhibit noncompetitive inhibition of the enzymatic function of BoNT/A. More importantly, preliminary data have shown partial protective effects of those RNA aptamers on mouse model against botulinum neurotoxin (Janardhanan et al. 2012). Due to their low toxicity, aptamers open a new avenue for much needed antidotes against botulism.

Staphylococcal Enterotoxin B

Staphylococcal enterotoxin B (SEB) is one of several heat-stable enterotoxins produced by gram-positive bacterium *Staphylococcal aureus.* SEB, a 28 kDa protein, is the second most common source of outbreak of food poisoning (Ahanotu et al. 2006). After administration/ingestion, the toxin elicits an emetic and diarrheogenic response. It produces symptoms in 1–4 h, and the duration of illness on average lasts for about 20 h (Greenfield et al. 2002). In addition to causing the symptoms related to gastroenteritis, SEB is also a bacterial superantigen that binds to monocytes at major histocompatibility complex II receptors of T-helper cells and stimulates the proliferation of T-helper cells and induces cytokine release (Ahanotu et al. 2006).

SEB is stable to heat, proteolytic digestion, and pH change (pH 4–10). The estimated 50 % lethal dose (LD_{50}) of SEB is about 0.02 μg/kg and 50 % of effective dose (ED_{50}) is as low as 0.0004 μg/kg by aerosolized exposure (Rusnak et al. 2004). While no data exist on the LD_{50} and ED_{50} in human by other routes of exposure, the minimum level of SEB causing gastroenteritis in human is approximately 1 ng/g of food (Rusnak et al. 2004). When inhaled, SEB results in different clinical symptoms, characterized as respiratory diseases, including fever, headache, myalgia, nonproductive cough, and retrosternal chest pain. This would be caused by the activation of proinflammatory cytokine cascade in the lung, leading to pulmonary capillary leak and pulmonary edema (Greenfield et al. 2002). While it is rarely lethal (unless massive amount is ingested or inhaled), SEB can cause rapid debilitation of a fighting force or civilian population if used by bioterrorists. Due to its inherent stability, high morbidity rate, high intoxication effect, and ease of dissemination, SEB is an attractive choice of biological aerosol weapon for potential terrorists (Ahanotu et al. 2006).

There are no specific treatments of pre- or postexposure prophylaxis for SEB intoxication. The treatment mainly involves supportive medical care, including fluid and electrolyte management (Ahanotu et al. 2006). Antibiotics are not recommended unless there is evidence of active infection. Patients are usually expected for full recovery, although it takes 1–2 weeks. Active research is being carried out to find the effective vaccine against SEB (Ahanotu et al. 2006).

There are no reports on therapeutic aptamers against SEB; however, aptamers have been selected for SEB (Degrasse 2012). The DNA aptamer identified by Degrasse (2012) has been used to develop an ultrasensitive fluorescence resonance energy transfer (FRET)-based aptasensor to detect SEB with limit of detection at 0.3 pg/ml (Wu et al. 2013). This ultrasensitive aptasensor took the advantage of easy modification of aptamer with fluorescence probe and was coupled with upconversion nanoparticles (UCNP) sensing.

Epsilon Toxin of *Clostridium perfringens*

Epsilon toxin of *C. perfringens* is produced by *C. perfringens* types B and D and causes a rapidly fatal acute toxemia. Epsilon toxin is a pore-forming protein, causing potassium and fluid leakage from cells and leading to cell swelling, blebbing, and lysis. It binds to vascular endothelial cells and causes severe vascular damage and edema in various organs (including the brain, heart, lung, kidney). Epsilon toxin mostly affects cattle, sheep, goats, pigs, and horses (Greenfield et al. 2002). The naturally human cases caused by epsilon toxin are very rare (Institute for International Cooperation in Animal Biologics 2004). However, epsilon toxin has the potential for effective mass dissemination through aerosol route. The rat IV lethal dose is 100 ng/kg. Inhalation of epsilon toxin might result in damage to pulmonary vascular endothelial cells, causing high-permeability pulmonary edema, followed by circulatory spread, leading to renal, cardiac, and central nervous system damage. Treatment is more likely to be supportive medical care, and no vaccine is currently available for human use.

While there is no report on aptamers against epsilon toxin of *Clostridium perfringens*, given the advantages of aptamers, aptamers would have great potential to be used against epsilon toxin. In fact, aptamers have been selected for many other biological toxins (protein and small molecule toxins), and much applications have been focused on their potential for development of rapid detection platform. Readers can refer the most recent review by Lauridsen and Veedu (2012).

Challenges in Developing Aptamers as Therapeutic Countermeasures for Biothreat Agents

Many protein toxins are A-B-type toxins, with their pathogenic effects happening at the intracellular level. Those include botulinum neurotoxin and ricin on the CDC and NIAID priority list of biodefense agents. While it is straightforward for

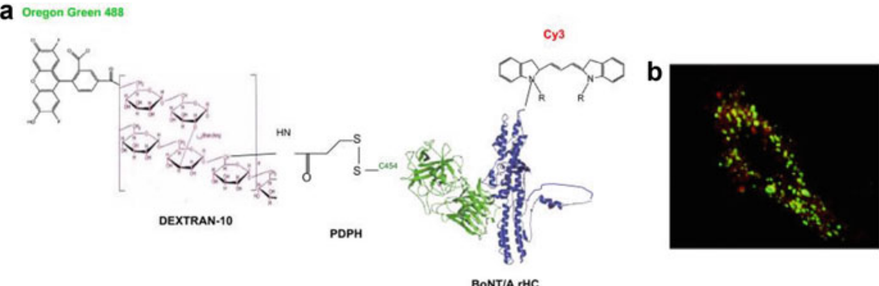

Fig. 6 Drug delivery vehicle consisting of recombinant HC linked with 10 kD dextran and Oregon Green 488 (*green*) and Cy3 (*red*) on rHC. *Panel A* showed that a 200 nM drug delivery vehicle was delivered into the mouse spinal cord neurons, as demonstrated by both green fluorescence and red fluorescence (*Panel B*) (Zhang et al. 2009)

aptamers to target the extracellular targets, for intracellular targets, like A-B-type toxins, it will need a special delivery system for aptamers to reach their intracellular targets. The relative low effects of aptamer against ricin in cellular model (Fan et al. 2008) and low/moderate protection of aptamer against BoNT on mouse model (Janardhanan et al. 2012) are mainly due to the low access of aptamers to their intracellular targets. Development of special delivery vehicle for aptamers is critical for intracellular targets in general and A-B-type toxins in particular.

In terms of A-B-type toxins, the binding domain (B domain) by itself does not pose any toxicity and yet provides a highly specific binding vehicle for the intoxicated cells. This domain therefore could offer a highly targeted delivery vehicle for aptamer-based antidotes against these toxins and minimize the effects on other healthy cells. For example, it has been shown that a heavy chain of BoNT can effectively deliver the cargo into neurons through receptor-mediated endocytosis (Fig. 6, Zhang et al. 2009).

Liposome and other biodegradable polymeric nanoparticles have been used for delivery of nucleic acid-based therapeutics, such as RNAi. Functioning of the surface of both liposomes and polymeric nanoparticles with binding domain of A-B-type toxins will provide a unique targeted drug delivery vehicle (Fig. 7), and this vehicle can be used for delivery of therapeutics into BoNT or other types of A-B toxin-intoxicated cells. The robustness of aptamers' structural stability makes the encapsulation of aptamer-based cargo more feasible. This will help the development of aptamers as true therapeutic countermeasures against biothreat toxins.

Conclusion and Future Directions

Biothreat toxins pose great risk for national security due to their ability for mass destruction and their lack of the specific treatments. Nucleic acid aptamers are a new class of biomolecules, with a range of advantages that sometimes make them superior to traditional antibodies and small molecules. Due to their high specificity

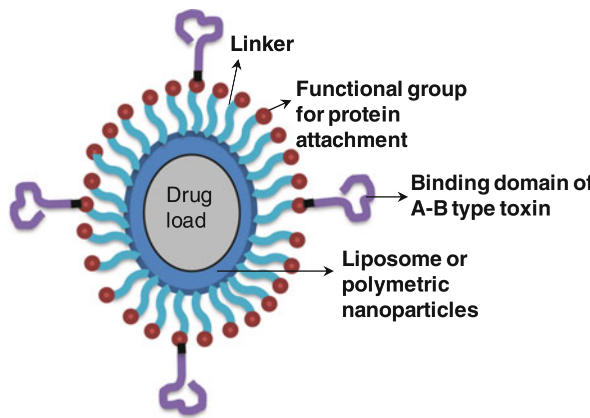

Fig. 7 A graphic illustration of targeted drug delivery vehicle functionalized with binding domain of A-B-type toxins

and affinity to their targets, this class of molecules offers a new avenue to develop specific therapeutics against biothreat agents. When combined with targeted drug delivery technology, aptamers will be highly effective for toxins with the intracellular targets and thus live up to their full therapeutic potential. In addition to protein targets, aptamers also can be screened for small molecule biological toxins and serve as inhibitors and antidotes against small molecule, non-immunogenic toxins. The unique flexibility of SELEX process for identification of aptamers and their availability on demand provide a unique position to aptamers as countermeasures against biological toxins. Aptamers not only provide strong therapeutic potential for biological toxins but also can be integrated into the rapid detection platform for toxins and thus open a new avenue for tackling the growing problem of bioterrorism.

Cross-References

▶ Antidotes to Botulinum Neurotoxin
▶ Botulinum Toxin: Present Knowledge and Threats
▶ Basic Chemistry of Botulinum Neurotoxins Relevant to Vaccines, Diagnostics, and Countermeasures
▶ The Biowarfare Agent Ricin

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Section V

Evolutionary and Historical Perspectives of Biotoxins as Weapons

Evolutionary Traits of Toxins

23

Raj Kumar, Tzuu-Wang Chang, and Bal Ram Singh

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Abstract

Protein toxins produced by bacteria are some of the most potent toxins known to mankind. Most of them utilize certain critical cellular processes of the host to invade and impair other important cellular processes. Different domains of these proteins have a number of homology groups with varying protein structure and

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functions. Some of them have sequence similarities across all three cellular forms of life – Bacteria, Archaea, and Eukarya – suggesting a possibility of horizontal gene transfer among these organisms. Considering these traits, the protein toxins provide useful tools to study host–pathogen interactions as well as the biological evolution of species. This chapter emphasizes two aspects of evolution: genomic organization and protein molecular characteristics. Evolution is not only a reflection of change in the characteristic of biological species, but it is also related to diversity at every level, including species, individual organism, and even at the molecular level. Study of evolution in terms of molecular properties such as folding, flexibility, and dynamics provides us with another very unique and necessary dimension to examine molecular and submolecular mechanisms involved in the evolutionary process. Such information can be used to develop countermeasures in the case of protein toxins and perhaps utility of highly evolved toxins as medicines, as in the case for botulinum neurotoxins.

Introduction

Toxins are poisonous substances produced by microbes (including bacteria), parasites, fungi, viruses, animals, and plants, which are poisonous to other species. In general, toxins can be small molecules, peptides, or proteins. These molecules are toxic because they are capable of evading protection mechanisms of the infected host cells and affecting important cellular processes such as protein synthesis, inhibiting cellular process, and promoting cytolysis. Toxins can be generally grouped according to their source (bacterial toxins), their purpose (hemotoxins, which cause hemolysis), and site of action or affected organs (hepatotoxins). Toxins can be considered as a biological warfare agent used by toxin-producing species in their struggle for survivability. Natural toxins, which are present now, may possibly be a result of coevolution of species. In the process of evolution, somehow they learnt to selectively hit key molecular targets in the cellular machinery of a host, e.g., bacterial toxins. As an example, botulinum neurotoxins target exocytosis machinery in neuronal cells.

Among different kinds of toxins, bacterial protein toxins are some of the most prominent known to mankind. They may be naturally present in host cells or organisms to carry out some essential cellular functions, but it is possible that these organisms started using these molecules for their protection as part of evolution. This may be one of the reasons why these molecules could be an interesting subject with which to study protein evolution. A general outline of protein evolution is as follows. In general, proteins evolve through a variety/combination of mechanisms, which involve gene duplication coupled with mutation and selection. Most of the proteins have twofold symmetry, for example, a/b proteins. During evolution, these two segments have enough sequence information to allow tracing back their ancestral origin. Many of these proteins have remarkable

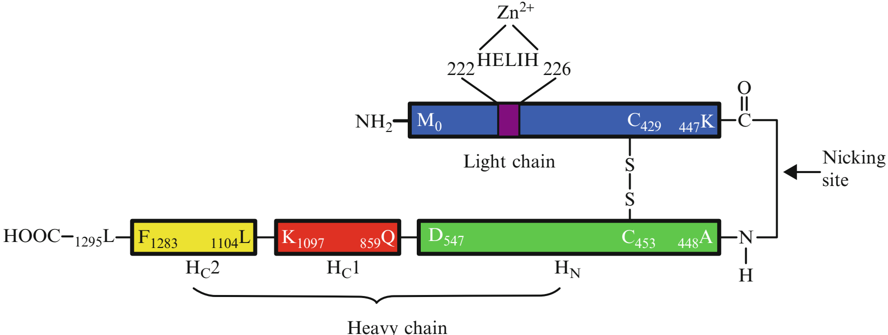


Fig. 1 Schematic representation of different domains of botulinum neurotoxin. BoNTs are produced as a single chain (progenitor or protoxin) which undergo proteolytic cleavage at a nicking site (exogenously or endogenously) to a biologically active dichain toxin linked by a disulfide bond. Botulinum neurotoxin is multidomain protein, comprising of two important chains – heavy chain and light chain – with heavy chain further subdivided into N-terminal (H_N) and C-terminal (H_C) halves; HC itself contains two domains, H_C1 and H_C2

sequence identity, with some even being similar at the higher structural levels. Structural-level identity leads to the search for folding identity, which involves mostly four mechanisms: insertion/deletion/substitution of secondary structural elements, circular permutation, β-strand invasion/withdrawal, and β-hairpin flip/swap (Grishin 2001).

Generally, the protein folds and functions are dictated by their primary sequences, and cumulative changes in these sequences lead to the evolution of protein structures and functions. However, examples exist whereby very similar or even identical sequences fold into different structures (Allison et al. 2011; Alexander et al. 2007; He et al. 2008). It is possible that protein structures are more conserved than sequences and these structural changes can generate new folds and topologies, which when coupled with the above mechanisms leads to the evolution of new functional and naturally selected proteins.

In general, protein toxins are multidomain, multifunctional proteins. It is interesting to learn how these different domains/structural units come together in the process of evolution. What is the role of different domains/structural units in the evolution of the present fold and function of these complex structures? Botulinum neurotoxin is one protein toxin with multidomain assembly (Fig. 1) and the most biologically effective (toxicity) molecule known to mankind. This chapter explores evolutionary traits of these most lethal toxins focusing mainly on the primary sequence and different functional conformations. This chapter also attempts to answer the question of whether there is any relationship, or a possibility of any relationship, between structures and evolution of these proteins.

Botulinum Neurotoxin and Its Toxicity: Botulinum neurotoxins (BoNTs), category A select agents as defined by Centers for Disease Control in the United States, are the most poisonous toxins (mouse LD_{50}, 10^{-8} mg/Kg for type A) known (100 billion times more toxic than cyanide). Each of the seven serotypes (A–G) of

BoNT blocks the release of acetylcholine at nerve–muscle junctions, leading to severe flaccid muscle paralysis, which can be fatal without medical care (Arnon et al. 2001). Due to their extreme toxicity and stability, BoNTs are considered as a group of the most dangerous biological warfare agents (Arnon et al. 2001). The toxins are also used as therapeutic and cosmetic agents at very low doses, placing this group of proteins in a unique position as immunization against the toxin is not advisable. One of the major concerns of botulism is its long-term muscle paralysis, which can last for months. The molecular basis of this process is poorly understood. Hence intense chemical characterization of BoNTs providing structural information of the biologically active molecule is crucial for understanding its toxic activity. Because of the long-lasting paralytic effect of BoNT/A, several efforts are underway to develop inhibitors for rescuing paralyzed nerves, in contrast to developing prophylactic drugs which have limited utility given the uncertain nature of detection and forewarning in BW (biological warfare) conditions. However, as of yet, no successful candidates have been developed. Several inhibitor candidates have been identified within the past 5 years, but most of these have consistently failed the general in vitro, cellular, in situ, and animal testing. For example, Eubanks et al. (2007) identified several inhibitor candidates from screening with in vitro endopeptidase activity, but they were ineffective in cell-based assays, and even those that were effective exhibited poor efficacy in mouse bioassay.

A major question that arises from the consistent failure to develop inhibitors against botulism is whether there is any design on the part of toxins which defies typical approaches. Have these toxins evolved to avoid countermeasures, and if so, how is that evolution coded? Is the structural flexibility part of evolutionary coding?

Evolutionary Traits of Bacterial Toxins

There are four major classes of bacterial toxins: (1) toxins that bind to the cell surface of a host cell via plasma membrane receptors and modify host cell physiology by affecting intercellular signaling; (2) toxins that bind and disrupt the plasma membranes; (3) AB toxins where the A domain possesses enzymatic activity, while the B domain binds and enables host cell entry; and (4) toxins possessing enzymatic activity that are delivered into the host cell by bacteria. Targets for these toxins includes actin, Rho GTPases, cellular protein degradation system, cell translational machinery, SNARE machinery, and cAMP signaling (Barbieri 2013). In general, it is believed that bacterial pathogens have evolved over a long period of time that allows a large number of random interactions of pathogens and host species, possibly leading to coevolution. Clearly, the mechanism of action of bacterial toxins involving their targets as point of interactions strengthens the idea of coevolution. The existence of toxin families with common properties and sequence homologies in the relevant genes indicates that they may derive from common ancestors. There is increasing evidence suggesting evolution of toxin genes involved horizontal gene transfer (Groisman and Ochman 1996) and recombination events mediated through phages, plasmid, chromosome, and

regulatory elements such as microRNA (Alouf 2006). Not only is the host environment responsible for toxin evolution, but there must also be some effect from independent factors such as the environment (e.g., soil, aquatic, gut) and from other vectors, upon evolving these molecules.

Organism evolution is a complex process, requiring interactions of not only environment and biological processes but also molecular and submolecular processes which provide the chemical and physical basis for biological adaptation and function.

Evolution: Evolution is a widely accepted hypothesis, but its principles are not clearly understood. Given that the evolutionary concept being examined here is for toxins, here we will provide a background of the basic evolutionary concepts before examining the structure and functions of toxins. Recent developments in the field of evolution, coupled with anthropology, genetics, and molecular biology, have underpinned the following inferences in recent years: (a) all humans are descended from a single individual, who lived about 270,000 years ago (Powledge and Rose 1996), (b) humans and monkeys share a common ancestor from 7 million years before present (Pontzer 2012), (c) the ancestor to both humans and mice lived about 50 million years, and (d) life arose on earth from a primordial single cell organism that lived 3.5 billion years before today (Deamer 1999). A few schools of thought about the origin of life; i) life appeared spontaneously, ii) life evolved from some common simple organism, iii) life originated from outer space, and iv) the original primordial organism is “unknowable”, and have no scientific evidence.

