SUMMARY OF THE
NIAID EXPERT PANEL ON BOTULINUM DIAGNOSTICS

May 23, 2003
Bethesda, Maryland

EXECUTIVE SUMMARY

The highly potent botulinum neurotoxins (BoNTs) expressed by the anaerobic bacterium Clostridium botulinum pose a significant bioterrorism threat, and as such are listed as Category A agents of bioterrorism by the National Institutes for Allergy and Infectious Diseases (NIAID) and the Centers for Disease Control (CDC). There is an urgent need for improved methods to diagnose human intoxication with BoNTs so that appropriate therapeutic and preventive measures can be rapidly applied. Diagnosis at the earliest stage of pre-symptomatic illness is critical to the administration of appropriate and effective clinical treatment, as well as implementation of preventive public health measures. A fully integrated end-to-end system, from sample preparation to data analysis, is needed to screen clinical samples.

Recognizing the need for better diagnostic methods for BoNTs, NIAID convened a meeting of experts in Bethesda on May 23, 2003. The purpose of this meeting was to:

1. provide an overview of the current status of BoNT diagnostics and their limitations;
2. evaluate the scientific and technical opportunities to develop improved, validated detection methods for clinical samples and food;
3. identify potential hurdles; and
4. provide recommendations for the development of improved BoNT diagnostics as priority civilian biodefense products.

This report is a summary of the discussions addressing these objectives.
**Introduction/Background**

The botulinum neurotoxins (BoNTs) are the most potent biological toxins known. Based on their exceptional lethality, their ease of production, and the high degree of disruption to national emergency medical care that even a small number of cases of botulism would cause, the BoNTs are listed by the National Institutes for Allergy and Infectious Diseases (NIAID) and the Centers for Disease Control (CDC) as Category A biothreat agents. The most comprehensive and recent publication on BoNTs as biological weapons evolved from the Working Group on Civilian Biodefense organized by the Johns Hopkins Center for Civilian Biodefense Studies (Arnon et al. 2001. *JAMA*. 285 (8), 1059-1070).

The most common route of entry of BoNTs is through the ingestion of contaminated foods. BoNTs have, however, been weaponized, and in that context their aerosolization and subsequent inhalation should be considered a possibility. In either case, BoNTs enter the circulation and eventually bind to and enter cholinergic nerve endings. The toxin gains access to the cytoplasm of nerve cells, where it acts as a zinc-dependent endoprotease that cleaves polypeptides, which leads to the blockade of transmitter release and paralysis. Current therapy for botulism consists of supportive care and passive immunization with equine antitoxin. Antitoxin therapy is most effective if administered early while toxin remains free in the circulation, and has not bound to nerve endings. The average time between toxin ingestion and onset of symptoms ranges from 18 to 36 hours for food borne botulism. Victims of naturally acquired botulism frequently require mechanical ventilation and intensive supportive care for 6 – 8 weeks at a cost of $250,000 - $500,000, while toxin is cleared from intoxicated neurons. 

Current diagnostic methods for botulism include the clinical assessment of neurological symptoms, which may also resemble Guillain-Barré or Miller-Fisher syndrome, myasthenia gravis, or certain other diseases of the central nervous system. The mouse protection bioassay is an auxiliary confirmatory diagnostic tool and is both highly sensitive and serotype specific. Unfortunately, this assay is also time-consuming, complex, and labor-intensive method and is performed in only a few laboratories nationwide. In the event of an intentional exposure of a large number of people, the availability of a rapid and specific presymptomatic diagnostic will:

- maximize the effectiveness of anti-toxin treatment,
- limit the need for extended intensive care, and
- allow health care providers to distinguish between the truly exposed and the so-called “worried-well”, thereby preserving limited supplies of anti-toxin for future use.

On May 23, 2003, the Division of Microbiology and Infectious Diseases (DMID) of NIAID convened a one-day meeting in Bethesda, Maryland to bring together some of the leading scientists in BoNT and medical diagnostics research.
Participating in this meeting were representatives from the Food and Drug Administration (FDA), the CDC, the Department of Energy (DOE), the Department of Health and Human Services (DHHS), the Department of Defense (DOD) and several universities and public health laboratories.

Two recent workshops provided background for the presentations and discussions at the meeting. NIAID convened a Blue Ribbon Panel on February 4 and 5, 2002, to discuss and propose a research agenda for the Category A threat agents. This meeting led to specific recommendations for immediate, intermediate and long-term research and development activities for the Category A pathogens and toxins, including the BoNTs. The recommendations were published and are available at: http://www.niaid.nih.gov/dmid/pdf/biotresearchagenda.pdf. On November 20, 2002, the NIAID convened a follow-up meeting with an invited group of BoNT experts from academia, industry, and government. The goal of the November 20, 2002 meeting was to identify the issues related to the development of the next generation of countermeasures against BoNTs. A summary of this meeting is available at: http://www.niaid.nih.gov/dmid/pdf/bot_toxins.pdf.

