



National Institute of Allergy and Infectious Diseases

SUMMARY OF THE NIAID EXPERT PANEL ON BOTULINUM TOXINS

November 20, 2002

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INTRODUCTION

The highly potent neurotoxins produced by the spore forming anaerobic bacteria *Clostridium botulinum*, *C. butyricum*, and *C. barati* pose a significant threat as weapons of bioterror. The most comprehensive and recent publication on botulinum neurotoxins 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).

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 toxins of *Clostridium botulinum*. This document was published and is available at (<http://www.niaid.nih.gov/dmid/pdf/biotresearchagenda.pdf>).

On November 20, 2002, the NIAID Division of Microbiology and Infectious Diseases convened a follow-up meeting with an invited group of botulinum toxin experts from academia, industry, and government, including the Food and Drug Administration. The purpose of the meeting was to engage expert opinion on issues related to the development of the next generation of countermeasures against botulinum toxins.

THERAPY

Current therapy for botulism consists of supportive care and passive immunization with equine antitoxin. A licensed trivalent antitoxin that contains neutralizing antibodies against botulinum toxin types A, B and E and an investigational heptavalent (ABCDEFG) antitoxin are available, but supplies are limited. The expert panel noted that although second generation therapies are needed, their use may reduce but will probably not eliminate the need for extensive supportive care (mechanical ventilation) for long periods (weeks to months). Most cities do not have the intensive care respiratory unit capacity to cope with any significant demand for this type of care.

ANTIBODY-BASED THERAPY

The expert panel agreed that a next generation monoclonal antibody therapy against all 7 botulinum toxin serotypes is technically feasible. Although a final heptavalent product is envisioned, product development should proceed by developing and evaluating monovalent products as they become available. The ideal next generation product was described as fully human/human-compatible lyophilized whole IgG. This product would be equally appropriate for use as either therapy or prophylaxis. It was recognized that more than one monoclonal might be required to neutralize any single serotype. Firstly, sub-serotypes A1 and A2 of type A toxin have been identified and additional sub-serotypes are likely to be discovered once a more thorough characterization of serotypes B, C, D, E, F and G is done. Development of broadly protective monoclonal antibodies is dependent on understanding the diversity between the representative clinical and environmental isolates and their botulinum neurotoxins. Secondly, even within a distinct serotype no single monoclonal antibody has been found to effectively neutralize toxicity, rather a combination of three monoclonal antibodies was required to neutralize one serotype. The priority for development of each monospecific product, from highest to lowest, is serotype A, B, E, C, F, G and D.

The cost of producing a series of monovalent products or a heptavalent product that potentially may contain a mixture of many different monoclonal antibodies will be considerable. A major cost will be associated with production using currently available fermentation technology, i.e. mammalian cells, bacteria or yeast expression systems. The expert panel emphasized that emerging, less-expensive expression systems (e.g. plants, humanized small and large animals) should be considered for production as they become available. However, caution must be taken to consider safety issues and FDA-related requirements that might pertain to production in these less-well documented systems. The expert panel recommended that the identification of the ligands proceed with urgency and that decisions related to scale-up and production be informed by the technology that is available and validated.

In light of the challenges of developing human/human-compatible monoclonal antibodies that neutralize at least seven serotypes of toxin, polyclonal antibodies are very attractive. Animals that produce human/human-compatible antibodies may be available within a few years. The application of these large animal polyclonal antibody production systems to the development of polyclonal antibody therapies against all seven serotypes of botulinum toxins has the potential to yield therapies that are effective and possibly less expensive. Polyclonal antibody therapies are likely to be less vulnerable to the deployment of genetically engineered toxins. Similar to the novel expression systems discussed above, specific safety issues will have to be addressed in systems where product is derived from the sera of other animals.

The expedient development of antibody-based therapies will require:

- Sufficient resources,
- Access to and characterization of representative isolates and neurotoxin complexes of each serotype,

- A well thought out plan,
- Management of intellectual property issues,
- Partnerships between botulinum toxin experts and industrial antibody manufacturing entities,
- Working relationship with the FDA,
- And a central laboratory facility to evaluate efficacy.

VACCINES

Toxoid Vaccine

An investigational pentavalent (ABCDE) botulinum toxoid vaccine to protect laboratory workers and military personnel is available in limited quantities. There is currently no available vaccine for toxin type F and G. It is essential that there is no gap in the availability of botulinum toxin vaccine to protect these populations as well as to respond to scenarios of intentional exposure. The timeline for developing a next generation recombinant heptavalent botulinum toxin vaccine is 5-7 years and thus it may be necessary to produce new lots of the toxoid vaccine.. Manufacturing new toxoid vaccine would allow improvements upon its current formulation, a crude cell extract, by removing more cellular contaminants and thus further purifying the toxins. However, it was recognized that any change to the manufacturing process could cause delays in the production and these risks and benefits would need to be assessed. Moreover, although there are no technical barriers to the production of this very old product, the lack of an appropriate cGMP manufacturing facility is likely to limit the feasibility of its production.