But recent developments in genetics, molecular biology, and related fields have improved our understanding of evolution. All living organisms have a genetic code generally represented by the sequence of nucleotides in their DNA which has been compared to a blueprint specifying the design of an organism. The genetic code represents a detailed historical record of the development of an organism. If there is significant sharing of the code sequences with another organism, they are very likely to have a common ancestor. Changes in nonfunctional DNA can be used to estimate the time since that ancestor existed. Comparison of genomes of various species allows construction of a “family tree” of life from which biologists infer the likely characteristics of the most recent common ancestor at the root of the tree of life (Fig. 2). The ancestor of this last common ancestor is unknown, which must have had ancestors of its own. To learn about them, one must turn to other lines of evidence. Up until the early 1980s, biologists had a “chicken-and-egg” problem. In all modern organisms, nucleic acids (DNA and RNA) are necessary to build proteins, and proteins are necessary to build nucleic acids. So which came first, the nucleic acid or the protein? This problem was solved when a new property of RNA was discovered: certain types of RNA can catalyze chemical reactions, meaning that RNA can both store genetic information and catalyze the chemical reactions to copy itself, the RNA world hypothesis. This breakthrough tentatively solved the chicken-and-egg problem: nucleic acids, and specifically RNA, came first with life later switching to DNA-based inheritance. Another important line of biochemical evidence comes in the form of surprisingly common molecules. As one might expect, many of the chemical reactions occurring in one’s own cells and that of

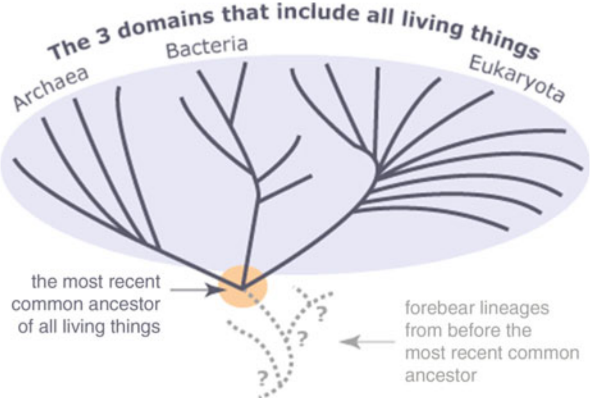


Fig. 2 Family tree of all three life forms rooted in some common ancestor. What existed before that ancestor? http://evolution.berkeley.edu/evosite/evo101/IIE2bStudyorigins.shtml

fungi and bacteria are quite different from one another. However, many of the reactions, such as those that release energy to power cellular work, are identical and rely on the exact same molecules. Because these molecules are widespread and are critically important to all life, these are thought to have arisen very early in the history of life and have been nicknamed "molecular fossils." ATP, adenosine triphosphate, is one such molecule and is essential for powering cellular processes in all modern life. Studying ATP and other molecular fossils has revealed a surprising commonality: many molecular fossils are closely related to nucleic acids. However, recent evidence suggests that this may not always be the case. It has been reported that (a) compositional analysis of comets and meteorites suggests the presence of α-amino and α-carboxylic acids, with typically greater abundance than nucleobases or riboses; (b) the first polypeptides were likely composed only of amino acids freely available in the environment, able to fold and perform simple metabolic and biosynthetic reactions; and (c) aromatic amino acids were absent in prebiotic environments (Longo and Blaber 2012; Longo et al. 2013). Proteins are easier to produce than nucleic acids, but it is possible that the first protein was much shorter than the proteins in current life forms. It is also possible that pre-RNA or peptide–RNA molecules have some molecular properties such as enzymatic activity to start life on this earth (Bohler et al. 1995; Li et al. 2013). So, the "chicken and the egg" conundrum is not completely resolved. But one thing is clear from these observations, and that is considering molecular characteristics may solve this problem, because it is the molecular property which allows RNA to perform multiple jobs, ATP to have a similar role in different organisms, and proteins to evolve before the cell (Zuckerkandl 1975). It is also hypothesized that most protein classes originated before the major elements of translation of DNA to the protein evolved in the modern cell (Zuckerkandl 1975; Li et al. 2013).

As a definition, evolution is change in characteristics of biological species between different generations. More inclusive definitions will point to diversity in biological species at every level, including species, individual organisms, and even at the molecular level. Molecular evolution is generally connected with biological molecules such as DNA and proteins. Despite the above definition being widely

accepted, it does not explain convincingly the variation in the rate of molecular evolution at all levels of biological organization. Part of the reason is that the widely accepted definition does consider molecules, but not the properties of molecule such as atomic organization, folding/shape of different atoms/molecules, molecular flexibility, and motions. At a basic level, all things present in nature are made of molecules, with the only difference being the role/shape/composition/organization/motions of these molecules in different systems. This view is counterintuitive because it considers the molecular properties as evolutionary traits rather than simply molecular descriptors. For instance, currently the dominant view is that the more rigid the molecule, the more restricted will be its motion and more stable. But another view could be that the more flexible the molecule is, the more easily it can adapt to the changing environment, possibly increasing its survivability and thus making it more prone to evolutionary change.

In this chapter, our focus is on proteins only, more specifically toxic proteins, as a model to examine the evolutionary aspect of these proteins relevant to their functions. An understanding of highly evolved molecular behavior could also allow developing more effective countermeasures, as well as the use of these proteins as effective therapeutics.

Botulinum Toxin

Botulinum toxin (BoNT) is synthesized as a single and relatively inactive (about 100 fold) polypeptide chain, which undergoes a proteolytic cleavage at its nicking site to form a biologically active dichain structure linked by a disulfide bond. The toxins cleave selectively and specifically SNARE (soluble NSF attachment protein receptor) proteins – syntaxin, synaptobrevin (also known as vesicle-associated membrane protein or VAMP), and SNAP-25 – hydrolyzing the target protein, blocking neurotransmitter release, and paralyzing the victim. While BoNTs remain in the nerve terminal, the tetanus toxin is transported through retrograde axonal transport into the cell body. Tetanus and botulinum toxins affect both somatic and autonomic nervous systems, with autonomic features more sensitive to tetanus.

All seven serotypes of BoNT, A through G, are structurally similar. They are synthesized as a single polypeptide chain of 150 kDa, each which is processed by endogenous or exogenous proteases into an activated dichain form (Singh and DasGupta 1989). In each case, the dichain neurotoxin is composed of a 100 kDa heavy chain (HC) linked by a disulfide bond to a 50 kDa light chain (LC). The heavy chain consists of two domains of 50 kDa each: H_C and H_N, with H_C the binding domain for binding to neuronal cells and H_N the membrane translocation domain that translocates L chain across endosomal membrane. Structurally, each toxin molecule has all three domains: binding domain (H_C; BD), translocation domain (H_N; TD), and catalytic domain (LC) (Figs. 1 and 3). Hc comprises two sub-domains – a β-sheet domain (H_{CN}) and a β-trefoil (H_{CC}). The HC and LC domains in the active toxin are bound together by a disulfide bond (Singh 2000).

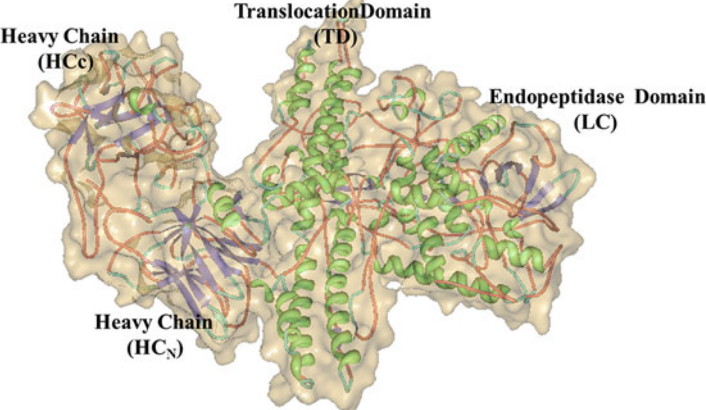


Fig. 3 The crystal structure of BoNT/A with all of the different domains (Coordinate source: PDB ID: 3BTA)

The three domains of BoNTs appear to be naturally designed for a toxin delivery system, with the binding domain (BD; HC) acting as a targeting unit, while the translocation domain (TD) delivers the toxic cargo into the cell (Montal 2010), which is the catalytic domain (LC; Singh 2000). The arrangement and function of BoNT subunits are analogous to those in the diphtheria and anthrax toxin complexes (Schiavo et al. 2000). In a BoNT structure, in addition to the disulfide link between HC and LC, there is a protein loop from the HC that wraps around the LC to hold the two chains together (Lacy and Stevens 1999). The LC active site is partially occluded by this loop in the unreduced toxin (Brunger et al. 2007) and is predicted to be the intermolecular chaperone for the light chain (Brunger et al. 2007). All of the domains work together with high precision and accuracy which probably explains why BoNTs are highly effective biologics having evolved into the most toxic substances known to humanity.

BoNT is synthesized in bacteria as a protein complex. In this complex, the toxin is associated with a group of neurotoxin-associated proteins (NAPs) consisting of a nontoxic–nonhemagglutinin (NTNH) or neurotoxin-binding protein (NBP) and hemagglutinin (HA) proteins. The genes of this complex are arranged in a cluster with different composition and organization of toxin and NAPs. The gene encoding the NBP component of the complex is found immediately upstream of the BoNT gene in all serotypes. BoNTs (A to G) belong to the Zn metalloprotease family and share a similar active site (HEXXH) and trimodular molecular structure comprising a binding, translocation, and endopeptidase domain (Singh 2000; Montal 2010), although they recognize different substrates. BoNT/A, BoNT/C, and BoNT/E cleave SNAP-25 (synaptosomal-associated protein, 25 kDa), whereas BoNT/B, BoNT/D, BoNT/F, and BoNT/G cleave VAMP (vesicle-associated membrane protein), also known as synaptobrevin (Schiavo et al. 2000; Montal 2010). BoNT/C can also cleave syntaxin. Even though more than one BoNT serotype

recognizes a given substrate, each of the BoNTs cleaves its substrate at a unique site. In multicellular species, especially vertebrate animals, cell exocytosis is an important process for the release of neurotransmitter, and BoNTs inhibit this process through proteolytic cleavage of different SNARE proteins [SNAP-25, syntaxin, and VAMP]. How did this kind of substrate diversity but high specificity evolve? Is this specificity the consequence of pathogen–host coevolution? While the answers to these questions are not yet clear, it is believed that the high specificity, extreme toxicity, and broad diversity between enzymes and their neuronal substrates have arisen as a consequence of natural selection over a long period of evolution (Schiavo et al. 2000).

Genomic Organization

The genomic organizations of BoNTs and their associated proteins are summarized in Fig. 4 (East and Collins 1994; Fujita et al. 1995). No characteristic signal peptide sequences are observed in any of the progenitor neurotoxin genes, and they are closely grouped as operons on the chromosome, phage, or plasmid. The NBP gene is located upstream of the BoNT gene by only 26 bp (type A, strain 667Ab or NCTC 2916 or CDC 3281) (Hutson et al. 1996) or 44 bp (type A, strain ATCC 3502) or 15 bp (type C, strain C-6814 and C-Yoichi) or 15 bp (type D, strain D-4947) or 11 bp (type F, Langland) or 12 bp (type F, strain F202) or 14 bp (type F, *C. baratii*, strain ATCC 43256) or 85 bp (type G, strain ATCC 27322). There are two neurotoxin gene promoter regions, with one overlapping the NBP gene C-terminal and the other shared with the NBP genes controlling co-transcription of the two genes. Neurotoxin gene expression is critically important in botulism diagnosis, as pathogen identification, and for evoking different cellular pathways in host cells. The neurotoxin genes are transcribed either alone or along with NBP genes via a polycistronic mRNA, and thus these two genes form an operon (Fig. 4). HA gene clusters form another operon transcribed in the opposite directions from the BoNT and NBP genes. HA gene clusters are located further upstream of the NBP gene by 262 bp (type C, strain C-6814 and C-Yoichi), 261 bp (type D, strain D-4947), 925 bp (type A, strain Hall A-hyper), 698 bp (type A, ATCC 3502), and 811 bp (type A(B), NCTC 2916), respectively. Each HA gene contains its own transcription terminator, which is predicted to form a stem and loop structure in the mRNA (Henkin 1996).

Neurotoxin Gene Cluster

Botulinum neurotoxins exist in complex forms. The genes encoding neurotoxin-associated proteins and the neurotoxin itself are colocated to form a continuous gene cluster in the bacterial genome (Fig. 5). Two major neurotoxin gene cluster types have been identified and named as type I “ha plus/orf-X minus” and type II “ha minus/orf-X plus” (Fig. 5). The gene loci for botulinum neurotoxin clusters

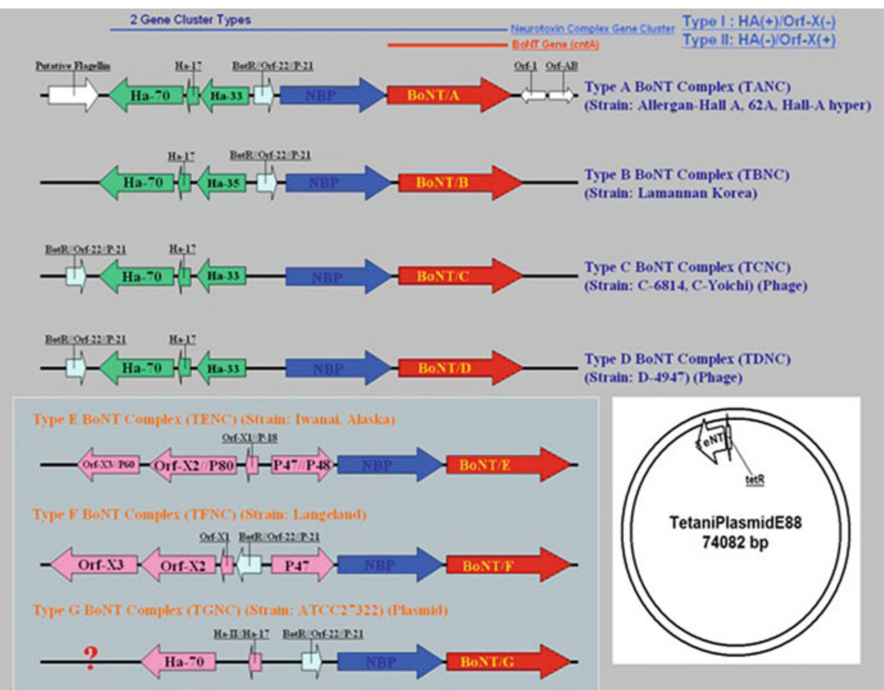
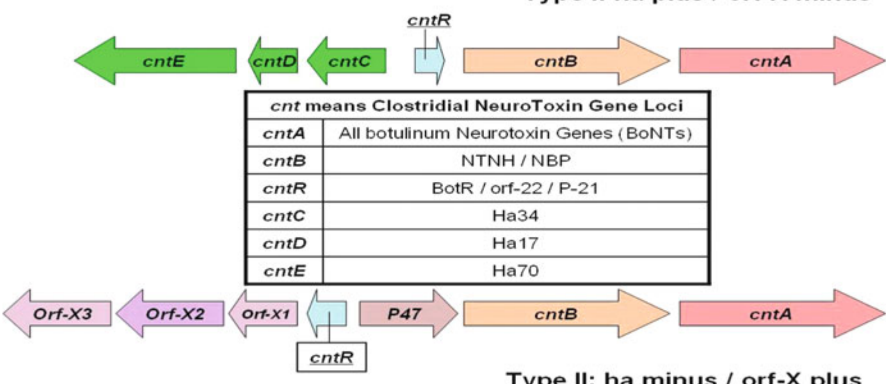


Fig. 4 The BoNT gene and its associated protein together form a polycistronic transcription unit. *Red* represents the locus of BoNT gene and *blue* the locus of NBP which are co-transcribed in the same direction. *Green* represents the hemagglutinin property and the loci of NAPs. *Pink* means their hemagglutinin attribution has not yet been confirmed. *Light blue* indicates the locus of transcription regulator for BoNT gene cluster. There are two types of BoNT gene cluster. Type I lacks for Orf-X family (Orf-) protein but contains hemagglutinin (HA+). Type II is the complementary form of type I



cnt means Clostridial NeuroToxin Gene Loci	
cntA	All botulinum Neurotoxin Genes (BoNTs)
cntB	NTNH / NBP
cntR	BotR / orf-22 / P-21
cntC	Ha34
cntD	Ha17
cntE	Ha70

Fig. 5 The *cnt* naming system of botulinum neurotoxin gene clusters and table translating updated with earlier names

Table 1 Percent amino acid identities (lower left-hand triangle) and similarities (upper right-hand triangle) of Orf 22

	Orf 22/A	Orf 22/B	Orf 22/C	Orf 22/D	Orf 22/F	Orf 22/G	TetR
Orf 22/A		**97.5**	72.5	72.0	78.5	83.0	84.5
Orf 22/B	**95.5**		66.5	66.0	71.0	78.0	79.5
Orf 22/C	52.0	48.0		89.0	**58.0**	60.0	65.0
Orf 22/D	52.0	48.0	86.0		66.0	68.0	71.0
Orf 22/F	60.5	55.0	45.0	53.0		83.0	81.5
Orf 22/G	59.0	54.0	**42.0**	44.0	60.0		83.5
TetR	66.5	61.5	48.0	52.0	66.5	65.0	

have been designated as *cnt* which means "clostridial neurotoxin" as the suffix for all genes located inside that cluster (Sebaihia et al. 2007). For example, the type A neurotoxin gene (BoNT/A previously) will be designated as *cntA*/A, type B neurotoxin gene (BoNT/B) as *cntA*/B, and so forth. Genes encoding NAPs are designated as *cntB*, *cntC*, *cntD*, and *cntE* as seen in the following table (Fig. 5).

The homology among all types of Orf 22/cnt R genes ranges from 97.5 % to 58 % in terms of sequence similarity and is listed in Table 1. The highest homology is between the A/B pair (97.5 %) and the lowest between the C/F pair (58 %). Interestingly, the Orf 22 type C has only shown higher homology with Orf 22 type D (89 %) which suggests the possible homologous relationship between types C and D.

The homology among all types of BoNT genes ranges from 55.2 % to 98.2 % and is listed in Table 2. The homology of tetanus gene to other BoNT genes ranges from 55.3 % to 62.1 %, which is lower than that among BoNT genes. The homology of serotype E between *C. botulinum* and *C. butyricum* has reached the highest value (98.2 %), and the lowest homology has appeared between serotypes C1 or D and A (see Table 2). Overall, the homology is generally higher within the same serotype (such as BoNT/B, BoNT/E, and BoNT/F) than that of among different serotypes even though it was compared from either different species (*C. botulinum*, *C. butyricum*, and *C. baratii*) or different proteolytic strains (pB vs. npB). The highest homology among different serotypes is located between BoNT/B and BoNT/G (74.2–74.9 %) and BoNT/E and BoNT/F (76.8–77.3 %). These results strongly suggest the existence of common ancestors of "BoNT/B–BoNT/G" group and "BoNT/E–BoNT/F" group during molecular evolution. In Table 2, BoNT/npB is non-proteolytic type B neurotoxin, BoNT/E is neurotoxin produced by *C. butyricum* type E, and BoNT/F is neurotoxin produced by *C. baratii* type F.

Blast P searching suggests that BoNT and TeNT genes derive from a common ancestor and the variations among different BoNT genes probably resulted from gene transfer among *Clostridium* species and subsequent DNA evolution (Elmore et al. 1995). There are several points of conclusion about the sequence comparisons of BoNTs and TeNT.

Table 2 Comparison of clostridial neurotoxins showing percent identity (lower left) and percent similarity (upper right). Note: BoNT/E is neurotoxin produced by *C. butyricum* type E, BoNT/npB is non-proteolytic type B neurotoxin, and BoNT/F neurotoxin is produced by *C. baratii* type F

	TeNT	B_0NT/A	B_0NT/pB	B_0NT/npB	B_0NT/C1	B_0NT/D	B_0NT/E	B_0NT/E	B_0NT/F	B_0NT/F	B_0NT/G
TeNT		55.3	61.8	62.1	56.2	55.3	57.8	58	59	57.9	60.8
B_0NT/A	35.4		60	60.2	**55.2**	**55.2**	61	60.5	61.8	63.2	58.8
B_0NT/pB	42.4	44.1		96	59.1	57.3	61.6	61.3	61.7	62.5	74.9
B_0NT/npB	42.7	40.7	92.8		58.4	56.6	61.9	61.3	61.8	62.8	74.2
B_0NT/C1	35.1	**33.9**	36	35		69.1	55.9	56	56.3	56.4	57.4
B_0NT/D	34.1	34.3	36.6	36.4	53.8		57.1	57.1	57.1	56.8	57.6
B_0NT/E	37.9	41.3	40	40.2	34.3	35.6		**98.2**	77.3	77.2	60
B_0NT/E	37.9	41.5	40	40.1	34.8	35.4	**96.9**		77.1	76.8	60
B_0NT/F	38.5	41.4	41	41.7	34.6	36.4	62.2	62.1		80.7	58.6
B_0NT/F	40.5	44.1	42.8	43.3	35.1	35.5	63.9	63.6	70.5		59.5
B_0NT/G	41.6	41.2	58.4	57.7	35	37.1	39.5	40.6	39.3	40.7	

(a) The G+C content of BoNT (26.3 %) and TeNT genes (27.8 %) is similar, as is that of *C. botulinum* genomes (28.2 %) (Fairweather and Lyness 1986).
(b) The percentage identity among BoNTs and TeNT at the amino acid level ranges from 33.9 % to 96.9 % and is unrelated to other known protein sequences (Table 2).
(c) The fact that some *C. botulinum* strains contain more than one type of neurotoxin genes (A (B), A (F), or B (F)) supports the gene transfer hypothesis and suggests that additional genetic events (e.g., mutation, deletion, recombination) have occurred. This hypothesis is supported by the results that some type A strains contain a silent type B gene (Hutson et al. 1996).
(d) The gene transfer among *Clostridium* strains probably involves a mobile DNA element on the chromosome or mobilization of a large plasmid harboring the BoNT gene (Hill et al. 2009).
(e) The sequence homologies of NBPs are higher than BoNT genes which suggest that NBPs are more conserved than BoNTs.