The goals of the May 23, 2003 meeting were to focus specifically on diagnostics and: (1) provide an overview of the current status of BoNT diagnostics and their limitations; (2) evaluate the scientific and technical opportunities to develop improved, validated detection methods for clinical samples and food; (3) identify potential hurdles; and (4) provide recommendations for the development of improved BoNT diagnostics as priority civilian biodefense products. This report is a summary of the discussions addressing these objectives.

**Biology of Botulinum Toxins**

The BoNTs are a family of protein toxins elaborated by *C. botulinum*, (Types A, B, C, D, E, and F), and neurotoxigenic *C. barati*, (Type F) and *C. butyricum* (Type E). The BoNTs are structurally related and possess 30 – 40 % sequence homology at the amino acid level. Each toxin comprises a heavy (100kDa) and a light (50kDa) chain linked by a disulfide bond. The heavy chain possesses three domains. The heavy chain C-terminal fragment (Hc) contains two domains that are required for binding to the neuronal membrane. The amino-terminal 50kDa domain (Hn) is required for translocation of the enzymatically-active light chain across the neuronal membrane. Although the BoNTs bear structural similarities, they are antigenically distinct.

BoNTs can be produced in large quantities as a crude biologically active extract. This crude material contains toxin aggregates, cellular material, bacteria, spores, RNA and gDNA, all potential targets for detection in environmental or food samples. Complex matrices may, however, exert an inhibitory effect on detection by masking epitopes, active sites and ligand binding regions of toxins. In
addition, toxin complexes adsorbed onto a solid phase can possess specific activities that differ from soluble toxin, complicating the interpretation of results based on spectra.

Detection in clinical samples is likely to be limited to the detection of the neurotoxin itself in blood and possibly stool. In human blood, BoNTs exist in soluble form, and although diluted from their source material, the BoNTs are very stable and are maximally accessible to analysis. These considerations combine to make development of diagnostics for serum analysis a problem of sensitivity limits and rapidity within a relatively consistent matrix, rather than a challenge of addressing the interference imparted by a range of very diverse test materials as is the case in food and environmental samples.

Once inside the nerve terminal, the toxins exert their effects through a zinc-dependent endopeptidase activity on small proteins required for the fusion and release of synaptic vesicles. BoNT cleave specific sequences within either of two classes of vesicle-binding proteins. BoNT serotypes A and E specifically cleave the 25kDa synaptosomal associated protein (SNAP-25). BoNT serotype C cleaves the membrane protein syntaxin and SNAP-25, and BoNT serotypes B, D, F and G act on vesicle-associated membrane protein (VAMP is also known as synaptobrevin). Cleavage of VAMP or SNAP-25 prevents vesicle fusion, neurotransmitter release, and therefore synaptic signal transduction. The cellular turnover of VAMP and SNAP proteins is slow, and the BoNT within the neuron continues to exert a neuroparalytic effect until the BoNT itself loses activity: a process that takes from weeks to months in human cases of botulism.

**Current Detection and Diagnostic Methods**

The standard method for BoNT laboratory determinations is the mouse protection bioassay (MPB). This assay monitors for the presence of specific botulism symptoms after intraperitoneal injection of the suspect material into mice. The MPB can take up to 4 days, and is dependent on the quantity of toxin, its activity, type, subtype, and the other material present in the sample. Sensitivity is generally of the order of 2 mouse LD\textsubscript{50} units per ml of sample (0.5 ml sample volume), which corresponds to approximately 10 pg of active toxin. The sample can be pre-incubated and injected with specific antisera to any or all of the BoNTs, so the antigenic subtype of the BoNT present in the sample can be determined. Unlike methods based on immunological binding alone, (e.g. ELISA) the mouse protection bioassay is specific for active toxin. The bioassay has been in use for several decades in the CDC, FDA, and numerous U.S. and international public health laboratories. The Association of Analytical Chemists (AOAC) has validated the test for the analysis of microbiological culture broth and various food matrices, but not serum.

Considered the ‘gold standard’ against which proposed improved methods will inevitably be compared, the current mouse protection bioassay has several
significant shortcomings. For example, mice can die non-specifically during the testing process, and this effect can be exacerbated when poorly defined sample types are being analyzed for the presence of toxin. The MPB is labor-intensive, requiring animal facilities with highly trained, immunized staff, who comply with strong administrative requirements for biohazard safety and select agent registration for control and check samples. In addition, the mouse protection bioassay does not possess any ‘surge capacity’ in the event of a real or suspected biodefense deployment, and is unsuitable for routine quantification of samples because of the large numbers of animals needed for statistically significant results.