Recombinant Vaccine

A recombinant vaccine to protect against all 7 serotypes of botulinum neurotoxin is considered technically feasible. The DOD has recombinant vaccine against serotypes A and B under advanced development. Stability, manufacturing, and formulation issues still exist for serotypes C-G. The product development pathway may proceed through a series of monovalent or multivalent products before a final heptavalent product is achieved. The advantage of this approach is to make recombinant vaccine for those toxin types that are considered the highest threat available sooner. In addition, research to determine whether antigenic competition will be an issue in a heptavalent product is needed. Finally, monovalent products will allow the flexibility to deliver vaccine in response to a specific defined threat while retaining the therapeutic potential of other botulinum toxin serotypes. Other laboratories outside of the DOD have botulinum toxin vaccines under much earlier stages of development.

“OTHER” THERAPIES

Panel members were asked to consider the use of small molecules that might be used to block the protease activity, the transport of the toxin into the blood stream, or the binding

and translocation of the toxins into the neurons. The panelists agreed that there were three potential sites for clinical intervention: 1) at the site of entry (intestine or lung epithelium), 2) in circulation, and 3) at the level of neuronal binding.

Little is known about the receptors on intestinal or lung epithelium that bind toxin. It is uncertain whether the botulinum neurotoxin complex, which contains a variety of accessory proteins, is the entity that is transported into the blood. Shedding of these accessory proteins occurs prior to neuronal binding but the details of this process are poorly understood. Thus the receptors on lung or intestinal epithelium may be quite different than those on neurons. There is a need for future research in this area. One possibility that was discussed was that toxin fragments (perhaps the same fragments used as vaccines) could have activity as inhibitors of transport in the intestinal milieu. Animal model testing may be warranted in this case. Much basic information is needed in this area before an informed approach can be developed.

Inhibition of the toxin in circulation can best be accomplished by antibodies (from vaccination or delivery of therapeutic antibodies) as discussed above. It may also be possible to deliver small molecule inhibitors that could bind to and inhibit protease activity of the toxins while they are in circulation. The affinity of such inhibitors must be sufficiently high, essentially irreversible, *in vivo* so as to remain bound during the transport and trafficking of the toxins in neurons. Molecules that are essentially molecular mimics or “false acceptors” could be used to capture neurotoxin while it is still in circulation.

The expert panel identified substantial technical challenges to the design and delivery of small molecule inhibitors of protease activity or molecules to the interior of affected neurons that could effect the removal of toxin from its SNARE substrates. There are several reasons for this, including lack of a method that could specifically target neurons. Research is underway to develop such delivery systems, and NIAID could encourage more of this research. For example, the targeting of neurons by the toxin itself could be utilized as a delivery system. This approach would require much more information about the toxin receptors, mechanism of translocation into the neuron, and mechanisms of toxin delivery to its SNARE ligands. It would require the design and production of non-toxic derivatives of botulinum neurotoxin containing or attached to the “inhibitor”. Another obstacle is the improbability of developing an inhibitor that would have sufficiently high affinity or that could be accumulated inside neurons at sufficiently high concentrations to effectively compete with the very strong binding affinity that botulinum neurotoxins demonstrate for their protein ligands. In addition, inhibitors would have to be highly specific for the botulinum neurotoxin class of endoproteases. Due to the long-lasting activity of botulinum neurotoxins within neuronal cells, therapies may need to be delivered over a period of weeks to months, demanding a high level of safety for any potential therapeutic compounds. Another approach considered was to deliver altered forms of SNARE proteins, particularly SNAP-25, that are uncleavable, to restore neuronal activity. Despite these obstacles, some work is underway, particularly at two Army facilities, USAMRIID and USAMRICD, a few academic laboratories, as well as small biotechnology companies on identification of small molecule inhibitors.

Recommendations for future research:

- Identification and characterization of the receptor(s) utilized by the toxin or toxin complex in intestinal and lung epithelium.
- Structural studies of progenitor toxin.
- Structural studies of the toxins (different serotypes) alone and in combination with neutralizing monoclonal antibodies, SNARE substrates and ligands, and inhibitors of protease activity. Such studies are required before rational drug design of inhibitors can take place.
- Study of the mechanisms of transport and cellular trafficking of the toxins in epithelial cells and neurons.
- Study of the enzyme activity and ligand specificity of the seven known many serotypes of botulinum toxins.
- Studies to identify if proteins other than SNAREs are cleaved by botulinum neurotoxins
- Genetic (microarray) studies of the mouse and human response to botulinum neurotoxins . This could start with determination of the response in cultured neuronal cells.

DIAGNOSTICS

Current methods to diagnose individuals suffering from the early stages of botulism intoxication, initially center on an observational assessment of characteristic symptoms of botulism as a neuropathological disorder. As such, accurate and expeditious identification of cases requires well-informed physicians knowledgeable about possible additional local cases that may form a focus of exposure. It is likely that a delay will occur in recognizing the first cases due to the imprecise symptoms and possible alternative diagnoses early in disease manifestation, e.g. double vision. A symptom-based database was proposed as a potentially very useful resource to identify botulism intoxication. Distributing a list of alternative diagnoses confused with botulinum poisoning might be a simple, immediate way to speed emergency room identification of botulism cases.