Evolution of Botulinum Toxins

The dendrogram shows that BoNT/C and BoNT/D are ancient offshoots of an unknown ancestor with BoNT/E being the youngest toxin. Assuming that all different serotypes of BoNT have evolved at the same mutation rate, BoNT/E should have the least sequence variation (highest conservation) in its cleavage site. BoNT/A being earlier than BoNT/E in molecular evolution is also consistent with biochemical observations. Puffer et al. (2001) observed that BoNT/E cleaved both SNAP25a and SNAP25b (from rat) at equal rate, while the BoNT/A cleaved SNAP25b much at higher rate than SNAP-25a. It is because BoNT/A perhaps appeared early enough in the evolution before SNAP25 split into two isoforms that it can recognize the isoform which is closer to the original SNAP-25 (means SNAP-25b here; Bark et al. 1995). BoNT/E might have evolved after the SNAP-25 isoforms split, and therefore, BoNT/E coevolved under the interaction with both SNAP-25 isoforms. Also, according to the “evolutionary arms race” theory, later pathogens are more likely to develop the ability to recognize the earlier hosts no matter how many isoforms those hosts evolved (Kim et al. 2008). Equal cleavage of both SNAP-25a and SNAP-25b by BoNT/E is thus consistent with this theory.

Other molecular evidence for differential evolution of BoNT/A, BoNT/C, and BoNT/E is provided from the observation of the genetic organization of BoNTs and their NAP gene loci. The gene clusters of BoNT/A, BoNT/B, BoNT/C, and BoNT/D belong to the same group when compared with BoNT/E and BoNT/F. BoNT/C and BoNT/D further formed their own subgroup, and BoNT/A and BoNT/B also formed their own subgroup. The relationship among all these subgroups is compatible with the prediction from the phenogram (a branching diagram representing phenetic relationship between organisms based on degree of similarity) which strongly suggests that BoNT/A and BoNT/D share the same common ancestral BoNT, while BoNT/E does not (Fig. 6).

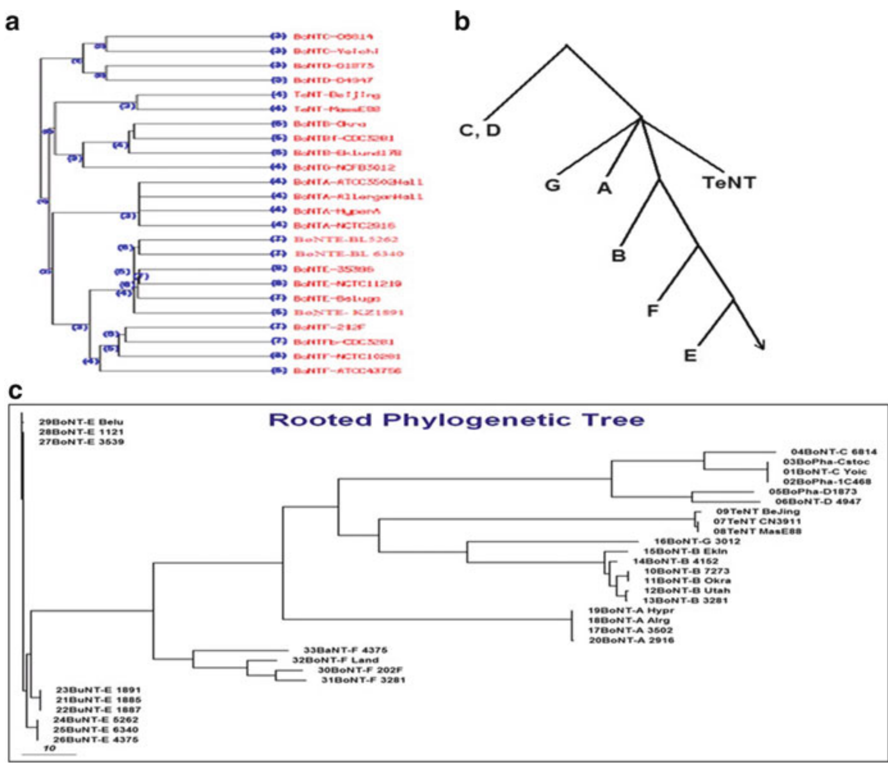


Fig. 6 Dendrogram (**a**) and the predicted evolution tree (**b**) constructed by the data of 22 botulinum neurotoxin and 2 tetanus neurotoxin sequences. Dendrogram was created by using PileUp program (Stanford University Web-based computer service). The method used here is similar to the method described by Higgins and Sharp (Higgins and Sharp 1989). The scoring matrix adopted in PileUp is blosum62. Gap creation and extension penalty were set as 8 and 2, respectively. The dendrogram here does not show the corresponding phylogenetic distance (**c**) of the evolutionary tree. Instead, it shows the number of branch degrees at each clade, with the smaller number at the branch point, meaning the closer distance to the root of dendrogram. The *left-hand* side shows the dendrogram, and the *right-hand* side shows the molecular evolution tree of all 7 serotypes of botulinum neurotoxin. The predicted evolution tree here is based on the full length of protein sequence alignment result

Folding Similarities of Botulinum Toxins with Other Toxins

CNTs are produced by four phylogenetically distinct groups (I–IV) of *C. botulinum* and also by strains of *C. tetani*, *C. baratii*, and *C. butyricum*. It appears that CNT genes have undergone significant lateral transfer among different strains of *Clostridium botulinum*. This is also supported by the discovery of plasmid-encoded neurotoxin genes in numerous *C. botulinum* strains (Marshall et al. 2007). Diversity of domains, folds, composition, and sequence are common characteristics of

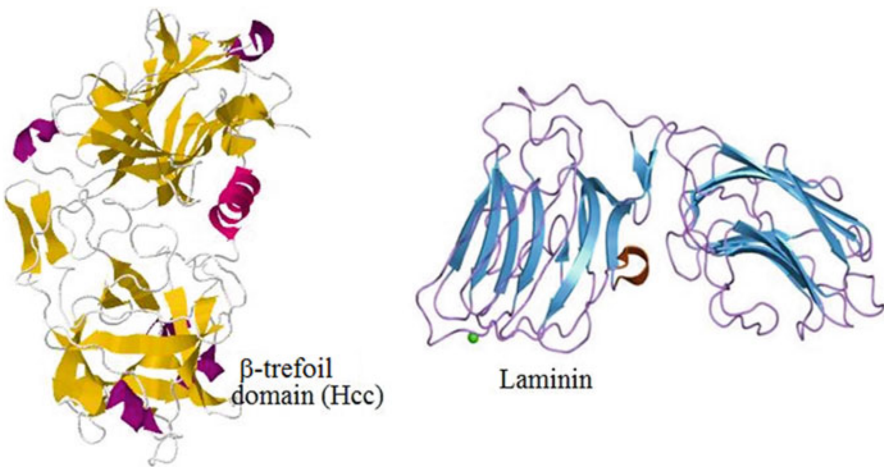


Fig. 7 C-terminus of heavy chain and laminin. The beta-trefoil, a receptor-binding domain of botulinum toxin, is common to interleukins, ricin-like lectins, laminin, and fibroblast growth factors

bacterial toxins. It is possible that bacterial toxins may have undergone considerable sequence rearrangements because of rapid sequence evolution. While CNTs appear to have very little sequence similarity outside of the CNT family and form a distinct protein family, there are some structural and functional similarities to other domain families. The beta-trefoil, a receptor-binding domain (Hcc; Fig. 7) and associated hemagglutinin component, is common to interleukins, ricin-like lectins, laminin, and fibroblast growth factors (Murzin et al. 1992; Doxey et al. 2008). HA-70 has a conserved domain related to the enterotoxin secreted by *Clostridium perfringens* (sequence similarity 44 %) and is a major virulence factor and the main reason for food poisoning and other gastrointestinal illnesses caused by *C. perfringens*. Interestingly, this domain might be responsible for the disintegration of tight junctions between endothelial cells and the intestine. HA-33 has a conserved domain in C-terminus with ricin B-related lectin (Villafranca and Robertus 1981). HA-33 contains another conserved domain which has similarity with a eukaryotic protein pierisin (Watanabe et al. 2001), an apoptosis-inducing protein, although HA-33 counterintuitively has antiapoptotic property (Kumar et al. 2012). HA-33 also has a whole sequence similarity with a mosquitocidal toxin secreted by *Bacillus sphaericus*. The fact that HA-33 has sequence similarity with different organisms, eukaryotes and prokaryotes, shows that it might play significant biological roles in both coevolution and pathogenicity between these organisms. NBP is believed to be a pseudo-toxin (Gu et al. 2012) and probably originated from the early genomic duplication event. The N-terminus of heavy chain (H_{CN}) has sequence similarity with laminin globular G domains (Lacy et al. 1998). The translocation domain (TD) of botulinum toxin resembles translocase-like domain in other toxins (Sun et al. 2011), indicating a common

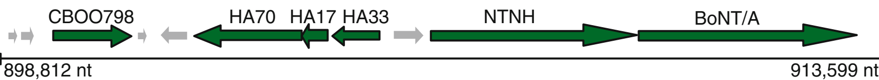


Fig. 8 Organization of clostridial neurotoxin gene cluster (Hall strain)

functional relationship. Lastly, the endopeptidase domain is functionally similar to Zn-like metalloproteases. It is notable that the active site of BoNT is located in a very deep and narrow crevice, whereas in thermolysin, the other Zn metalloprotease, the active site is broad and very near to the surface. In the case of the BoNT/A LC active site, the crevice travels through half of the enzyme structure (20 Å deep) and is negatively charged (Brunger et al. 2007). So, it is possible that botulinum neurotoxin originated initially as a metalloprotease, but because of coevolution with its substrate, it later became a neurotoxin.

A comprehensive analysis of pairwise sequence similarity of all proteins encoded within *C. botulinum* (strain Hall A, ATCC 3502) shows clear similarities between BoNT/A, NBP, HA, and the adjacent CBO0798 gene encoding a flagellin protein (Doxey et al. 2008; Fig. 8). CBO0798 flagellin gene is located immediately upstream of the neurotoxin gene cluster. Sequence similarity between CNTs and CBO0798 is between 20 and 24 %. The strongest similarity of CNTs to CBO0798 is of the translocation domain as well as HCN domain (PDB ID: 1IO1, 2ZBI, 2D4X). Flagellins are known to contain conserved regions at the N- and C- termini, as well as the hypervariable central region that is exposed to the flagellar surface, and this highly divergent central region was structurally similar to HC_N (jelly-roll fold). Interestingly, flagellin protein, FliA(H), is a relatively close homolog of CBO0798's C-terminal region. The PSI-Blast search of the hypervariable region of FliA(H) possesses significant similarity to microbial collagenases (Doxey et al. 2008). Notably, microbial collagenases and the collagenase-like region of the FliA(H) contain a HEXXH motif, similar to endopeptidase domain of CNTs. Therefore, multiple links to collagenases and flagellin genes (CBO0798) point toward a possible evolutionary precursor of CNTs.

Evolution of SNAREs

Since SNAREs are the substrates for BoNT endopeptidases, it is important to also discuss their evolution. This will also help us to analyze the possibility of pathogen–host coevolution. One of the critical steps in the evolution of animals was the transition from unicellular protozoa to multicellular organisms. Multicellularity brings several characteristics to cellular organization and function, such as intercellular cohesion, communication, differentiation, protein domain organization, and vesicle trafficking. In brief, secretion in prokaryotes is possible by translocation of polypeptides across the plasma membrane. Early eukaryotic cells are capable of limited modes of endocytosis, vesicular transportation, and phagocytosis (Fig. 9). Elaboration of this basic pattern and subsequent evolution yielded

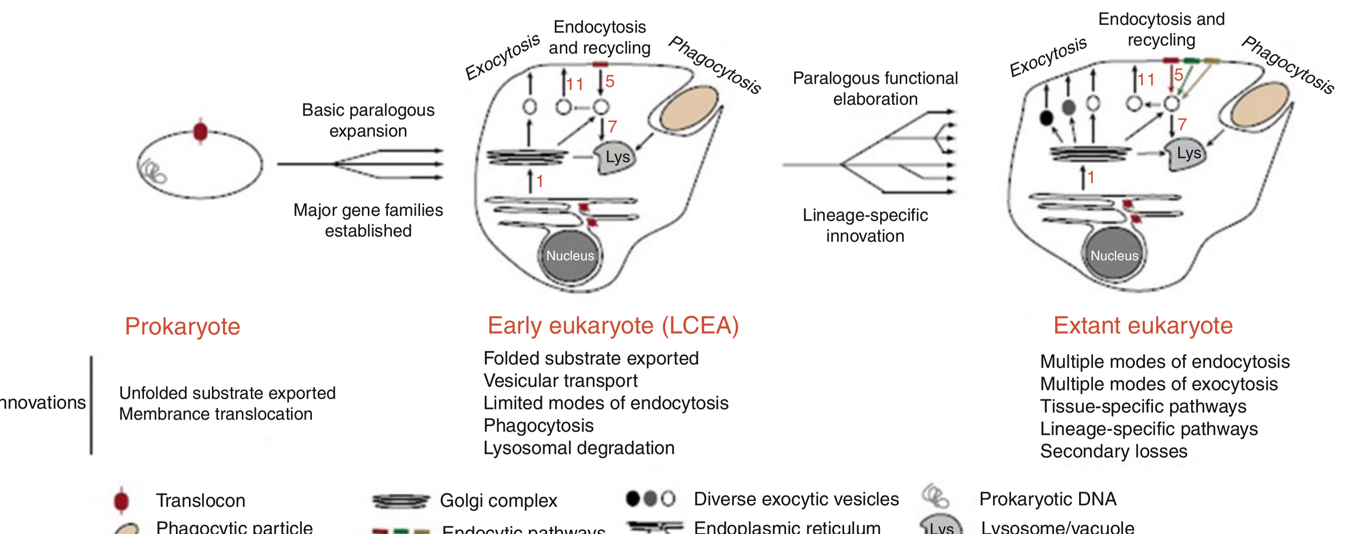


Fig. 9 Major transition in evolution of the endomembrane system. In prokaryotes, secretion occurs just by translocation of unfolded peptides, whereas in early eukaryotes (last common eukaryotic ancestor or LCEA), folded substrates were transported. This cellular system included complex vesicular transport, phagocytosis, limited mode of endocytosis, and lysosomal degradation. Subsequent evolution leads to diversification of endomembrane systems, which include multiple modes of exocytosis and endocytosis and development of various pathways (Adapted with permission from Dacks and Field 2007)

multiple modes of well-developed (late eukaryotic organisms) exocytosis, endocytosis, lysosomal system, and various cellular pathways involving multiple proteins/ protein complexes in eukaryotic cells. Eukaryotic cells possess various internal membrane-bound components, and this characteristic is the fundamental difference to their prokaryotic counterparts. Appearance of these organelles enabled membrane trafficking in the cellular machinery for internalization and digestion of extracellular material, targeted intracellular/extracellular transport, and surface remodeling. Several experiments have established the role of small GTPases, adapter proteins, and coat protein complexes as a cargo carrier, whereas SNAREs, syntaxin-binding proteins, and Rab GTPases ensure specificity of cargo proteins with the correct target membrane. Many of these proteins can be divided into subfamilies in which each paralog performs a function similar to that of the other members but at a specific location or in a very distinct transport pathway. Therefore, these proteins can be a very good model to study the origin and evolution of eukaryotic cellular machinery. Since SNARE proteins are involved in exocytosis of neurotransmitter, this section will focus on the possible origin and evolution of SNARE proteins. Their evolution may give some insight into the evolution of botulinum neurotoxin.

Directed transport of vesicles is a unique feature of eukaryotic cells. Each of these internal compartments has very specific and vital cellular functions. The identical unique feature of vesicle trafficking is achieved by various specific and regulated protein machineries. Primitive endomembrane differentiated into separated compartments, in which material exchange is mediated by fusion of cargo-loaded vesicles. These processes lead to the development of exocytotic and endocytotic pathways. The abovementioned proteins (SNAREs, GTPases) are examples of some of these important protein machineries. Vesicle trafficking is tightly regulated, and one of the players involved in this is the SNARE protein family. Most SNARE proteins orient toward cytoplasm. As a common mechanism of molecular association, the 60–70 amino acid long peptide segments of heterologous sets of SNARE proteins (SNARE motifs) assemble in a zipper-like fashion into a tight 4-helix bundle between two membranes. The formation of this 4-helix complex initiates fusion of vesicular and plasma membranes. The SNARE machinery has diversified only modestly over the course of eukaryotic evolution. It is already established that a basic set of 20 types of SNARE proteins is conserved in all eukaryotes (Kloepper et al. 2007). SNAREs can be divided into vesicle or v-SNAREs, which are incorporated into vesicles during budding, and target or t-SNAREs, which are located in membranes of target compartments. Together, these SNAREs form a nanomachine, consisting of four parallel helix bundles. In its interior are hydrophilic centers consisting of three glutamine (Q) residues and one arginine (R) residue. Proteins with glutamine as the central residue in the SNARE complex are known as Q-SNAREs, whereas R-SNAREs are those with a central arginine. The three Q helices can be classified as Qa, Qb, and Qc, and the whole complex is called as QabcR complex (Bock et al. 2001). Almost all SNARE groups found are highly conserved in eukaryotes. High conservation probably suggests that the early eukaryotes are equipped with an elaborate endomembrane system and

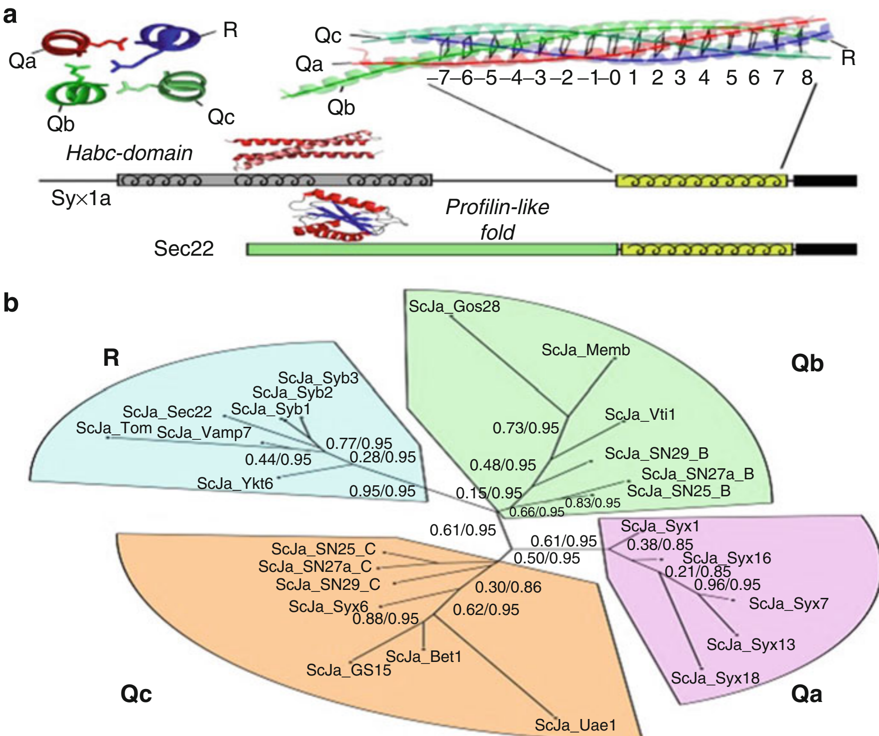


Fig. 10 Schematic representation of the different domain arrangements found in SNARE proteins. The four-helix bundle structure of the neuronal SNARE complex is shown as ribbon diagram on the *top right* (*blue*, *red*, and *green* for synaptobrevin 2, syntaxin 1a, and SNAP-25a, respectively). The domain architecture of two most important types of SNARE proteins are depicted below with the highly conserved SNARE motif and adjacent transmembrane domains indicated by a *yellow* and *black* box, respectively. (**b**) General outline of the phylogenetic tree of SNARE proteins. All SNAREs split into four groups (Qa, Qb, Qc, and R) representing the four different positions of the four-helix bundle SNARE core complexes. Each of the four main groups segregates into the distinct subgroups. Notably, two SNARE motifs of Qbc-SNAREs are indicated by a B or C suffix for the Qb-helix or the Qc-helix. Basically, all SNAREs split into these four classifications (Adapted with permission from Dr. Dirk Fasshauer; Kloepper et al. 2007)

vesicle trafficking system. The phylogenetic tree of SNARE proteins also suggests the fundamental splitting of the four branches of Qa, Qb, Qc, and R-SNAREs (Fig. 10). The existence of these four branches reflects the common structural arrangement of SNARE complexes, further strengthening the idea that all SNAREs assemble into four-helix bundle structures. Notably, each of the SNAREs in eukaryotes possibly evolved from secretory SNAREs (Kloepper et al. 2007) with each of the four branches further divided into subgroups. This also strengthens the view of all SNARES having a common structural arrangement, namely, the four-helix bundle.