The current understanding of BoNT structure and activity provides the basis for the development of future diagnostics. However, with the current bioassay detection in the low picogram range, improvements in sensitivity to the femto and attogram range present a real technical challenge. Successfully achieving this goal for the BoNTs in serum may have significant spin-off benefits in the detection and quantification of other proteases and antigens.

New Methods for Detection and Diagnosis

Detection of the BoNTs can exploit a range of features. Structural features of the toxins include specific epitopes or ligand binding sites, whereas functional characteristics center on the protease activities. Improved toxin-specific antibodies can benefit a range of methods, including capture, concentration or purification systems utilizing flow cells: ELISA, time-resolved fluorescence, surface plasmon resonance, electro-chemiluminescence, immuno-PCR, color-coded bead arrays, and ligand sandwiches.

An intriguing potential for BoNT diagnostics development lies in exploiting the protease activities of these enzymes. Each BoNT subtype has a unique peptide sequence specificity that can be used as a substrate for subtype-specific tests. This approach leverages the inherent amplification associated with assaying an enzyme target. However, the human VAMP and SNAP-25 sequences are not the optimal sequences for BoNT enzyme activity, and studies using short synthesized peptides have identified substrates that are digested more efficiently. Optimized substrate can be combined with conjugation chemistries to tag the cleavage product for identification using any of a number of high performance instruments, e.g., FRET, mass spectroscopy, etc.

The complexity of matrices for detection of BoNT presents challenges for sample preparation from clinical, food and environmental samples. Inhibitors, such as endogenous proteases, and the low concentration of the proteins are issues to be addressed. Because it is likely that endogenous proteases in blood, gastric lavages, stool, or any number of other matrices may cleave the native target peptide sequences of the BoNT, a comprehensive survey of naturally occurring protease activities in the sample types under consideration may need to be
Inhibition of endogenous protease in clinical samples by steric hindrance or by using protease inhibitors may greatly increase the selectivity of the substrate for the specific BoNT in diagnostic assays.

**Electrochemiluminescent (ECL) assays** have been developed by applying BoNT-specific antibodies to commercially prepared assay reagents. ECL assays are simple, easy to use, and sensitive to 1 mouse LD₅₀, which is of the order of 100 - 1000 times more sensitive than that routinely achieved with chromogenic ELISAs. The ECL assay has been demonstrated with pure toxins, and with artificially contaminated matrices, including serum. As with all tests, issues relating to the availability of toxin in matrices, aggregation of toxin, and matrix inhibition of assay reactions need to be addressed.

**Development of new ligands** can provide a high affinity link to toxin that can be combined with new detection technologies to produce assays in the femtomolar range. The known crystal structures of BoNT/A and the defined enzymatic activity of the toxins allow both rational and empirical development of ligands. New approaches to the molecular evolution of monoclonal antibodies as therapeutics will improve the sensitivities of monoclonal antibody-based methods down to the 150 fg range; well beyond the 15 pg range obtained with antibodies derived using conventional immunization.

**Time-resolved fluorescence** methods are similarly sensitive as ECL technologies and have also been demonstrated with pure toxins and artificially contaminated matrices. Background fluorescence can be problematic with certain matrices.

**Color-coded bead array** is a flow cytometer based method developed by commercial vendors and have been applied to the detection of BoNTs with some success. The system shows excellent promise because it is well suited to the multiplexing. Sensitivity approximates that of ELISA. This assay could play a role in screening. Internal sample controls can be included due to the multiplexing capabilities of the system.

**Mass spectroscopy** offers some rapid, highly resolving technologies that are well suited to the study of BoNT. There are potential problems with complex matrices, sample preparation, and inhibitors, however, methods such as electrospray or matrix-assisted laser desorption time-of-flight (MALDI-TOF) have some significant attributes. They are exceptionally rapid, highly resolving, and capable of analyzing large numbers of samples. The power of these techniques and the declining cost and complexity of the instrumentation is leading to increased acceptance of mass spectroscopy in many settings, including clinical medicine. Problems with the sensitivity of these systems need to be addressed.

Optical methods based on **fluorescence resonance energy transfer (FRET)** have made recent progress and hold promise for future advances in diagnostics.
Immuno-PCR has been explored as a generic approach to combine antibody reagents with the installed base of PCR instrumentation. The appearance of major background problems has led to this approach being shelved.

Micromachine platforms developed by DOE/Sandia National Laboratory and commercial vendors have been evaluated for use with BoNTs. This system utilizes a hand-held instrument containing a microfluidics chip capable of concentrating samples and separating targets according to migration times. Samples are tagged with a fluorescent dye and chemical signatures can be determined with low nM sensitivity in a 10-minute assay.