Tests for botulinum neurotoxin in the serum and in possible vehicles center on the mouse bioassay; the precise toxin type is determined with neutralizing goat antitoxin. The mouse assay is very sensitive and well established. The CDC has deployed reagents and expertise around the country, and has a stockpile of additional reagents for use in an emergency. The Panel agreed that bolstering the infrastructure and improving response time represents a logical and helpful approach to swiftly increase the national defensive capability. Particular needs include more and higher quality serotype-specific antisera for confirmatory assays.

There is a range of more modern approaches for the detection of botulinum toxins under various stages of development. Important applications beyond the capability of the mouse model include essentially all the specifications of diagnostics, except for

sensitivity, in which the mouse bioassay is the “gold standard”. Important parameters not well addressed by the mouse bioassay include attribution, high volume, rapidity, and deployability. These may be improved using methods such as fluorescence resonance energy transfer (FRET) systems currently at a late stage of development, or tests based on the catalytic activity of toxins, which is a potentially promising area. It was pointed out that several high-performance molecular detection methods could easily surpass the sensitivity of the mouse bioassay, and provide serotyping or mass data that would quickly identify very low toxin levels. Detection of toxins and resolving among the numerous variants represents a cutting-edge research challenge that could lead to many spin-off applications in the detection of specific proteins in clinical samples, and thus potentially be a highly productive research area.

It was agreed that toxin preparations will likely be contaminated with DNA, allowing a range of important tools to be used, including PCR, which is available in many generic and specific formats, and microarray assays, which may be forthcoming after the *C. botulinum* genome sequence is published and annotated. Developing a set of methodologies for the useful application of PCR represents an achievable medium-term goal. Microarrays have significant theoretical potential to evaluate a set of toxin sequences and relate the result to a sequence database derived from geographically diverse *C. botulinum* strains. This could be highly valuable for attribution purposes as well as determining appropriate immunotherapeutics. These methods will have to be employed with judicious caution as *C. botulinum* is ubiquitous and thus PCR-based assays to detect its DNA might produce too many positives to be meaningful.

Diagnostics for spores and vegetative cells also represents an important capability, which requires significantly different sample handling modules. The natural distribution of *C. botulinum* in the environment needs to be determined to minimize false interpretation of results. Models describing the likely dispersal of spores or cells from a deliberate release would be valuable.

Diagnostics based on host responses is an attractive research area. Due to the shortage of ICU beds, it will be important to recognize the earliest signs of intoxication and recovery so that resources are used most efficiently. The Panel encouraged the development of interdisciplinary teams for the development of diagnostics, and emphasized the importance of a thorough understanding of the biology of botulinum neurotoxins to be represented within the research teams

The critical need in developing new diagnostics is in careful evaluation and validation of methods. The Panel identified this as an essential point for researchers, developers, reviewers, and point-of-care operators to appreciate. Until recently, validation was not considered a very productive or even legitimate component of research proposals. A role for NIAID in ensuring accurate validation was proposed.

Many spin-off applications will stem from improvements in botulinum toxin diagnostics. A successful platform for the resolution of protein isoforms has broad importance to clinical diagnostics, and microarray-based sequence detection methods referenced to a

substantial geographically indexed strain database uses the same intelligent data procurement analysis modules as is needed for many epidemiological and biodefense trace-back studies. Methods for the identification of spores and vegetative cells will be beneficial for the early diagnosis of infant botulism, and applications in veterinary medicine and the food industry were starkly exemplified in the recent Finland fox botulism outbreak. There is also a clear opportunity to relate advances in clinical and environmental detection for the mutual benefit.

In conclusion, diagnostics for botulism represent a fertile area for research into a subject of enormous biodefense significance. There are good prospects for major, broadly significant advances in the medium term.

RESEARCH RESOURCES

The panel agreed that there was much that NIAID could do in the area of research resources to serve the community. Among the most pressing needs is the establishment of a strain and reagent repository. Individual strain collections of *C. botulinum* exist in research laboratories and at the CDC. Some of the individual collections are in jeopardy due to retirement of researchers and new regulatory requirements. NIAID was strongly encouraged to add *C. botulinum* to its repository plans. The location, contents and status of current collections will be determined. Plans should be made to have an essential number of isolates moved to the repository or supported where they exist until a move is possible.

Once the repository is established it should be characterized to develop “fingerprints” of all strains. Information databases that were identified as desirable in the repository include:

- Genetic information: restriction maps, multi-locus enzyme electrophoresis, sequencing of selected regions including the toxin gene and accessory proteins in the neurotoxin complexes
- Structural information: X-ray crystallography of the holotoxin and the toxin complexes (all 7 serotypes), cryoelectron microscopy, complexes with receptors, complexes with antibodies, etc.
- Immunologic characterization
- Reagents: microarrays, detection systems

The issues of accessibility, shipment of strains etc. were discussed but these are issues that are not *C. botulinum* specific. They are issues that must be addressed at the Department level and above.

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