Evolutionary Correlation Between Botulinum Neurotoxins and Their Neuronal Substrate

Figures 11 and 12 show the dendrograms for both selected SNAP-25 (52 hits) and collected syntaxin (90 hits) (Blast P; Altschul et al. 1990). The SNAP-25 dendrogram suggests the existence of major SNAP-25 clusters/groups: (1) protozoa, plants, and yeasts (see top and bottom regions in Fig. 11); (2) invertebrates; and (3) vertebrates. The SNAP-25 of vertebrates split further into two isoforms (paralogous event), SNAP-25a and SNAP-25b (Nagy et al. 2005) with the sequence of SNAP-25b being more closely related to invertebrate SNAP-25, suggesting it evolved earlier than SNAP-25a. Biochemically, SNAP-25a is more readily cleaved by BoNT/A (Puffer et al. 2001). Combining the above two reasons, it can be

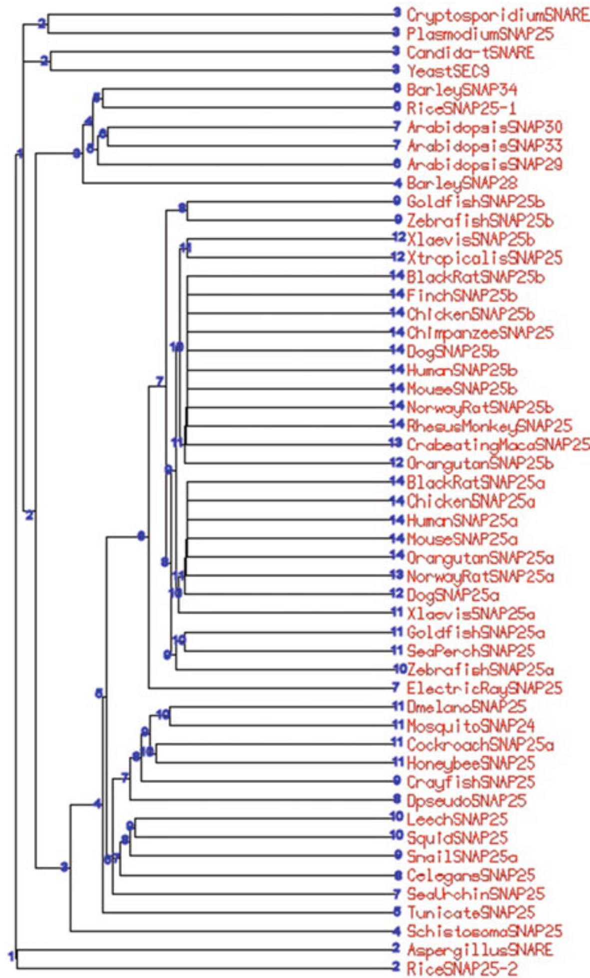


Fig. 11 Dendrogram of 52 selected SNAP-25 proteins

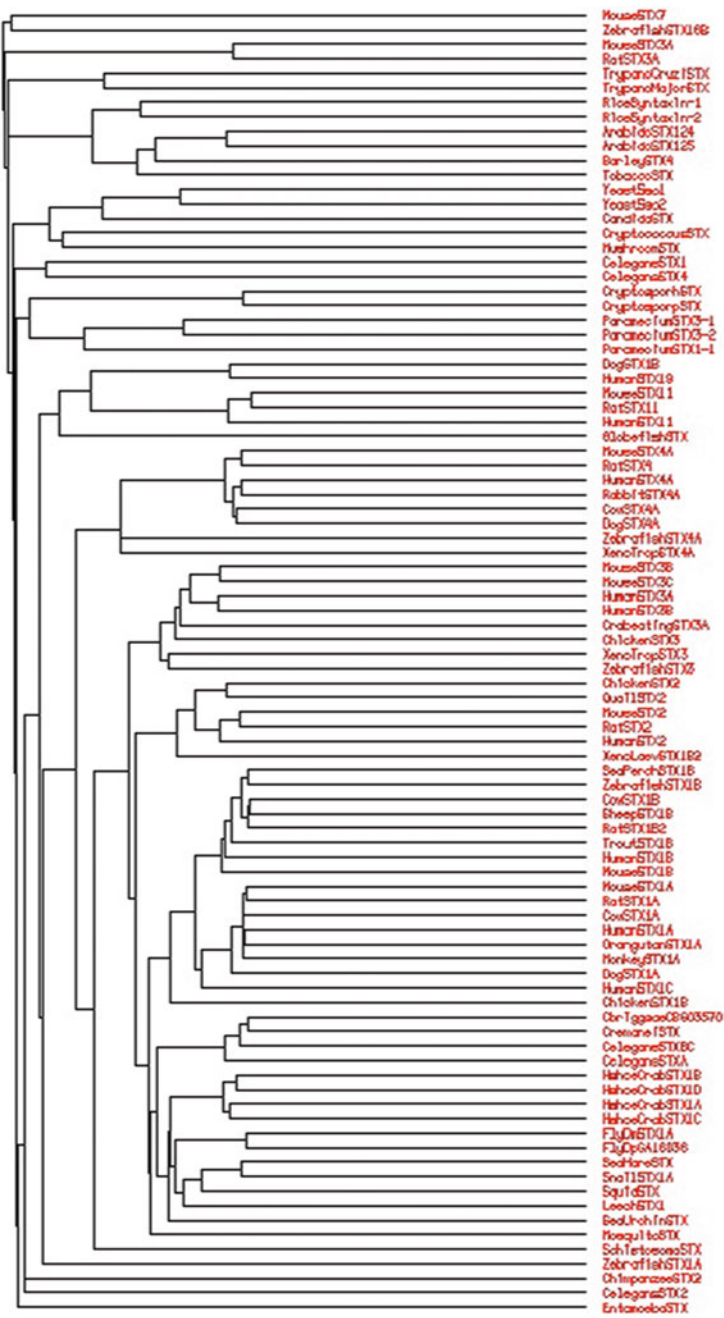


Fig. 12 Dendrogram of 90 selected syntaxin proteins

Fig. 13 Multiple sequence alignment of SNAP-25 that shows the cleavage sites of BoNT/C, BoNT/A, and BoNT/E

predicted that the SNAP-25b is the ancestral form of SNAP-25a, as the ancestral SNAP-25 must have longer time to be recognized by different enzymes during the evolution.

The BoNT/A cleavage site contains five different sequence patterns, while that in BoNT/C contains 13 (after combining the cleavage sites in SNAP-25 with syntaxin) and BoNT/E contains only two cleavage sequence patterns. Among the 13 sequence patterns in the BoNT/C cleavage site, R/A, K/A, and N/A are the three that appear in both syntaxin and SNAP-25, H/A and Q/A appear only in SNAP-25, and the remaining eight patterns can be seen only in syntaxin. In other words, the BoNT/C cleavage site in syntaxin contains more sequence variation patterns than in SNAP-25 (Figs. 13 and 14). It should be pointed out that in obtaining the data related to BoNT/A, BoNT/C, and BoNT/E endopeptidase cleavage sites on SNAP-25, the data belonging to protozoa, fungi, and plants were excluded because their

Fig. 14 Multiple sequence alignment of syntaxins that shows BoNT/C cleavage sites. Diversity of sequences is explained in the text

SNAP-25 family protein sequences are much longer (249–526 a.a.) and more diverse (below 26 % sequence homology).

The unique characteristics of BoNT endopeptidases include exclusive substrate and highly specific cleavage sites (Li and Singh 1999). Of the seven known serotypes of BoNT, only BoNT/C cleaves more than one substrate (SNAP-25 and syntaxin). SNAP-25 is also cleaved by two other serotypes of BoNT (type A and E), although at mutually exclusive sites, which are different from the BoNT/C cleavage site. Protein sequence alignments of SNAP-25 showing cleavage sites of BoNT/A, BoNT/C, and BoNT/E from 52 different sources are shown in Fig. 11. Current public domain databases hold 76 SNAP-25 sequences, but cleavage sites have only been documented in 52 (Puffer et al. 2001).

A multiple sequence alignment of 86 known syntaxin sequence in the vicinity of BoNT/C cleavage site is shown in Fig. 14. The most common BoNT/C cleavage site on syntaxin is K/A, where R in SNAP-25 cleavage site is conservatively replaced with K. Notably also, the cleavage site of BoNT/C contains alanine in the P_1' position (first amino acid of the cleavage site), in all 86 syntaxin and 36 SNAP-25 sequences which contain BoNT/C cleavage sites. Alanine residues could be critical for the BoNT/C cleavage activity, as it is common to the cleavage sites of BoNT/C in both of its substrates. The conservation of alanine in P_1' position is maintained in syntaxin without exception in all organisms ranging from protozoa, fungi, and plants to advanced vertebrates. This sequence alignment which resulted in showing the P_1' position of BoNT/C cleavage site being highly conservative is consistent with the fact that biochemically BoNT activity does not tolerate amino acid substitutions adjacent to the scissile bond at the P_1' position (Vaidyanathan et al. 1999).

The BoNT/C cleavage site is only one base downstream from the BoNT/A cleavage site (Q_{197}–R_{198} for BoNT/A vs. R_{198}–A_{199} for BoNT/C). The conservativity of P_1' site at the BoNT/A cleavage site directly leads to the conservativity of P_1' site at BoNT/C cleavage site. This is therefore an appropriate model for studying the sequence microevolution of a specific gene under different selection pressures imposed by dissimilar pathogens (bacterial enzymes/BoNTs). The fact that BoNT/C can cleave two different substrates (SNAP-25 and syntaxin) could also provide additional information to examine the coevolutionary relationship between two different substrate genes under the same selection pressure (BoNT/bacterial enzyme). Currently, there is no available literature to explain if the molecular evolution of these neuronal substrates is parallel (paralogous event) or sequential (orthologous event). It is believed that syntaxin evolved earlier than SNAP-25 (if they both are based on the same mutation rate) because the BoNT/C cleavage site in syntaxin contains more sequence variation (more amino acid substitution events) than in SNAP-25. The dominant BoNT/C cleavage site sequence (R/A: 83.3 %) in SNAP-25 is maintained or restricted by the conservativity of P_1' position of BoNT/A cleavage site (Q/R), which exerts a different selection pressure. BoNT/C exerts a differential selection pressure on SNAP-25 and syntaxin. Not only is the frequency of the dominant cleavage site sequence different, but also the sequence pattern is not the same (R/A in SNAP-25

vs. K/A in syntaxin). K and R both belong to the basic amino acid group, suggesting a similar molecular recognition mechanism exists for BoNT/C to bind to its two different substrates. Similar molecular recognition means similar selection pattern. Therefore, it is possible to directly compare the magnitude of selection pressure between SNAP-25 and syntaxin to the same agent (BoNT/C) by calculating the sequence entropy at these cleavage sites.

Molten Globule Conformations Coupled with Unique Folding

The most essential question in protein folding studies is how an unstructured peptide folds into its unique active state. To get to this state, a protein has to go through an astronomically large number of possible conformational states (Levinthal's paradox; Levinthal 1968; Zwanzig et al. 1992). The presence of folding pathways in which some intermediates are well populated could be the answer to this paradox and could help us to conformationally select those intermediates. A typical small protein can fold reversibly, in vitro, undergoing first-order transition between the folded and unfolded state. But under certain conditions, a protein can also exhibit another state which is a thermodynamically different state than the unfolded state and the native state, which will have secondary structure similar to the native state and have a dynamic tertiary structure. This particular state is termed as molten globule, which corresponds to the narrowing region of folding funnel (indicative of limited number of energy states of the conformation), and is higher in energy than the native protein but lower in energy than the unfolded protein. The structural characteristics of molten globule state are:

1. Presence of substantial content of secondary structure
2. Absence of most of the tertiary structure associated with light packing of the side chains
3. Dynamic feature of the structure with motions on a time scale longer than nanoseconds
4. Compactness of the protein molecule with a radius only 10–30 % larger than that of the native state
5. Presence of a loosely packed hydrophobic core that increases the number of hydrophobic residues accessible to solvent

Evolution of protein results because of a compromise between rigidity and flexibility allowing for various combinations. The molten globule state provides a good model as it has less rigidity and more flexibility than the native state. It is believed that molten globules retain their functional active sites even though the other sites may have fluctuating or misfolded configurations. Therefore, the existence of functional molten globules can support the evolutionary scenario in which the active site will fold into the functional configuration. This semiflexible nature of a molten globule provides a unique flexibility in different environments to facilitate interactions. In this respect, botulinum neurotoxins provide a very good model to

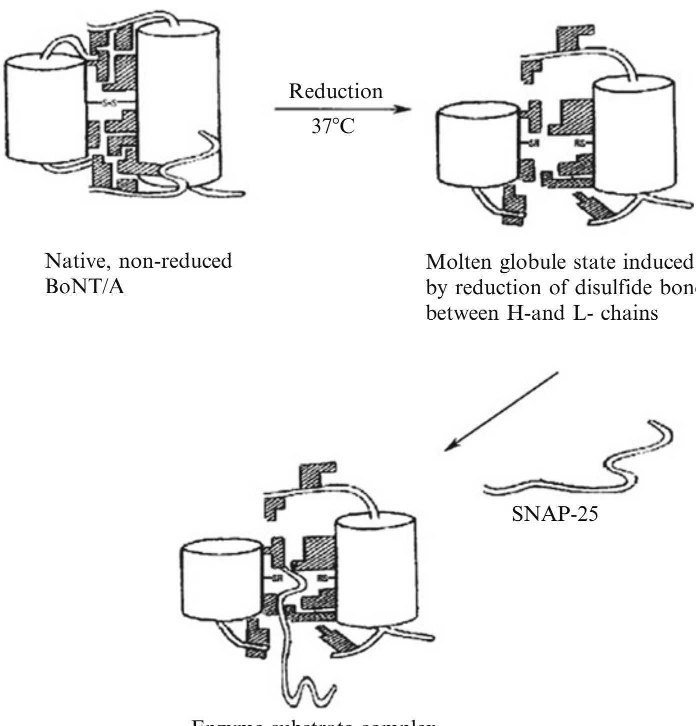


Fig. 15 Pictorial representation of binding of SNAP-25 to the molten globule conformation of BoNT/A

study the correlation between molten globule and evolution. The heavy chain of botulinum neurotoxin is connected to the light chain through a disulfide bond. Reduction of this disulfide is necessary for the activation of the protein. However, reduction of the disulfide bond brings conformational changes in the protein molecule, resulting in a molten globule conformation (Cai and Singh 2001; Fig. 15). Structural differences between the reduced and nonreduced form of BoNT/A were observed, and a biologically active molten globule state was observed in the reduced form of BoNT/A (Cai and Singh 2001). Considering the fact that the substrate for BoNT/A toxin, SNAP-25, is unstructured in solution (Puffer et al. 2001), it is possible that the molten globule conformation of BoNT/A (which exists at 37 °C in reducing conditions) might facilitate the binding of such a large substrate to the toxin's endopeptidase domain.

Interestingly, Kukreja and Singh (2005) reported the existence of active PRIME (pre-imminent molten globule enzyme) conformation in BoNT/A light chain at physiological temperature (37 °C) (Fig. 16). This is important because light chain is the catalytic moiety of BoNT which enters the neuronal cells and cleaves SNARE proteins. It was also reported that the BoNT/A light chain also exists in an active molten globule form (Kukreja and Singh 2005). The molten globule conformation

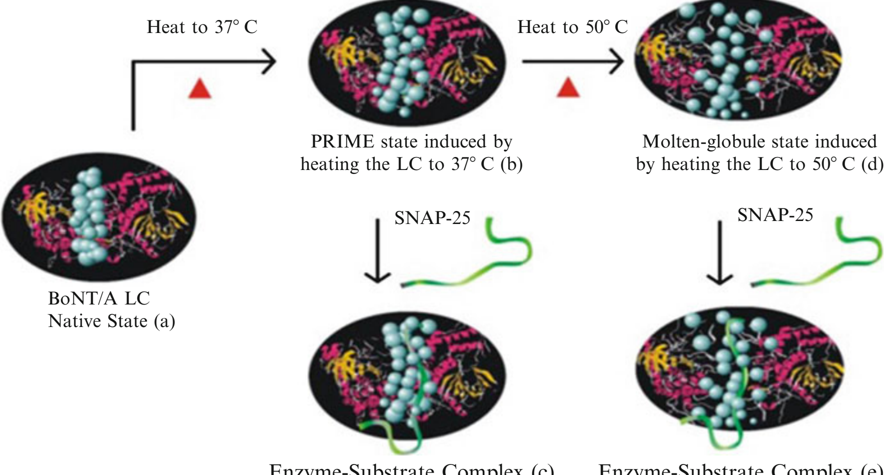


Fig. 16 Schematic model representing the PRIME and molten globule states in BoNT/A LC, facilitating binding of substrate SNAP-25. The *blue balls* represent the nonpolar side chains, which are tightly packed in the native conformation (**a**). Upon heating to 37 °C, there are significant alterations in the polypeptide folding, and the protein core becomes slightly loosened as compared with the native state and forms the PRIME state (**b**), facilitating binding of SNAP-25 to form the enzyme–substrate complex (**c**). BoNT/A LC exhibits optimum activity in the PRIME state. Further heating of BoNT/A LC to 50 °C leads to the formation of the molten globule folding intermediate, which is represented by a loose packing of side chains in the protein core and partial unfolding of loops (**d**). This structure is slightly more expanded than the PRIME state. In this state, although BoNT/A LC binds to SNAP-25 forming the enzyme–substrate complex (**e**), the binding is suboptimal, resulting in 61 % of the optimal enzymatic activity

generally has a fluidic structure, and its dynamic role may contribute to the extreme specificity of the BoNT toward its substrate.

As already stated, folding and flexibility of proteins govern protein evolution. To understand the dynamic behavior and folding of the BoNT molecule in solution, it is necessary to shed light on the structural element and its folding, which may explain the extraordinary intracellular longevity and biological action. Unfortunately, a crystal structure is not adequate to do so, as is apparent by comparing the molecular folding in the crystal and solution structures. Published crystal structures of three light chains (A, B, and E, PDB ID: 2ISE, 2ETF, and 1T3A, respectively) showed no difference in their secondary and tertiary structural arrangements (Fig. 17), whereas solution structures of all three proteins as monitored by circular dichroism spectroscopy showed a different picture (Kumar et al. 2013; Cai and Singh 2001; Kukreja and Singh 2005). The tertiary structures of LCA and LCB are similar, whereas LCE completely differs.

In addition, folding of light chains appears to be different in solution (Kumar et al. 2013). All these observations suggest significant differences between the published crystal structure and the solution structure of these proteins. A careful

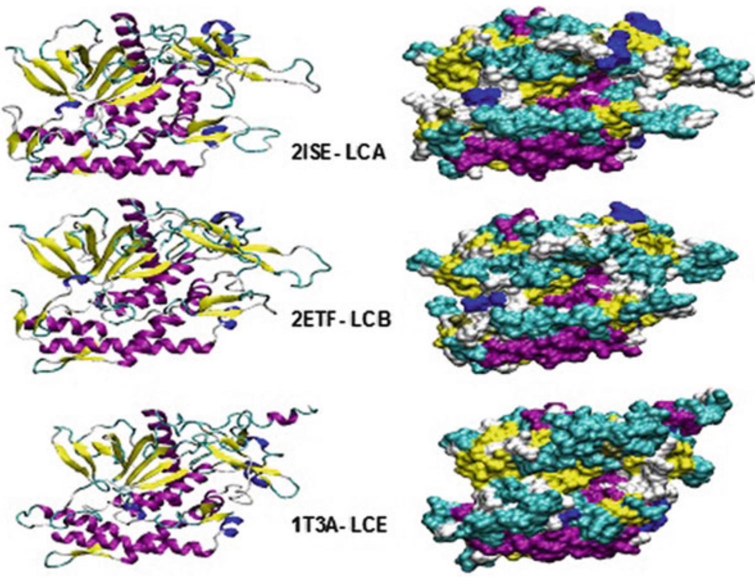


Fig. 17 Crystal structures BoNT light chain. PDB (protein data bank) ID is listed with each figure. On the *left* are representations of secondary structures (ribbon diagram), whereas tertiary structures are depicted on the *right* (surface diagram)

study of structural characteristics of these proteins is needed to resolve questions related to specificity, longevity, and evolutionary aspects of proteins.