Improved knowledge of BoNT binding activity should allow the development of improved methods based on spectral shifts observable during the binding event of toxin molecules to high specificity ligands or receptors. The e-tag reporter system and the ‘Biobriefcase’ may have application in the BoNT area.

Development of both nonspecific and specific measures of the host response to exposure and intoxication with BoNTs has generated considerable interest. Some early stage research is currently being undertaken to study the pre-symptomatic gene expression profiles of intoxicated neurons. Specific molecular biomarkers of a neuronal response to intoxication may provide early information of exposure, however, this requires the identification of specific biomarkers, careful integration with clinical medicine, excellent communication to physicians, and a syndrome clearinghouse or bioinformatics database that will readily associate clustered cases and flag them as a single event.

**Recommendations**

**Produce BoNT pharmacokinetic data based on oral and inhalational ingestion in small animal models and non-human primates.**

The importance of pharmacokinetic studies on oral and inhalational ingestion of BoNTs was detailed in the November 20, 2002 meeting, with respect to the development of new therapies and vaccines for botulism. Similarly, characterizing the nature, persistence, and trafficking of the target in the material to be tested was identified as a critical step in developing molecular diagnostics. NIAID has initiated studies to collect data on the pharmacokinetics of BoNTs in small animal models.

**Accelerate target characterization at the level of gene sequence, antigenic variation, and enzymatic activity and make data available in an annotated database.**

Limited information is currently available to describe the range of BoNT targets that must be recognized by a future diagnostic. For example, toxin complexes contain both accessory proteins and RNA. Furthermore, the data that does exist
is not available in one annotated searchable database. Characterization of *C. botulinum* bacteria and neurotoxins between and within serotypes at the level of sequence, antigenic variation, and enzymatic activity is needed. Comparative genomic studies among the BoNT-bearing *Clostridia* are encouraged. The research community should agree on the identity of the target and the breadth that is required to conservatively detect a broad range of BoNT serotypes and possible subtypes.

**Develop higher affinity BoNT specific antibodies.**  
The specificity and sensitivity of many of the current technologies are dependent on the affinity of the monoclonal antibody employed as a target capture. It is encouraging that contemporary antibody engineering technology offers the opportunity to significantly improve the affinity of monoclonal antibodies and thus the specificity and sensitivity of these assays.

**Improve sample preparation.**  
Significant advances are needed in the area of sample preparation applicable to a wide variety of matrices in flowed systems.

**Expand scientific expertise in BoNTs.**  
Until recently, botulism was considered a public health threat of minor significance, which is reflected in the relatively small number of scientists with expertise in the field of BoNTs. Increasing the number of qualified researchers is hampered by the rather onerous restrictions and requirements of working with select agents under appropriate security and biosafety controls. In response to this concern, NIAID has announced numerous opportunities for training, multi-disciplinary research support and funding for capital improvements to satisfy the needs of researchers moving into the biodefense field. The new Regional Centers of Excellence will provide a significant level of capability. In May 2003 the CDC and FDA conducted a joint one-weeklong class that trained 16 scientists in the detection and characterization of BoNTs and use of the MPB and a modified ELISA method.

**Make a variety of standardized reagents available.**  
The availability of standardized reagents will promote uniformity of assay evaluation and reduce duplication of effort in development of reagents. NIAID has funded a new reagent repository capable of handling *C. botulinum* strains and toxins for use by the research community. Additional reagents could include ligands, MAbs, nucleic acids, (e.g., primers, and gDNA), peptides and other substrates, protease inhibitors, standard negative control matrices, defined artificially-contaminated materials, crude culture precipitates, clinical specimens and check samples.

**Access pathways to product evaluation and validation.**  
The area of biodefense products generally includes problematic issues including a low to non-existent level of naturally occurring endemic disease, unusual routes
of exposure, and the unpredictability of the agent itself. These complications can stymie the accumulation of the data needed by the FDA to evaluate the diagnostic. There is, however, the ’05 exemption’ for validated diagnostic products for use in emergencies. The Center for Devices and Radiological Health of the Food and Drug Administration has authority over new diagnostics for human botulism. It is particularly valuable for researchers and those involved in product development to contact the FDA at an early stage in order to gain an understanding of the diagnostics approval process.

**Encourage the development emerging genomic and non-genomic technologies for supporting the next generation of diagnostics.**

Diagnosis of botulism has centered on the currently available mouse protection bioassay, which is based on neutralization by specific Abs and can take up to 4 days to complete. There is a need to develop a variety of new diagnostic platforms to achieve diagnostic tests that will surpass the mouse assay in sensitivity, speed, and high volume capacity, since samples must be obtained before the antitoxin treatment has begun.
**Botulinum Neurotoxin Diagnostics Meeting**

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