Existence of active molten globules, PRIME conformations, and unique solution conformations/folding provides a very different and unique substrate–ligand relationship. These conformations are possibly the result of distinctive molecular characteristics of these proteins, which allow them to have exclusivity, stability, and adaptability to different environments. More detailed understanding of these properties will provide new knowledge about the evolutionary origin of botulinum neurotoxin.

Conclusion and Future Directions

Although considerable progress has been made to understand the structure and function of these toxins, their evolutionary origins remain unclear. While evidence suggests frequent horizontal transfer of clostridial genes among different *Clostridium* species, no CNT homologs have been identified outside the *Clostridium* genus. The existence of sequence similarities of botulinum neurotoxin to other proteins (section "Folding Similarities of Botulinum Toxins with Other Toxins"), as well as the existence of active molten globule, PRIME conformation, and unique solution conformation/folding of botulinum neurotoxin, indicates unique evolutionary characteristics. Molecular characteristics, potential coevolution with its substrate, the

possibility of horizontal gene transfer among different species, and association of different protein domains for a coordinated cellular function are some of the critically unique characteristics of bacterial protein toxins. Complete understanding of these characteristics coupled with flexibility and molecular dynamics could not only allow design of better countermeasures but also reveal fundamental molecular and submolecular mechanisms involved in evolution, particularly of proteins.

Cross-References

▶ The Biosecurity Threat Posed by Biological Toxins
▶ Biotoxins and Food Safety
▶ Botulinum Toxin: Present Knowledge and Threats

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Pheromones and Amphibian Behavior

24

Michael J. Tyler

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Abstract

Pheromones were first discovered in the Amphibia less than 20 years ago: newts in 1995 when the compound named sodefrin was located in male cloacal glands and frogs in 1999 when a compound named splendipherin was located in the male rostral and parotoid glands. The ability of an alarm compound released by a traumatized toad tadpole to disrupt schools of tadpoles of the cane toad, *Bufo marinus*, is being explored as a possible control mechanism. A significant number of tadpoles exposed to the alarm chemical metamorphosed at a smaller size, while the survival rate was reduced by 50 %.

Odors released by frog dermal glands also drew the attention of chemical biologists and herpetologists. Most resemble recognizable compounds such as nuts (like cashews and peanuts), cut-grass, and curry. The role of these volatile

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aromatics is not known, but their species-specific nature is strongly indicative of their being pheromones.

Finally it was demonstrated in 2013 that male nuptial pads in the breeding season release proteins related to salamander pheromones which are believed to modulate courtship behavior. Formerly the role of nuptial pads was considered solely to maintain a secure hold of the female during amplexus, but now they are likely to also be pheromone-secreting glands.

Introduction

In his "Dictionary of Herpetology," Lillywhite (2008) defines a pheromone as "A chemical substance produced by an individual and released into the environment for the purpose of signalling between (among) individuals of the same species. Examples include substances that are used to mark territories, attract mates, or provide scents used in trailing."

The examples cited by Lillywhite do not include the most significant for anurans, namely, the alarm pheromones whereby the presence of an injured tadpole causes conspecifics to flee. The fundamental issue is that in the absence of adequate means of auditory or visual communication, such as localities where there is considerable background noise, some frogs employ tactile means, but by far the most common is chemical communication which Agosta (1992) termed "the language of pheromones."

One of the first hypotheses of the existence of pheromones is that of Zeller (1905) who suggested their presence in the abdominal gland of the newt *Triturus carnifex* (as *Triturus cristatus carnifex*).

Pheromones are present in diverse invertebrate and vertebrate animals, inevitably in minute quantities. Agosta (1992) likens their concentration as being equivalent to a teaspoonful in an Olympic swimming pool.

Rajchard (2005) prepared a review of amphibian sex pheromones and predicted their highly significant role in sexual relationships and warned that the infochemical system can be disturbed chemically by environmental impacts.

It is less than 20 years since the first pheromone was detected in the Amphibia. In the last decade, the proposal that the *Bufo* alarm pheromone might provide a means of controlling the cane toad, *B. marinus*, in Australia (Tyler 2006) coincided with intense studies by Professor Shine and his colleagues at the University of Sydney which are summarized here.

Odorous pheromones are readily detectable by humans and commonly resemble well-known smells such as spices, cut-grass, cashew nuts, or many other distinctive aromas which can be elicited by specific chemicals. Their role and significance to amphibians is yet to be clarified.

Sex Pheromones

Anura

The only record of a sex pheromone in the Anura is the presence of a sex attractant (splendipherin) in the Magnificent Tree Frog *Litoria splendida* of northwestern Australia (Fig. 1). Wabnitz et al. (1999) reported that female frogs gave a positive response to a secretion of the rostral glands of males obtained by the electrical stimulation technique developed by Tyler et al. (1992). Splendipherin comprises 25 amino acid residues with the sequence GLVSSIGKALGGLLADVVKSKGQPA-OH. The tests on the impact of splendipherin upon behavior were conducted in a glass chamber (aquarium) measuring $0.65 \times 2.0 \times 0.75$ m. Water was added to a depth of 2 cm – sufficient to keep a large frog wet but enough for it to be able to maintain a hold on the floor.

A female *L. splendida* was placed in the center of the tank. Within 20 s, it would move towards a pad containing the pheromone. If the pheromone was less than 4 ng, nothing happened. Similarly, nothing happened if the dose was above 40 ng. At a higher dose, the observers concluded that the frog was "confused."

The movement of the pheromone upon the surface of the water was assumed to be due to it being a surfactant, but no tests were undertaken to confirm this. However, this conclusion was reached before Willaert et al. (2013) reported the existence of aerially dispersed pheromones, whose action would presumably also be rapid.

Caudata (Urodela)

Kikuyama et al. (1995) at Waseda University, Japan, reported that the cloacal glands of the male newt *Cynops pyrrhogaster* contained a decapeptide which attracted females. They named the peptide sodefrin and observed that this was the

Fig. 1 Adult Australian hylid frog *Litoria splendida* from Lake Argyle, Western Australia, exhibiting a vast gland upon the rostral and parotoid areas

first pheromone to be identified in an amphibian and the first peptide pheromone identified in a vertebrate. The source of the pheromone had been assumed to be the cloaca (Kikuyama et al. 1977; Malacarne et al. 1984), and, prior to the identification of the pheromone, it had been reported that the water in which male newts were kept attracted conspecific females (Toyoda et al. 1994). Clearly the presence of a pheromone had been established before its identity was known.

Yamamoto et al. (2000) reported the existence of a similar sex pheromone in the congener *C. ensicauda*. They named it silefrin and found that it was operative within the range 0.1–1.0 pM. Beyond that concentration it does not appear to have an effect.

Aquatic Pheromones

When tadpoles of the bufonid genus *Bufo* and some other genera are exposed to an extract of a crushed conspecific tadpole, they rapidly disperse in all directions. This phenomenon has been known for many years and is assumed to involve an unknown pheromone.

Hrbacek (1949) described this response as "chaotic flight" in *Bufo vulgaris* [probably what is now called *B. bufo*], and surprisingly, the reaction persisted, even when the extract had been boiled for 15 min. Few biological chemicals, particularly proteins, would retain their activity after exposure to such heat for this period of time.

Perhaps the most significant of Hrbacek's observations was that flight reactions could be induced by material other than crushed tadpoles. He obtained a similar flight response from exposure to aquatic extracts of skin from different regions of the body of adult *B. vulgaris, B. viridis*, and *B. calamita*. He also observed that similar flight reactions were produced when tadpoles were exposed to extracts of digitalis plants. Previously it had been assumed that the flight response was only induced by exposure to a crushed tadpole of the same species. It is not clear why the authors chose to introduce digitalis, but it seems to be part of the alarm material. It is difficult to imagine a compound common to both the skin and digitalis except perhaps histamine or 5-hydroxytryptamine (5-HT).

Hews (1988) examined the alarm response in tadpoles of *B. boreas* induced by the attack of giant water bugs on members of the shoal. This was probably the first demonstration of the purpose of a flight reaction, whereby the sacrifice of an individual to a predator leads to the escape of congeners in the vicinity.

Shoaling is common to *Bufo* species but is not confined to them. The Australian hylid *Cyclorana australis* is an example. In deep water, the tadpoles form large congregations which maintain their position, hovering together in a mass as large as $1.0 \times 0.5 \times 0.5$ m.

Aggregations of literally hundreds of millions of *Litoria dahlii* tadpoles have been observed in the Kakadu National Park in the Northern Territory of Australia. Aggregations have been seen moving as a continuous column two meters wide slowly down the Magela Creek for more than 12 h (Fig. 2). Clearly the tadpoles

Fig. 2 Aggregation of tadpoles of the hylid species *Litoria dahlii* in Kakadu National Park, Northern Territory, Australia (Photo: Greg Miles)

Fig 3 The limnodynastid frog *Limnodynastes dumerilii* from Adelaide, South Australia. The tibial gland is orange (Photo: Stephen Walker)

need to be able to communicate to maintain their position. The pheromone involved is unknown, as is the purpose of the migration. Of the 23 species of frogs in the Kakadu National Park (Tyler et al.), *Litoria dahlii* is the only one found in flowing water. For that reason, there is no possibility of other species competing with it by entering the school.

Among the enlarged dermal glands (macroglands of Duellman and Trueb 1986) is the tibial gland on the dorsal surface of the tibia in *Bufo* and *Limnodynastes*. Crook and Tyler (1981) examined the structure and function of the tibial glands of the Australian species *L. dumerilii* (see Fig. 3). They found that laboratory rats that fasted for 48 h would consume the entire body of a freshly killed frog except the tibial glands. Subsequently, in another trial, the glands were compressed forcing the contents to the outer surface of the glands. The rats then failed to devour any part of the frogs. Those that licked the glands attempted to wipe the secretions away and exhibited distress. Many rats approached the frog but then moved away, presumably detecting an odor released by it that they found bitter or in other ways unpleasant. The rats did not exhibit any long-term effects from exposure to the chemical.

Odorous Secretions

Numerous species of frogs emit an odor when handled and there is evidence that some of these odors are used for communication. Smith (2001) and Smith et al. (2004) examined the extent that odors derived from granular glands provided protection from predators.

The human olfactory senses are inferior to those of domestic and other animals. Bauer et al. (1990) listed the most common descriptive terms used to characterize odors. Smith (2001) employed the same definitions and these are reproduced here in Table 1.

Among the odor definitions, the one termed "spicy" and described as "generic term for odors of various spices" is very much a catchall. Various studies have associated the compounds with different spices and herbs, such as curry, thyme, and parsley.

All of the frog odors emitted are species specific. Their various roles in communication have yet to be resolved (Smith et al. 2000).

Practical Application of Pheromones

In Australia, considerable effort and millions of dollars have been devoted to find a means of controlling the cane toad, *Bufo marinus*. Introduced in 1935 in a failed attempt to control two beetle pests of sugarcane in Queensland, its geographic

Table 1 Common descriptive terms used to characterize odors secreted by frogs

Animal(ic)	Typical notes from the animal kingdom, e.g., musk, castoreum, skatol, civet, ambergris
Balsamic	Heavy, sweet odors, e.g., cocoa, vanilla, cinnamon, Peru balsam
Citrus	Fresh, stimulating odor of citrus fruits such as lemon or orange
Curry	Resembling a sweet curry dish
Earthy	Humus-like, reminiscent of humid earth
Floral, flowery	Generic term for odors of various flowers
Fruity	Generic term for odors of various fruits
Green	Typical odor of freshly cut-grass and leaves
Herbaceous	Non-characteristic, complex odor of green herbs with, e.g., sage, mint, thyme eucalyptus-like, or earthy nuances
Medicinal	Odor reminiscent of disinfectants, e.g., phenol, lysol, methyl salicylate
Metallic	Typical odor observed near metal surfaces, e.g., brass or steel
Minty	Peppermint-like odor
Mossy	Typical note reminiscent of forests and seaweed
Nutty	Resembles peanuts or cashews
Powdery	Note associated with toilet powders (talcum), diffusively sweet
Spicy	Generic term for odors of various species
Woody	Generic term for the odor of wood, e.g., cedar wood, sandalwood

Source: Bauer et al. (1990); only those reported for frogs are included. Additions are curry and nutty

distribution is expanding exponentially and it now occupies approximately one million square kilometers. As indicated below, the latest control method explored has involved examining the pheromone responsible for the alarm response of tadpoles exposed to damaged conspecifics. The method was attractive because the pheromone has no effect on native species.

Hagman and Shine (2009) demonstrated that repeated exposure of *B. marinus* tadpoles to the alarm chemical reduced tadpole survival rates by 50 % and also reduced body mass at metamorphosis by 20 %. This effect has not been reported in pheromones in other taxa. The impact of the pheromone upon post-metamorphic body structure is clearly extremely profound. Hagman et al. (2009) reared tadpoles in the presence of crushed conspecifics and found that they increased the amount of the parotoid toxin bufalin, a bufadienolide, but reduced the total bufadienolide content. The interpretation was that the cane toads metamorphosed at a smaller size as a response to the pheromone, and at the same time, they changed their reliance upon particular defensive toxins in the parotoid glands.

Hagman and Shine (2008a) demonstrated that metamorphing toads exhibited behavioral responses similar to those of tadpoles, and Hagman and Shine (2008b) showed that tadpoles of six native species of the families Hylidae, Limnodynastidae, and Myobatrachidae did not exhibit aversion to the same extract. Instead they, “either ignored the stimulus or tended to approach it rather than to avoid it.”

Nuptial Pads

Male nuptial pads are black excrescences of keratin on the first or first and second digits of the hand. Their position is such that they come into contact with the skin of the female when the pair are in amplexus. There are three forms of amplexus which influence the positions of the nuptial pads: axillary, where the male rides upon the back of the female and grasps each side behind the head; inguinal, where the male clasps the female around the waist; and dorsal, where the rotund *Breviceps* species of Africa adhere to an adhesive pad on the back of the female whilst standing behind her.

Until as recently as early 2013, the function of nuptial pads was considered to be solely a means of securing the male and female during amplexus. Noble (1931) stated “It is well known that the nuptial pads of frogs are used for maintaining a firm grip on the back of the female during egg laying.” However, a report by Willaert et al. (2013) reveals that the nuptial pads have a much more significant role in the European ranid frog *Rana temporaria*: they are linked to a source of secreted molecules that reach the surface during amplexus and are absent at other times. Using transcriptome and proteome analyses, they identified the presence of proteins of the Ly-6/uPAR family for which they coined the term “amplexins.” Although the function or functions of the amplexins remain unknown, the authors draw attention to the fact that they structurally resemble plethodontid modulating factors which are

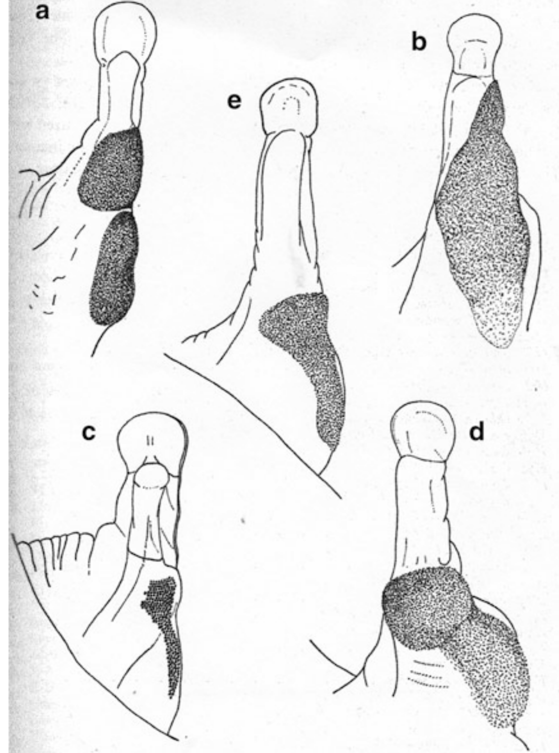

Fig. 4 Nuptial pads of Papuan species of *Litoria*. (**a**) *Litoria infrafrenata*; (**b**) *Litoria thesaurensis*; (**c**) *Litoria graminea*; (**d**) *Litoria arfakiana*; (**e**) *Litoria spinifera* (From: Tyler (1968))

proteins that impact upon the duration of salamander courtship such as increasing female receptivity to advances from a male.

Because the nuptial pads are the delivery system for pheromones, it becomes important to examine their structure. For many years, their diversity has been recognized, and therefore, illustrations are included in papers describing new species.

Nuptial pads are presented in two formats. By far the most common are line drawings which indicate the form and extent in relation to the underlying phalangeal and metacarpal bones. Examples of these are illustrations of Papuan hylid species of the genus *Litoria* (Fig. 4) at that time referred to *Hyla* by Tyler (1968) and a study of 16 species and subspecies of frogs related to the European *Rana temporaria* by Herrmann (1996). Lynch and Ruiz-Carranza (1996) employed differences or absence of nuptial pads to define the centrolenid genera *Centrolene, Cochranella*, and *Hyalinobatrachium*.

With the aid of a scanning electron microscope, it is possible to examine the surface of a nuptial pad in considerable detail. Early studies failed to recognize that mature pads are regular in form and commonly extremely elaborate in design, and always with sharp edges, whereas those postmaturities are irregular and smooth. Unfortunately visual examination of a pad will not distinguish the perfect form

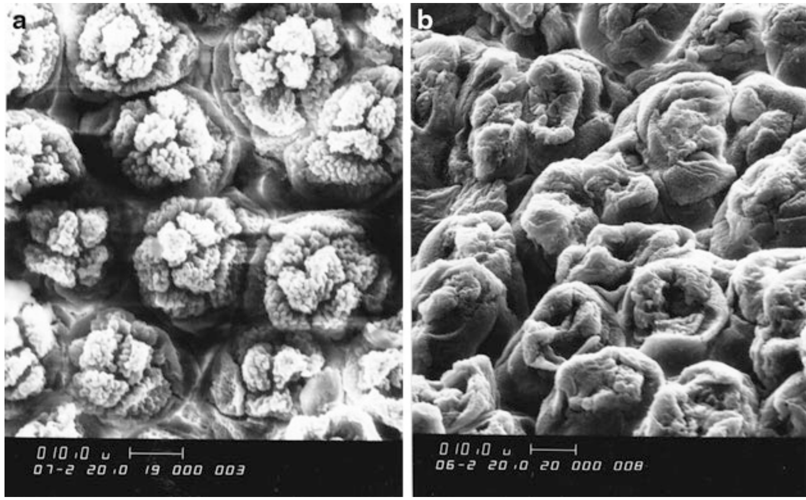

Fig. 5 SEMs of *Litoria caerulea*. (**a**) Mature pad; (**b**) degenerating pad (From: Tyler and Lungershausen (1986))

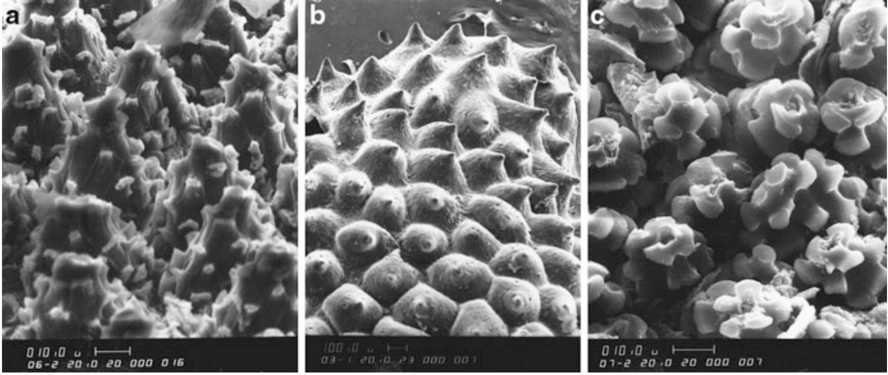

Fig. 6 SEMs: (**a**) *Lechriodus fletcheri*; (**b**) *Litoria nannotis*; (**c**) *Platyplectrum ornatus*

from one which is deteriorating (see Fig. 5). It is evident that most published SEMs have not been recognized to be deteriorating and bear no resemblance to the elaborate form of the mature nuptial pad.

In Fig. 6 there is a selection of SEM views of nuptial pads picked to give an idea of the incredible diversity of architecture upon the surface. Elsewhere Zweifel (1983) figured *Nyctimystes disrupta* and *N. trachyderma*. Although each of the pads involved is slightly past its perfect state, it is possible to see that each closely resembles the form in *Litoria caerulea* (Fig. 5a).

Finally, it needs to be noted that Kurabuchi and Inoue (1981) have demonstrated that the nuptial pad of *Xenopus laevis* consists of small spiny projections, which are also found on other parts of the body (Kelley 1996).

Conclusion and Future Directions

It is ironic that the extraordinary diversity of anuran pheromones and their delivery systems via nuptial pads has only just been discovered. The very fact that nuptial pads deliver pheromones from glands beneath them was not known until 2013. Scanning electron microscope studies have demonstrated considerable variation in the surface of the pads, but, as yet, the significance of the morphological variation is not known. All that can be concluded is that the pads always terminate in sharp shapes that presumably perforate the skin of the female when the male grasps her in amplexus.

So little is known. For example, the curious inactivity when two species of different genera amplex together and remain together for as long as 24 h without anything happening. Is it the nature of the amplectic grasp? Or is there some other action that fails to induce the onset of egg deposition and fertilization?

These and many other questions remain to be answered. Studies of pheromones and the manner of their release will challenge the scientists of the future.

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Marine Biotoxins in History: Misuse and Mayhem

25

Lyndon E. Llewellyn

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Abstract

Saxitoxin (STX) and tetrodotoxin (TTX) have a colorful history. Two of the most toxic natural toxins known, they have been used as weapons to commit suicide, attempt, or successfully commit murder, and STX has been officially declared a chemical weapon by the world's governments. This meant that it was no longer a natural marine chemical of interest to toxinologists, pharmacologists, and at times seafood producers (because it can taint their product); it had

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now come upon the radar of many government agencies. TTX has its own mystique more renowned as the toxin of "fugu" fame, but also been blamed as the active agent in the zombification rituals in some Caribbean cultures. However, deliberate intoxications by these toxins remain far fewer than accidental deaths through eating poorly prepared fugu meals or saxitoxin-infested shellfish. Diagnosis, rather than detection, remains the best means of identifying whether a victim has been purposefully intoxicated. Treatment of intoxication by STX or TTX is still relatively crude, relying mostly upon gastric evacuation to remove unabsorbed toxin and symptomatic relief to nurse the victim through the acute phase of an intoxication and then relying upon the body's natural recovery mechanisms. Some potential pharmaceutical treatment options exist, but these have only been tested in small mammal studies, upon few test animals, and under limited conditions and would require significant investment in resources to progress them beyond the laboratory.

Introduction

Most toxins kill, save for a few more renowned for simply sickening their victims, be they human or other organisms, but generally, causing death at low doses is a hallmark of a toxin. This defining characteristic of lethality has motivated numerous studies to better understand how we manage their presence and the risk they pose or use them as tools to uncover the secrets of the pharmacological or biochemical mechanisms which they attack. But their lethality can also attract those with nefarious ambitions. This chapter will not deal with the accidental deaths that natural toxins can cause through ingestion or other forms of exposure, but their use as a weapon. Proteinaceous toxins are dealt with elsewhere so the focus here will be a pair of small organic toxins, one being saxitoxin (STX), the only natural, nonprotein toxin declared as a schedule 1 chemical weapon (Tucker 2002) and, as the reader will discover, has a long and interesting story in the field of chemical weaponry. The other is its pharmacological relative, tetrodotoxin (TTX), which along with STX acts upon the same site of the voltage-gated sodium channel (VGSC) to block passage of sodium ions and is more renowned as the "fugu" poison. Part of the mystique and culture of the "fugu" dining tradition is that a well-prepared meal, which involves cooking of pufferfish flesh that usually contains some TTX, will invoke symptoms of a mild poisoning but occasional deaths occur due to poor preparation. It too has been used maliciously but it has not received the honor of being an official "chemical weapon."

TTX and STX are two of the most iconic marine toxins known (Fig. 1), but marine creatures have successfully developed many potent toxins, which are both effective at low concentrations and efficient in being absorbed by humans; a feat many pharmaceutical companies continually try to emulate in their drug development. A number of highly toxic polyether marine toxins have caused numerous human poisonings, being highly potent in pharmacological experiments eliciting effects at nano- and picomolar concentrations. These toxins include palytoxin

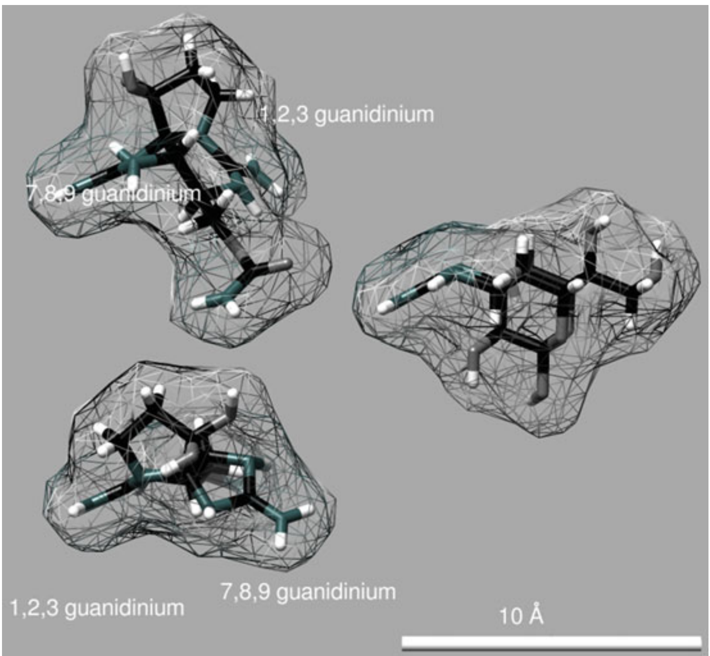

Fig. 1 Three-dimensional structures of saxitoxin (*left*) and tetrodotoxin (*right*). Carbon, nitrogen, oxygen, and hydrogen atoms are shown as *black*, *dark gray*, *light gray*, and *white*, respectively, and the solvent-excluded molecular surface is also shown as a mesh surface. The structures are aligned by their guanidinium moieties with the point of alignment to the left of each molecule. The STX structure is duplicated to show the possible alignments because it possesses two guanidiniums, noting the 7,8,9 moiety is the most critical for its bioactivity. Images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen et al. 2004)

(attacks cell membrane Na^+-K^+ ATPase), okadaic acid (an inhibitor of certain classes of protein phosphatases), as well as ciguatoxin and brevetoxin (both hypersensitize the VGSC). Domoic acid is another marine toxin that is a cyclized analogue of L-glutamate and stimulates mammalian glutamate-activated ion channels causing abnormal neuroexcitation. These toxins are widespread throughout the marine ecosystem, often originating in dinoflagellates and diatoms and being sequestered up through the food chain, sometimes into species which people eat where they sicken the unwary diner and, in some cases, kill them (Llewellyn 2001, 2009). How does one use a chemical as a weapon? After obtaining the toxin, it can be delivered to victims via food, water, or air. More directly it may be by using a penetrating device such as a syringe or some other traumatizing device such as a dart or other structure that damages the skin and allows the toxins to enter the bloodstream to then be delivered to its site of action. This limits the use of the weapons to a stochastic relationship between amount of toxin and how many victims can be attacked. This is different to a biological weapon where infection in the victim can increase the production of the weapon (i.e., the toxin) within the

victim who also acts as an incubator to produce more of the disease-producing biological agent, or its toxin, eventually overcoming the host, but in the process, it may be spread to other host individuals to then wreak their damage once again.

This chapter will describe the darkest side of these natural toxins where they have been intentionally used to cause harm, rather than by accidental intoxication. Intentional harm can be brought about by administration to another person or persons or to oneself. Suicide by STX or TTX is little known, although there may be unknown and unreported instances where persons have knowingly eaten toxic seafood. Homicide, or attempted homicide, by administering TTX or STX has occasionally been reported. As mentioned previously, STX has received greater attention with respect to weaponry because of its declaration as a schedule 1 chemical weapon, and TTX has been blamed as the agent used by Caribbean witch doctors to create zombies (Davis 1985). An aspect little considered has been their potential as economic weapons with naturally caused STX intoxication events sometimes creating many millions of dollars of damage and from which some communities may take months, if not years, to recover. It is important to know how to diagnose such instances and what responses or treatments may be available to counteract their effects.

Suicide

Suicide by toxin ingestion or injection is relatively unknown for STX or TTX. The only reported suicide was a case reported during an epidemiological study in Brazil (Silva et al. 2010). In an investigation of a saxitoxin-caused fatality in East Timor, authorities were told that a group of crabs renowned to science for bearing both STX and TTX in the flesh were used to commit suicide (Llewellyn et al. 2002). No evidence beyond these stories was obtained, but if true, it clearly indicates community knowledge of the lethal potency of these toxins and their potential for self-harm.

STX was anecdotally reported as one of the toxins of choice for suicide pills with the CIA reportedly harvesting many tonnes of toxic clams during the 1960s for STX extraction, so it might be manufactured into pills or other devices for agents and spies to administer when captured. The most infamous such instance involved the pilot of the Lockheed U-2 spy plane, Gary Powers, which was shot down over Russia in 1960. Powers apparently possessed a silver dollar bearing a needle coated with STX, but as history shows, Powers did not use the device after capture. The potential utility of STX in this manner points to its stability over long periods of time while exposed to air, a property that contributed to its perceived potential as a chemical weapon for the military.

Murder

There have been no confirmed reports of STX being used in a homicide; however, TTX (Ohno 2006) was reported as one of the agents coadministered with aconitine to the victim. In this case, a woman died suddenly while traveling, but her spouse

was suspected of the murder because of his recent purchase of a very substantial life insurance policy. Initially, aconitine was believed to be the sole agent of her death which weakened the case against the suspect because the victim and her husband were physically separated at the time of her death and during the time it would take for the aconitine dose to cause her death. Later forensic analysis revealed that TTX was also in the victim's blood and tissues, and pharmacokinetic studies revealed that TTX delayed the toxic effect of aconitine adding to the time window within which the husband could have dosed his wife, enabling him to be well distant from his wife by the time the toxins achieved their purpose and strengthening his alibi (Ohno 1998). The outcome of the case against the husband is unknown, but if this were indeed how the murder was committed, it demonstrated a very sophisticated understanding of toxin and drug pharmacokinetics by the perpetrator.

An unconfirmed case of TTX murder occurred in 2009, with a former British military officer dying in mysterious circumstances in Sierra Leone. Postmortem analyses discovered traces of TTX in his body but conclusive evidence of his murder by this toxin was not uncovered. Despite the evidentiary absence, the coronial inquest concluded that the most likely cause of death in this case was TTX.

A less successful attempt to murder by using TTX was the case in the late 2000s where Edward Bachner IV was charged in the United States of misrepresenting himself so that he could purchase "at least 64 mg of TTX to defendant's UPS mailbox address" which included one single shipment of 50 mg of the toxin (Kapala 2008). The motive for the scheme was claimed to be his intention to receive the payout from a $20 million life insurance policy he had purchased for his wife. The scheme failed because the supply companies became suspicious of the large amounts of toxin being purchased and the size of some individual orders. Bachner was said to have established false companies in an attempt to legitimize his purchases and hide the fact that toxin was being bought by an individual and not as a business enterprise intending to use the toxin for valid purposes.

Military Use

STX is listed in schedule 1 of the Chemical Weapons Convention (CWC) (Anonymous 1993). Under this convention, some characteristics that define a chemical as a weapon are as follows:

- Been developed, produced, stockpiled, or used as a chemical weapon.
- Has high potential for use in activities prohibited under the convention because of one or more of the following:
- It possesses a chemical structure closely related to that of other toxic chemicals listed in schedule 1 and has, or can be expected to have, comparable properties.
- It possesses such lethal or incapacitating toxicity that would enable it to be used as a chemical weapon.
- It has no purposes other than as a chemical weapon.

The chemical synthesis of STX in 1977 (Tanino et al. 1977) raised the possibility that it may now be manufactured, at least in a laboratory, and along with its high potency attracted the attention of those negotiating the CWC. Furthermore, there had been a series of political events that had revealed the existence of a stockpile of STX held by the US Central Intelligence Agency (Regis 1999). In 1969, the then US President Richard Nixon announced that the "USA was renouncing the use of any form of deadly biological weapon that either kills or incapacitates" and later extended his bioweapons ban to include toxins (Tucker 2002). Approximately 10 g of STX had been produced by the CIA, a supply which survived Nixon's chemical and bioweapon destruction order. Later, it was rediscovered in a storage facility in 1975, triggering a US Senate investigation in the late 1970s, resulting in it eventually being made available for research.

Why is there interest in chemical weapons? One reason is cost. In 1969, the cost of generating a casualty per square kilometer was estimated to be US$2000 for conventional warfare, US$800 for nuclear warfare, and US$600 for chemical warfare (Danzig 1996). These all paled in comparison to biological warfare however which in the same analysis was estimated to only cost $1 per casualty per square kilometer. Times have changed of course since 1969 and these costs are no longer meaningful but it does give an insight into the motivation for military research and development of the day.

To understand another reason, unpleasant as it may seem, it is also important to consider the theory behind chemical weapons. Killing simply removes one soldier, or in some cases civilians, from the battle, whereas an injury not only prevents the victim from participating in the battle any further, if it is a soldier, but also removes others in the battle unit that help the victim; places demands upon medical aides, nurses, and doctors; occupies hospital beds; consumes drugs and other forms of treatments; and so potentially overwhelms scarce medical and evacuation assets. Only a few soldiers need to be victims to render their whole unit or squad dysfunctional by the requirements of the support needed for the injured or incapacitated personnel. In a civilian population, the potential for civil disruption can be profound simply from the fear that may be caused by only the threat of deploying chemical and biological weapons, and their actual use may lead to widespread panic.

What are the consequences of a chemical being recognized as a chemical weapon, particularly schedule 1 chemicals? This particular group of chemicals can only be shipped between signatories to the CWC after having received a permit. After an export, they cannot then be reexported to another country. This step limits the ability of suspect organizations or governments from obtaining these chemicals, or their precursors for the manufacture of the chemical weapon itself, by complicated pathways as a means of obscuring their activities. Countries that are signatories to the CWC also have to ratify the convention by enacting their own legislation to reflect provisions within the convention. This means that for STX, you need a permit from the governments of the country of origin and receipt, as well as one from the Organisation for the Prohibition of Chemical Weapons and the United Nations organization which administers the CWC. This had a negative impact on neurological and toxinological research for a period after the enactment

of the CWC because it limited the ability of researchers to obtain tritiated STX. The then supplier of this reagent sourced their STX from another country, which was then tritiated prior to its sale for medical and research purposes. The sale of the tritiated analogue was deemed a reexport after the initial purchase, and so sales of this research reagent were effectively stopped. Through the efforts of many, this hurdle has been overcome, partly by recruiting companies with radiolabeling facilities colocated in the same county as the means of toxin supply removing the initial international transfer. This has made the radiolabeled STX available for research as well as for use in bioassays for monitoring toxicity (Llewellyn et al. 2001; Robertson et al. 2006; Van Dolah et al. 2012) to prevent toxic seafood from entering the supply chain.

Incapacitation

Death may not always be the intended outcome, but rather incapacitation. For instance, both STX and TTX cause muscular paralysis which may give the appearance of death or disable the person from performing any action or function. Incapacitation, rather than death, was claimed to be the intention of Caribbean witch doctors who included pufferfish livers, presumably including TTX, in powders used to create zombies (Davis 1985). The powders also apparently contained shards of glass to cut the feet of intended victims as they walked across the powder which had been spread across the ground, with the toxin then entering the bloodstream via these cuts. The toxin would cause a total, but nonfatal, whole-body paralysis from which the victim would recover, thereby giving the witch doctor great power. This claim by ethnobotanist Wade Davis in his book "The Serpent and the Rainbow" (Davis 1985) was controversial with the analysis of zombie powders by toxinologists not finding TTX or only trace amounts of the toxin (Kao and Yasumoto 1990) leading to a debate about whether an effective dose could ever be delivered to a victim.

The knowledge that the livers could be used to potentially affect someone such that the individual presented as dead but was able to recover is an interesting aspect in and of itself with respect to ethnomedicine or traditional knowledge (Albuquerque et al. 2012). Was there some traditional knowledge or experiences that formed the basis for the formulation of these powders based on the idea that it could be used as a social persuader to reinforce or substantiate the zombie legend and maintain an individual's cultural power.

Economic Attack

Humans may not be directly attacked by these chemicals but indirectly through the intentional death of agricultural livestock or companion animals with the former having an economic consequence. In 1991, a blue-green algal bloom along the Murray-Darling River in Australia caused the death of numerous sheep and cattle

(Bowling 1992; Negri et al. 1995), an event estimated to have cost the farming communities along that river many millions of dollars. While these were accidental deaths, this shows how contamination of water supplies may affect others apart from humans and still have a significant consequence. In this instance, the indirect costs were quite extensive where, in addition to the direct cost of losing valuable livestock and having to then replace them, communities had to purchase clean drinking water, tourism-generated income dramatically decreased, and if lost livestock were valuable breeding stock, this impacted upon farm-breeding programs. Removal of free toxin could counteract such an event and several strategies have been pursued to identify cost-effective methods, such as charged clays (Burns et al. 2009), for detoxifying water bodies, primarily for keeping drinking water safe after blue-green algal blooms, but these approaches could of course be applied to water bodies toxified by other means. Such tools might be maintained in storage until required in the event of premeditated poisoning of water supplies as well as toxification through blooms.

Toxin Potency

Nature has designed extraordinarily effective toxins in both STX and TTX. As little as 1 nmol/kg is enough to kill some animals when it is administered directly into their bloodstream (Cheymol and Toan 1969; Xu et al. 2003). Both have similar toxicities via intraperitoneal injection into mice of only 8–11 μg kg^{-1} (Wiberg and Stephenson 1960; Xu et al. 2003). Their toxicities via oral administration are several hundredfold less potent than either of these two routes of administration, but they remain in the range of hundreds of micrograms per kilogram.

Interestingly, while more known for its toxicity by ingestion, STX is significantly more toxic to mice by inhalation than other routes of administration having an LD_{50} of only 2 μg kg^{-1} (Franz 1997). The toxin also elicits its toxicity far more quickly when administered as an aerosol, often causing death within minutes of exposure, presumably because uptake into the bloodstream via the pulmonary system is rapid.

Toxin Procurement

To be used for nefarious means, one has to be able to produce or obtain the toxin in sufficient quantities prior to administering the toxin to the intended victims. There are three main methods for producing a chemical: extraction from a natural source, chemical synthesis, and biosynthesis.

Harvesting from Nature

Probably the most feasible method of obtaining sufficient toxin for weaponry is to harvest from nature. This was the method reputedly used in the 1960s by the CIA to

produce a stockpile of STX (see earlier) where molluskan shellfish made toxic by consumption of toxic algal blooms were collected and extracted and the toxin then purified. Pufferfish, which produce TTX, are farmed to supply various markets and they too could be a means of producing large amounts of toxin from a concentrated supply of toxic animals. Farmed pufferfish however are often much less toxic than their wild relatives and so many more fish would be required to produce a significant amount of toxin compared to what might be generated from wild-caught fish.

Another source of naturally occurring high levels of STX or TTX are xanthid crabs, with two species *Atergatis floridus* and *Zosimus aeneus* almost always highly toxic with STX or its chemical relatives and at times TTX (Llewellyn et al. 2006), sometimes containing many milligrams of these toxins per individual. These tropical and subtropical crabs are relatively easy to collect from coral reefs and, like pufferfish, provide a relatively simple means of toxin acquisition.

While we have referred to both TTX and STX as marine toxins, in truth they can be found outside the sea. TTX is also present in the skin of several species of amphibians, including salamanders, newts, and frogs (Hanifin 2010), and STX is produced by freshwater cyanobacteria (Carmichael et al. 1997). These sources add to the potential natural supplies of these toxins.

Chemical Production

Both STX and TTX present enticing challenges to the synthetic chemist. With the end result being a toxin that has proved valuable as a research tool for many decades, numerous attempts to chemically synthesize the toxins have been reported. The first chemical synthesis of STX was reported in 1977, requiring almost 20 steps and having a final yield of only 0.25 % of the starting material (Tanino et al. 1977). A more efficient synthesis requiring less chromatography followed (Jacobi et al. 1984), but in both of these syntheses, the STX analogue decarbamoyl STX was an intermediate which is then converted to STX in the final step. This approach of synthesizing one of the many bioactive analogues before conversion to the parent STX is now the most common strategy adopted (Iwamoto and Nagasawa 2010; Sawayama and Nishikawa 2011), but it is worth noting that many of these analogues can be as toxic as STX (Llewellyn 2006). The syntheses are becoming more efficient with recent syntheses having increasingly fewer steps (Bhonde and Looper 2011). Methods to chemically synthesize TTX are now quite numerous (Chau and Ciufolini 2011), but of interest is that some start with readily available and cheap chemicals like glucose (Sato et al. 2008) and levoglucosenone (Urabe et al. 2006) reducing the overall cost of their production.

Biological Production

Probably the preferred means of production by an unscrupulous person or persons would be biosynthesis by bacterial or other microbiological vectors. These vectors

may be naturally occurring producers of the toxins or ones that have been transformed with the biological machinery to manufacture the toxins. STX has long been known to be produced by dinoflagellate cultures (Proctor et al. 1975) and later by cyanobacteria (Carmichael et al. 1997), and these natural vectors are used to produce these toxins for use as calibration and reference standards (Watanabe et al. 2011).

Identification of bacteria resident, and potentially symbiotic, in saxitoxic dinoflagellates inspired the notion that bacteria might produce STX and its chemical relatives (Silva 1990). A group of sulfated analogues of STX were identified in trace amounts in bacteria purified from the cytoplasm of a highly toxic strain of the dinoflagellate *Alexandrium tamarense* (Kodama et al. 1990). Reports of STX production by bacteria have long been a topic of debate (Gallacher and Smith 1999), because absolute structural confirmation of the toxins is absent. Some bacterial fluorescent products that behave like STX peaks during HPLC analysis (Baker et al. 2003) have been proposed as being the chemicals previously, and incorrectly, identified as STX analogues being produced by bacteria. There have also been numerous reports of bacterial production of TTX (Maran et al. 2007; Campbell et al. 2009; Yu et al. 2011), but like with STX, there is an alternative view that the evidence is not yet absolute (Matsumura 1995b; Lehman et al. 2004) leaving bacterial biosynthesis of TTX an open question (Chau et al. 2011).

A complex gene cluster has been assigned as the enzymatic pathway that produces STX (Kellmann et al. 2008), and these clusters have now been found in toxic cyanobacteria species (Mihali et al. 2011). The complexity of this genetic machinery would once have been seen as a roadblock to producing toxins by transformation and introduction of the metabolic potential for manufacturing these toxins into a microbiological vector. However, transforming microbial vectors more amenable to large culture for production of complex metabolites is a growing field and has been successfully used to produce complex marine natural products in nonindigenous microorganism (Ongley et al. 2013).

Detection and Diagnosis

Detecting STX is a standard practice in the seafood industry to mitigate the risk of toxic product entering the market and sickening consumers or possibly killing them. The best form of detection is foreknowledge of potential toxicity and this is the more common mechanism for TTX where species, such as pufferfish, are well known to be toxic. STX in food species, such as oysters, clams, and mussels, is usually caused by their consumption of toxic microalgae, and so the presence and abundance of the source toxic organism in the waters where these mollusks live is used as an indicator for potential toxicity, but this is often married with sampling flesh samples and testing for toxicity. However, this is not a situation where there is an intention to cause harm and it is a monitoring tool to prevent accidental poisoning, and screening, either by chemical or bioanalytical means, is unlikely to assist in cases of intentional human poisoning.

There are few reports detailing the identification of TTX or STX toxins from victims of fatal poisonings, and a variety of methods have been used ranging from bioassays (Tsunenari et al. 1980; Llewellyn et al. 2002) to sophisticated chemical analyses that can deal with the complexity of human body fluids (Moriya et al. 1992; Cho et al. 2012). Often, tissue extracts or bodily fluids need to be pretreated by methods that remove many of the chemicals and biochemicals in humans that may interfere with these analytical methods. Pathological indicators specific to these toxins are few, but some have noted that victims display "purplish mouth and nails" with some "dilatation of stomach and intestines" (Chen et al. 1999).

In victims that have not yet succumbed to the toxin's lethality, it will depend upon persons around the victim to realize that the victim has been poisoned and to then correctly diagnose what the nature of the poison might be. People that accidentally eat TTX or STX can begin to sense the effects of the poison within 5–30 min after ingestion, initially experiencing a slight tingling in their digits progressing to numbness around the mouth, neck, and face. In a severe case, victims exhibit incoordination and respiratory difficulty, have trouble swallowing, and experience a sense of throat constriction, incoherent speech, headaches, dizziness, nausea, possibly vomiting, and reduced eye pupil size. Complete paralysis may result within 2–12 h of the poisoning event with death resulting from respiratory failure. If after 12 h death has not occurred, patients often start to recover gradually and have no residual symptoms a few days after the event. If however the toxin is administered as an aerosol or intravenously, routes by which the toxins are more toxic, this progression of symptoms can be quite rapid, and unless treatment starts almost immediately, victims may not survive very long.

Many of these symptoms can also be confused with allergies and other diseases or illnesses which may result in the victim or others misdiagnosing what they are experiencing, leading to inappropriate treatments or delays to the victims receiving any treatment at all which may prove fatal.

Treatment

If the victim is correctly diagnosed and able to be treated, what options are there? Only two possibilities exist: treating the victim to nullify the effects of the toxin or providing symptomatic relief during the acute phase of the poisoning until the victim's natural recuperative abilities can overcome the effects of STX or TTX.

Symptomatic Treatment

Victims of severe intoxication will need artificial respiratory support and benzedrine can be effective in aiding artificial ventilation (Kao 1993). Artificial respiration may need to be provided for some hours before the effects of a lethal dose begin to wane. In quite severe cases, the victim may expire by cardiac arrest and

respiratory support is not sufficient. Victims of oral intoxication should have gastric lavage as soon as possible to prevent further absorption of toxin from the gut contents. Activated charcoal can also be administered in an attempt to bind any freely available toxin within the stomach contents. Activated charcoal is known to bind these toxins and is often used as a cleanup step in the early stages of STX purification. Both STX and TTX possess guanidinium groups (Fig. 1) essential to their toxicity (Llewellyn 2009), and when these guanidinium groups are charged, they can also be bound by the activated charcoal. Noting that the stomach and intestines are of different pH, this will affect the charge state of the toxins and the efficacy of the activated charcoal, and so the efficiency of toxin binding to the activated charcoal will be different in progressive compartments of the human gut. But if the toxin has been introduced to the victim via other mechanisms such as injection or inhalation, other methods would have to be adopted to treat a victim, such as an antitoxin, because the effects can be so rapid and the toxin molecules have bypassed the gut and entered the bloodstream where they can gain access to its pharmacological target.

Antitoxins

Chelating STX or TTX with antibodies produced to the either STX (Davio 1985; Kaufman et al. 1991; Benton et al. 1994) or TTX (Matsumura 1995a; Rivera et al. 1995; Xu et al. 2005) is a possible mechanism to prevent STX or TTX intoxication. Antibody production requires the toxins to be conjugated to a carrier protein, creating an epitope on the carrier protein against which the antibodies will be generated. Several anti-STX antibodies have been demonstrated to protect experimental animals exposed to the toxin (Benton et al. 1994; Davio 1985), but these antibodies are often quite specific and do not bind other STX relatives, or if they do, it is usually with much lower affinity. This will be a problem because the affinity of STX and TTX for the voltage-gated sodium channel is in the nanomolar range and will likely outcompete the antibodies for the toxins. This would require any antibodies to be administered in very large doses to compete for the toxin by weight of numbers rather than by a stronger affinity. The ability of antibodies to be effective after a lengthy delay from the time of intoxication is also likely to be a hurdle to successful application of an antibody as an antitoxin. Once the administered toxin has translocated within the victim to where it can exert its maximum toxicity, any antitoxin would have to be administered in such a way that it can get to as much of the toxin dose as possible before it can take full effect. This approach will have a better chance of success in cases where the route of toxin administration is such that the onset of intoxication is slow, such as via a meal or some other oral dosing. Intravenous or pulmonary administration will be unlikely to provide a window where such interventions would be successful, unless they were administered within minutes of intoxication.

Alternative binding proteins to antibodies that have potential as antitoxins are the saxiphilins (Morabito and Moczydlowski 1994) and a family of TTX- and

STX-binding proteins found in pufferfish (Yotsu-Yamashita et al. 2010). Both of these groups of toxin-binding proteins are circulatory proteins more likely to tolerate the environment found in the bloodstream and remain effective as toxin chelators. They also bind STX and TTX in the nanomolar and subnanomolar ranges, significantly more tightly than the aforementioned antibodies, meaning they may effectively compete for the toxins against the voltage-gated sodium channel.

An STX antidote might be created from a chemical which displaces STX or TTX from their binding site on the voltage-gated sodium channel, but it would have to not also inhibit sodium ion flow through the sodium channel like STX and TTX do and allow the excitable tissues to function correctly. This may be more likely to be achieved if the displacer were an allosteric inhibitor that prevents toxin binding but from a site on the sodium channel distant from that of STX or TTX. Other pharmaceuticals may however be useful in counteracting the effects of STX or TTX. The developmental drug 4-aminopyridine has been found in animal studies to protect, if not counteract, the effects of both STX and TTX (Benton et al. 1998), reversing the loss of blood pressure in experimental animals and enhancing neuromuscular transmission to allow their diaphragm to work. Large doses were required in these experiments, enough to potentially cause serious side effects, which could be ameliorated if the treatment occurs in a hospital, eventually saving the victim's life.

Given that the effects of STX or TTX can be experienced within 30 min of a meal, but even more quickly if the toxins are given intravenously or by inhalation, unless diagnosis is immediate and the treatment given quickly and by a rapid acting pharmacological route, they are unlikely to be effective as the toxin will be well on its way to achieving its purpose. Pharmacological treatment is more likely to be useful in addition to other supportive measures that will allow the patient to survive the critical window of lethality and can last up to 12 h from intoxication.

Conclusion and Future Directions

While potentially titillating to some, but more likely depressing to most, that the previous words might be when one considers the negative side of toxinology, the reality is that the greatest risk to human life that STX and TTX pose is via accidental consumption of toxic food. In truth, these toxins have been used to intentionally harm more people in fictional television shows, movies, and books than in real life. Such television shows include Columbo which portrayed a murder where the perpetrator injected TTX into a bottle of wine by injection via the cork and the Australian-produced television series "Sea Patrol" where the villains were planning to use poisonous crabs as a source of toxin to contaminate a city's water supply.

While gruesome, this topic does raise a more benevolent side in that treatment for victims of intentional intoxication is equally beneficial to accidental victims. The challenges for these treatments are to be able to commence treatment as soon as

practicable after realization of STX or TTX intoxication. One of the hurdles to effective treatment is that the sooner medical intervention can commence, the greater is the likelihood of survival. If diagnosis is in a location far from primary healthcare facilities, then the victim will depend upon first aid treatment which is independent of pharmaceutical intervention or elaborate medical procedures. Manually applied artificial respiration is the obvious solution and this may allow the victim to be kept alive until they reach a facility where drug intervention and/or mechanical artificial respiration is possible. This approach is much like the preferred method for snake envenomation where pressure bandages and immobilization are used to inhibit snake venom spreading far beyond the bite location until the victim can be delivered to a hospital for treatment in an acute treatment room. Better first aid options, as well as diagnostic tools, are worthy of further attention by the medical fraternity and first responders who are the personnel who will have to deliver a practical solution to a victim in the field. Hospital-based treatment should not be ignored, and antitoxins, either antibody-based or other binding proteins, should be investigated, and pharmaceuticals that may increase the speed by which toxin is eliminated (e.g., diuretics which may increase urinary elimination) would reduce the length of the window where victims are exposed to acute toxicity, thereby reducing the risk that the patient may expire while their respiration is supported.

It should not be forgotten that like many other toxins, if we can harness their pharmacological effects without their toxicity, they may be effective pharmaceuticals. Both STX and TTX have been investigated as anesthetic leads, able to stop neural function without irreversibly damaging the nerve in the process (Llewellyn 2006) or being added to other anesthetics to supplement their action allowing lower doses of the more mainstream anesthetics to be administered for the same outcome (Barnet et al. 2004). Rather than attempting to design a nontoxic but bioactive chemical relative, strategies have been adopted such as applying it to a pharmacological compartment, such as the eye, that prevents its broader uptake into the body (Duncan et al. 2001) or encapsulating the toxins in controlled-release formulations (Martinov and Nja 2005). Metastatic breast cancer cells were discovered to overexpress TTX-sensitive sodium channels which enabled greater cellular motility and invasion. TTX inhibited these channels and consequently cellular metastasis, albeit in experiments rather than an in vivo model (Fraser et al. 2005).

Why someone with evil intent would go to the lengths of acquiring these toxins commercially or manufacturing them via complicated protocols rather than just turning to nature itself is a mystery. These toxins are a natural part of the environment and can occur in very large amounts within animals relatively easy to collect from accessible environments. It is this fact which often bemuses those in the marine toxinology field when faced by the regulations intended to prevent the use of these toxins as a chemical weapon. Their stability, potency, and lack of need to purify STX or TTX for them to be effective provide a pathway to stockpile enough toxin to be a threat to many people, allowing criminally minded persons to easily bypass these regulatory hurdles. It is probably the insights of others into the behaviors and mindsets of these persons who may pursue these easier pathways

to arming themselves with these chemical weapons that will probably prevent their use to harm others. This should not detract from efforts that should be directed to treating potential casualties of these toxins, especially as close to the scene of the intoxication as possible, because progress on this front will also assist victims of accidental poisonings of which there are many each and every year.

Cross-References

▶ The Public Health Response to Potential Bioterrorism by Toxin Attack

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Yesterday, Today, and Tomorrow: A Selective View of Toxins in Weapons and Medicine

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Abstract

A look at the past and a look at the future. What is the history of toxins used as weapons and where is the danger in the future?

Before 2001, there was recognition that biological weapons, including toxins, were weapons that required state sponsorship. But that is no longer true. As the asymmetries of weapons and politics have changed, so has the probable use of toxins as weapons. Our understanding and expectation of likely use of toxins in weapons has changed. Even the word toxin is commonly used inappropriately to mean toxic chemical rather than a chemical produced by organisms. The possibility of toxin use in weapons was evaluated on whether the toxin could be manufactured or purified from natural resources. The more complicated the toxin's structure, the less likely it was to be purified or created using laboratory synthesis. Many infectious disease specialists regard toxins as the eventual cause of the destruction of cells in most bacterial infections.

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In the spectrum of chemical and biological weapons, from the simple chemical structures of mustard and sarin to the complicated toxins, such as palytoxin, where do toxin weapons fit? Does the use of toxins as a weapon imply the use of biological weapons or, if the toxin is laboratory synthesized and purified is it a chemical weapon? Do toxin weapons need to have purified chemicals? Are toxins the ultimate dual use agents? Do toxinologists need to examine their research and publications for the potential of possible illicit use? Do restrictions on research and publication of biologically based materials, including toxins, really impede the development of weapons? How can these restrictions be implemented to guarantee security concerns?

Introduction

Prior to 2001, most people believed the manufacture and use of toxins and biological weapons required state sponsorship because of the technology and equipment to prepare and purify the agents. That is no longer true. Now it is generally recognized that agents do not need to be pure and they certainly can be prepared from a variety of sources using many different methods, including those that have been developed from new and evolving technologies.

The use of asymmetric weapons has enabled small groups and even individuals to threaten large countries. Biological weapons and toxins can be used to make political statements as well as cause inordinate amounts of defensive or protective measures. The expectation of the use of toxins in weapons has changed; a little bit of agent can go along way and have a considerable influence on politics. The media and politicians have made the use of the term toxin so common as to even dilute its meaning; now the word toxin is commonly used to be a toxic chemical rather than a chemical produced by an organism.

A toxin is defined in the Merriam-Webster Dictionary as

> a poisonous substance that is a specific product of the metabolic activities of a living organism and is usually very unstable, notably toxic when introduced into the tissues, and typically capable of inducing antibody formation. (Merriam-Webster)

Prior to the new developments in genetic engineering and protein synthesis, the possibility of using a toxin in weapons was evaluated on whether the toxin could be manufactured or purified using natural materials. In today's world, the laboratory synthesis of toxins is commonplace and not necessarily more complicated by the structure of the toxin.

Concerns about the use of chemical and biologically-based weapons have taken on new urgency and significance as politics and dwindling resources have pushed more societies into the use of asymmetrical warfare. In many ways both chemical and biologically-based weapons are the perfect instrument for smaller entities (individuals or groups) to either defend or attack themselves. In efforts to defend and preserve life, many organisms have evolved to make toxic chemicals, toxins,

and ways to disperse them for use against predators or to disable their prey to gain an advantage.

> In their struggle for life some animals grew fangs, others claws or tusks, while still others produced poisons. – Szent-Gyorgyi, Albert (M.D., Ph.D., Nobel Laureate for Medicine), The Crazy Ape, The Universal Library 1970

Humans have used these toxins developed by other organisms, e.g., plants, jellyfish, insects, or even mammals, for thousands of years, and humans have become quite adept in their use both for weapons and for medicines. At each new stage of technology, scientists have needed to evaluate the foreseeable uses of that technology, whether for good or bad. In many cases, the use of technology against society is an unintended or unforeseen consequence. The world is again at a stage for evaluations and this article encourages scientists and politicians to pause to think of the future and the role of counterbalances to regulate or curb further possible illicit and destructive use of toxins. The history of toxin studies and many of the facets of toxins are well presented in various chapters in this Handbook of Toxinology. Biological Toxins and Bioterrorism.

A Selective History

History abounds with examples of toxins used as weapons, and in most cases, the organism producing the toxin is included so that the history of toxins is also the history of biological warfare (see for example Globalsecurity.org and Christopher 1997). Humans have sought ways to gain advantages over their enemies for centuries, including using biological weapons as direct as hurling live poisonous snakes over walls and dumping dead and decaying cows and horses in wells and rivers. More sophisticated uses of biological weapons included dipping conventional weapons in material known to convey diseases (e.g., feces from populations infected with cholera). Some cultures used snake venom, venom from frogs, and even toxins from sea weed to make their swords, spears and arrows more effective. A selected list of toxins and venoms, which are believed to have either been used as weapons, including hunting, sabotage, assignations, etc., or could be used is presented in Table 1. These toxins include some of those naturally occurring naturally in plants (Magnuson (1997)).

Simplistically, toxins are proteins produced by animals, plants and bacteria and venoms are usually a mixture of toxins (Globalsecurity.org). Most readers already know that toxins are produced directly by organisms or indirectly, meaning they are metabolic side products, whose production is often increased in response to environmental conditions. The discussion of whether toxins are chemical or biological agents colors the history of toxins as weapons and the perception of their use.

In 1975, the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction (1972), otherwise known as the BTWC, entered into force (BTWC). It was an improvement on the 1925 Geneva Protocol, which prohibits the use of chemical and biological weapons in war. According to the US Code,

Table 1 ***Some possible bacterial, fungal, plant and animal toxins in weapons***. This table contains some toxins that have historically been used in weapons, including hunting. The list also contains some toxins considered to be possible to use in weapons. The list is not a complete list of all toxins. When possible the type of chemical, the active biochemical site and possible biological source, plant, fungi, bacteria, and animal are noted

Toxin	Type of chemical	Active site	Biological source
Ricin	Carbohydrate-binding protein	Inhibits ribosomal protein synthesis	Castor oil plant, *Ricinus communis*
Saxitoxin (paralytic shellfish toxin)	Nonprotein toxin	Neurotoxic alkaloids block Na and K channels in nerve cells	Marine dinoflagellates
Maitotoxin, palytoxin, ciguatoxin,	Nonprotein toxin	Disrupt ion channels	*Gambierdiscus toxicus*, dinoflagellate
Tetrodotoxin	Nonprotein toxin, aminoperhydroquanizole	Blocks Na channel	Bacteria, *Pseudoalteromonas tetraodonis*
Botulinum toxins	Single polypeptide chains	Blocks release of acetylcholine	*Clostridium botulinum*
Clostridium perfringens toxins	Proteins	Toxins specific to intestinal cells	*Clostridium perfringens*
Staphylococcal enterotoxin B	Protein	Toxin specific to intestinal cells	*Staphylococcus aureus*
Anthrax toxins	Protein	Disrupt cellular signalling	*Bacillus anthracis*
Plague toxins	Protein	Disrupt immune cells	*Yersinia pestis*
Aflatoxin	Difuranocoumarins	DNA damage, carcinogenic and protein inhibition	Fungi, e.g., *Aspergillus flavus*
Tricothecene	Fused ring compounds	RNA damage	Fungi, e.g., *Fusarium* and *Stachybotrys*
Snake venom	Proteins and polypeptides	Disrupt various biochemical processes; cytotoxins and neurotoxins	Many snake varieties, including cobras, rattle snakes, vipers, and sea snakes
Scorpion venom	Proteins, peptides,	Neurotoxins, channel blockers, enzyme inhibitors, etc.	Arthropods, including Brazilian scorpions, Emperor scorpions, Deathstalker scorpion
Spider venom	Proteins, peptides, polamines and other substances	Neurotoxic and cytotoxic (necrotic)	Arthropods, including Black widow, brown recluse and

> the term ‘toxin’ means the toxic material or product of plants, animals, microorganisms (including, but not limited to, bacteria, viruses, fungi, rickettsiae or protozoa), or infectious substances, or a recombinant or synthesized molecule, whatever their origin and method of production, and includes – (A) any poisonous substance or biological product that may be engineered as a result of biotechnology produced by a living organism; or (B) any poisonous isomer or biological product, homolog, or derivative of such a substance; (USC 2012)

The BTWC defined toxins as including “(both proteinaceous and non-proteinaceous) of a microbial, animal or vegetable nature and their synthetically produced analogues.” In those intervening years between 1925 and 1972 and 1975, science had advanced so that toxins that were originally considered biological products could also be produced in the laboratory. In 1990, Dr. Graham Pearson described the CBW Spectrum, which illustrated the range of people potentially affected varied from chemical agents used in weapons through bioregulators and toxins to genetically modified biological warfare agents, and thus to the traditional biological warfare agents, such as anthrax and plague (Pearson 1990).

By the time the Chemical Weapons Convention, CWC, entered into force in 1997, discussions sometimes wandered over whether or not toxins purified from biological sources should be considered toxins or a chemicals, or were chemicals only those produced synthetically in the laboratory (CWC) With advances in genetic engineering and cell cultures, such discrimination is pointless from a technology viewpoint but still perhaps useful from a political perspective and as a negotiation tool.

Toxins in Nature

Types of Toxins

Most toxins are proteins and their toxicity depends on disrupting one or more biochemical pathways necessary to metabolism. Toxins are very selective about the biochemical pathways they disrupt. This selectivity also means only a very small amount of toxin needs to be present, but it must be delivered to the point where the disruption can occur. This usually means the toxins must be delivered safely to the bloodstream and preferably avoid the digestion systems unless they are stable.

Most proteins are not very stable; they react with water, oxygen, light, and heat. They also react to changes in pH and with other chemicals. The effectiveness of toxins in nature depends on their delivery. Organisms using toxins for defense may inject toxins under the skin or in the blood system with teeth or barbs. They may rely on spraying the toxin in a concentrated liquid into eyes or other mucosal tissues so that the material can be easily transferred to the bloodstream. Many toxins are destroyed during digestion or diluted or reacted in water.

Animal toxins include neurotoxins, toxins that interrupt the transmittal of nerve signals, cardiotoxins and cytotoxins that act on membrane lipids/proteins of blood cells or heart cells or produce severe arrhythmia of heartbeat, myotoxins that act on

muscle cells, hemotoxins that affect the coagulation properties of blood, and vasoactive toxins that affect blood pressure.

The list of plant toxins is large and includes abrin, ricin, hemlock alkaloids, lectins, urushiols, cyanogenic glycosides, digitoxins, and fungal toxins (mycotoxins, tricothecenes, aflatoxin, etc.). Reviews in this Handbook include abrin (an toxic protein derived from the seeds of the plant Abrus precatorius), aflatoxin (mycotoxins that are produced by Aspergillus flavus), mycotoxins in general and ricin.

Bacterial toxins include exotoxins, secreted by the bacteria, and endotoxins, those parts of the cell structure included in the cell envelope. Most bacterial toxins are exotoxins, e.g., botulinum, shiga, tetanus and diphtheria toxins. Endotoxins are defined as the lipopolysaccharide (LPS) complex associated with the outer membrane of Gram-negative pathogens such as Escherichia coli. The LPS complex can interact with the immune system of monocytes and macrophages to induce releases of cytokines and eventually produce toxic shock.

Aptamers, single-stranded DNA and RNA proteins originally produced by both plants and animals, but subsequently engineered and grown in vitro, are used to target specific molecules and pathways. Although not necessarily fitting the classic definition of a toxin, these chemicals can be made to be much more stable than the original proteins and still offer specificity and discrimination as targeting drugs (Al-Shamy 2009; Toxin Targeting 2008). The specific targeting and interference with biological pathways conforms to more modern biotechnology definitions of toxins and may also be referred to as bioactive peptides.

Release or Use of Toxins

In nature, the organism generally releases a toxin when it is threatened, incapacitates its prey, or, as an aid in digestion. Scorpions, snakes, and many other animals release the toxins in a barb or sharp tooth that is hollow and delivers the toxin by breaking through the skin or shell of the attacker. Jellyfish use nematocysts, capsules containing toxin and a very small barb (Brinkman 2007; Brinkman and Burnell 2007). Some frogs and amphibians release toxins through their own skin into the skin or mucous membranes (mouth and gastrointestinal tract) of the attackers. As spiders bite into the attacker or prey they release venoms containing toxins as a digestion aid. Many animals use venoms containing a mixture of toxins and additives to help the toxins effectively reach their targets. Some snakes may spit into the eyes or mucous membranes of their attackers. All of these releases involve intimate contact between the organism and its attacker using a mode that breaks through natural defenses of skin and mucosal membranes. With the exception of ingestion, few natural methods of releasing toxins are passive; they require direct contact to be effective.

Bacterial toxins are released after infection by the bacteria. These toxins weaken the bacterial host so that the bacteria can more easily thrive and reproduce (Todar). Mycotoxins released from molds and fungi probably have similar roles to help

breakdown the infected plants, but the releases of mycotoxins are perhaps more complicated and may be triggered by stresses in the environment. Plants and algae that produce toxins have likely evolved to use their toxins for protection. Ingestion of plants and other organisms containing toxins requires the attacker to eat the toxin containing material. Bacillus thuringiensis, Bt, a bacterium found in many soils, produces crystal proteins, Cry toxins, which interfere with the normal gastrointestinal activities of many invertebrates eventually killing them. The attackers learn avoidance; those that do not eat that organism do not get sick, or only those attackers immune to the toxin survive.

The effectiveness of toxins depends upon the biochemical disruption they cause, but a key part of their effectiveness is the delivery to specific target pathways. Without an effective delivery, most toxins will react and decay so that less material reaches the target. Extracting a toxin from an organism and then putting it in water more often than not destroys the toxin. In more military terms, organisms using toxins must be able to weaponize the toxin for effective delivery. Whether for an individual plant or animal, synthesizing the toxin is only the beginning of the creation of a weapon: the extraction of the toxin and the delivery system are crucial components.

The Dual Nature of Toxins in Medicine and as Weapons

As human understanding of biology has grown, the science of toxins has been used in medicine to treat diseases and conditions. Examples of medicinal use of toxins include bacterial, animal and plant toxins. Botulinum and other toxins are well known to cause paralysis, and their specialized use via injections can prevent muscle spasms and smooth wrinkles. Anti-coagulants components of snake venoms can be used to treat and prevent blood clots (Rojnuckarin 2013). In another type of usage, Bt is used as an insecticide to protect plant crops.

Using genetic modifications, scientists are learning to select and modify genes to enable organisms to be less susceptible to toxin disruption. The converse is that the knowledge also can work to identify organisms more susceptible to toxin disruption. Genetic selection can be used to select drugs and biochemical pathways unique to individuals and groups to improve drug delivery and effectiveness. But, the same techniques can be used to improve toxin delivery and effectiveness when toxins are used as weapons.

Advances in science can be used to develop new methods and refine existing methods that deliver toxins to the bloodstreams of both animals and humans. Weaponizing a toxin can use the same methods to develop mass inoculations or quick and easy delivery of medicine. The fastest and most effective route to deliver medicine is often by inhalation; the drug can quickly enter the bloodstream because very thin walls separate the blood capillaries and the alveoli of the lungs. This works well with vaporized and aerosolized materials. Proteins (or vaccines) attached to nanomaterials can be delivered quickly via inhalation and then once in the bloodstream, these same materials can be used to speed toxins to their targets.

Technologies using viruses to deliver chemicals directly into tumors may also be used to target specific organs and bypass protective barriers or components of immune systems (Al-Shamy 2009). What works well in drug delivery, works well in toxin delivery. The same science used for defense and protection can be used to improve weapons.

Historically, using toxins as weapons (or for defenses, depending on perspective) does not need pure chemicals. Using toxins as weapons can be elegant or crude. Putting corpses in wells or upstream of towns or throwing snakes over city walls are crude uses, but effective. The more elegant, purified toxin weapons are really most effective as sabotage or terror weapons because the delivery system can be stealthier than the crude uses.

Toxins are at the nexus of many new techniques in biology, medicine, genetics, immunology, and nanotechnology. Toxinolgy can be thought of as the Janus of biology, a point where the beginning and changes in technologies cause scientists and societies to look back on their histories and look forward to the future. The study of toxinology necessarily looks at both toxicology and weapons.

Ethics Education

What can scientists, technologists and societies use as an aid to planning advances currently and in the future? The study of ethics and its application is often taught as a general philosophy. The application of ethics to the intersection of biology, technology and medicine, bioethics, is required in most medical schools (NIH, Bioethics Resources on the Web). However, ethics and bioethics are fundamental to societies even if it is not a designated course or training. It is also not unique to scientists, technologists, medicine, chemistry, physics or any science (Mazor 2012). A consideration of ethics, the benign or aggressive use of toxins, is more obviously critical to toxinology than many other sciences or perhaps toxinologists are more aware of these issues because of the history of toxins. Dual-use is a part of nature and the evolution of toxins. Most organisms make and use toxin for defense and survival.

An important point in this discussion of history and future of toxins is not who or what makes a toxic chemical, or what can improve a weapon, it is “Are societies imposing limits on the development and research in technology in order to solve political problems?” How are decisions made on which weapons are banned? Who decides which characteristics of the weapons determine such bans? The processes used to discuss these issues are not easy ones and the discussions need to involve both scientists and policy makers. However many times the participants in these discussions do have not the training required to understand the implications of the policies or the science.

It is incumbent on science education programs to include ethics (Green 2013) As in medicine, the creed of “first do no harm” should be the pivotal question when

applying technology or conducting research. It should also be the pivotal question in policy making. Ethics and biomedical ethics are often taught as part of medical training. Indeed most publications in ethics focus on the ethics in medical treatment and clinical practice. There are few programs in ethics for general science.

In the US, review boards evaluate studies proposed by scientists for dual-use potential in research. This is a time-consuming process that gives a false sense of security to those research projects successfully passing the review (US DURC 2012, 2013) In Europe, Dutch authorities recently ruled that publication of research by virologists in an international journal required an “export license” according to EU law and this now applies to 28 countries. Australia has suspended its Defense Department review of research on special pathogens while researchers and law-makers discuss how to modify their reviews so that science is not hindered but security is not deterred (MacKenzie 2013). A more general training in ethics in science, engineering and political studies may help to streamline this type of review. Indeed the need for communication between scientists, technologists, policy makers and lawyers has increased as progress and advances in science and technology have grown. At the same time the gap in understanding between science and non-science has widened.

Conclusion and Future Directions

In modern societies where success often includes rapid applications of new technologies, the recognition of the importance of reviewing the technologies against a set of ethics can be a new concept. Who determines the set of ethics, the guidelines for the reviews, and the process? How can this process be implemented? Is the process really needed? How can the processes be structured so that they do not impede progress?

There are few advances in science or technology that cannot be turned for malevolent use. Szent-Gyorgyi, a Nobel Prize winning anti-war scientist, isolated vitamin C (NLM). Certainly this was peaceful tool used to alleviate malnutrition? Yet, the Nazis used vitamin C to enable their ships to stay at sea longer. This is one example of how scientists cannot control or anticipate illicit or compromising uses of technology and science, even peaceful uses. Would a review board of scientists have considered the isolation and synthesis of vitamin C a potential dual-use area of research?

A review of the history of toxinology, pharmacology and even defense science emphasizes that training in ethics needs to begin early, at least in undergraduate science programs. The importance of ethics is not reserved for medical doctors or graduate programs or even scientists. Engineers and technicians with bachelor degrees are often very capable of applying and changing technology. And everyone in society participates in the use of technology, whether they study science or use results of technology. Ethics training should be for every individual.

Cross-References

▶ Botulinum Toxin: Present Knowledge and Threats
▶ Evolutionary Traits of Toxins
▶ Marine Biotoxins in History: Misuse and Mayhem

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Erratum to Chapter 5: Abrin and Ricin: Understanding Their Toxicity, Diagnosis, and Treatment

Hsiao Ying Chen, Ling Yann Foo, and Weng Keong Loke

Owing to an oversight on the part of the Springer Figure 1 and Figure 2 of this chapter were initially published with errors. The correct presentation is given here.

The online version of the original chapter can be found at http://dx.doi.org/10.1007/978-94-007-5869-8_1

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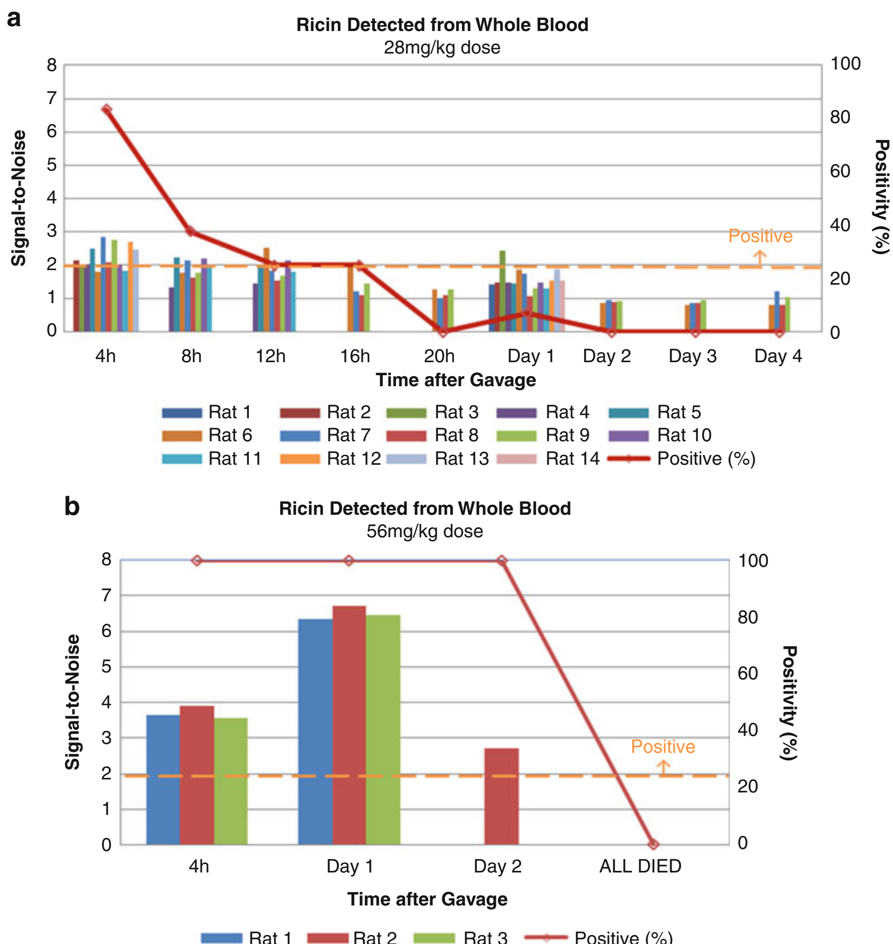

Fig. 1 Figure illustrates the number of whole blood samples gathered from intoxicated rat at various durations post-gavage that provided positive detection response (*right* y-axis) to ricin toxin at a signal-to-noise ratio (*left* y-axis) greater than 2 (S:N > 2). (**a**) Ricin detected in whole blood from intoxicated rats at a morbid ricin dose of 28 mg/kg; (**b**) ricin detected in whole blood from intoxicated rats at a lethal ricin dose of 56 mg/kg. Day 2: two rats died before testing

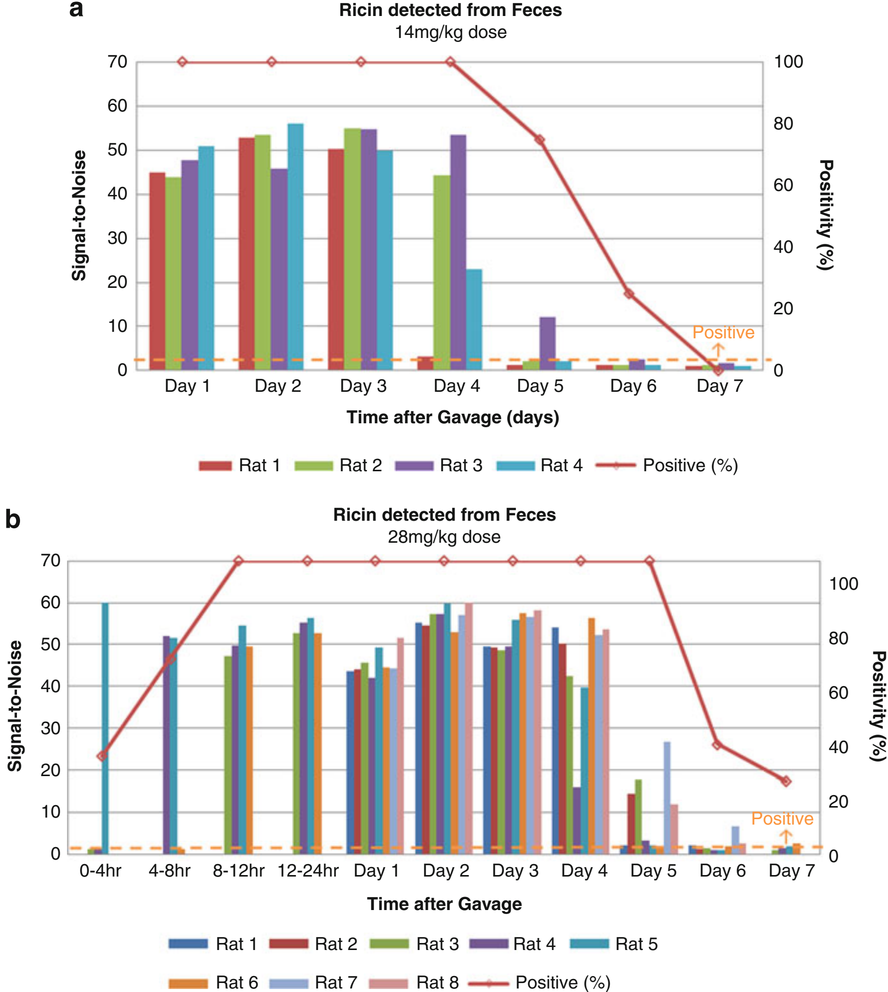

Fig. 2 Figure illustrates the number of fecal samples gathered from intoxicated rat at various durations post-gavage that provided positive detection response (*right* y-axis) to ricin toxin at a signal-to-noise ratio (*left* y-axis) greater than 2 (S:N > 2). (**a**) Ricin detected in feces from intoxicated rats at an asymptomatic ricin dose of 14 mg/kg; (**b**) ricin detected in feces from intoxicated rats at a morbid ricin dose of 28 mg/kg. 0–4 h and 4–8 h: three out of four rats produce feces

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