



SUMMARY OF THE NIAID EXPERT PANEL ON BOTULINUM NEUROTOXINS THERAPEUTICS

February 9 and 10, 2004

Bethesda, MD

EXECUTIVE SUMMARY

The botulinum neurotoxins (BoNTs) are the most potent and lethal toxins known to man. The toxins are both relatively easy to produce, as well as to deliver through intentional contamination of food or beverages. Current treatment consists of intravenous infusion of an equine antibody-based antitoxin available through the Department of Homeland Security National Strategic Pharmaceutical Stockpile. The use of equine-based antibodies has been associated with symptoms of hypersensitivity, including urticaria and serum sickness, and anaphylaxis.

Recognizing the need for improved post-exposure therapeutics for the BoNTs for use in civilian populations, NIAID convened an Expert Panel Workshop in Bethesda on February 9 and 10, 2004. The purpose of the meeting was to:

- ❖ discuss the most appropriate targets for clinical intervention,
- ❖ provide an overview of the current status of discovery of new therapeutics,
- ❖ evaluate the technical opportunities, as well as the constraints, to the development of the next generation of therapeutics,
- ❖ identify the knowledge gaps that constrain development of new therapeutics against specific targets, and
- ❖ identify research resources that are needed to advance discovery efforts.

This report is a summary of the discussions addressing these objectives.

Next generation antitoxins are likely to be derived from cocktails of human compatible monoclonal antibodies or from transgenic animals capable of producing high specificity human compatible polyclonal antibodies. This approach leverages the currently accepted relative safety of human compatible antibody-based therapies, an important consideration for the potential use of treatments in diverse populations of unknown exposure status. Although synthetic inhibitors (drugs) that target circulating BoNT have the potential advantage of being cheaper to produce than antibody-based therapies, there are currently no drugs under development. The discovery of such inhibitors requires a more focused and concentrated effort than is currently underway. A high throughput screening, medicinal chemistry structure-based iterative process is needed. Regardless of whether the inhibitor is an antibody or drug, a relatively short therapeutic window is available to inhibitors that are restricted to neutralizing neurotoxin in the bloodstream, before it is internalized and sequestered in peripheral cholinergic nerve cells.

The largest clinical and economic benefit would derive from the ability to prevent, reverse or block paralysis, and thus the need for long term (weeks to months) supportive care in a respiratory intensive care unit. This may be achieved by blocking the activity of the BoNT once it has reached its target, the cytoplasm of the peripheral cholinergic nerve cell, and in the presence of its natural substrate. The primary challenges associated with this approach are: a) to discover highly specific non-toxic inhibitors and b) to deliver those inhibitors to the cytoplasm of peripheral cholinergic nerve cells. The discovery of such inhibitors requires a more focused and

concentrated effort than is currently underway. Again, a high throughput screening, medicinal chemistry structure-based iterative process is needed. An alternative to blocking the activity of the BoNT light chain is a strategy to accelerate its intracellular catabolism/degradation. Understanding how the light chain is trafficked to the cytosol and persists in its active site environment is key to addressing this possibility.

In summary, there are still considerable gaps in our knowledge of the biology of the mechanisms of transport from the lumen of the gastrointestinal tract or lung into the plasma, endocytosis into peripheral cholinergic nerve cells, translocation to the cytoplasm of the nerve cell, substrate recognition domains and persistence within the cytoplasm. The two most attractive therapeutic targets are 1) neutralizing toxin in the blood, and 2) blocking/reversing paralysis by inhibiting enzymatic activity within the cytoplasm of the nerve cell. We are technically capable of developing bloodstream inhibitors, but basic challenges remain for blocking activity inside the nerve cell.

BACKGROUND

The highly potent neurotoxins produced by the spore forming anaerobic bacteria *Clostridium botulinum*, *C. butyricum*, and *C. baratii* pose a significant threat as weapons of bioterror (Arnon et al., JAMA, Feb 2001; 285: 1059 – 1070 (<http://jama.ama-assn.org/cgi/reprint/285/8/1059.pdf>)).

NIAID convened a Blue Ribbon Panel on February 4 and 5, 2002 to discuss and propose a research agenda for the Category A Agents of Bioterrorism. 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 botulinum neurotoxins (BoNTs). This document was published and is available at <http://www.niaid.nih.gov/biodefense/research/biotresearchagenda.pdf>.

On November 20, 2002 the NIAID Division of Microbiology and Infectious Diseases convened a meeting with an invited group of BoNT experts from academia, industry, and government to focus exclusively on the BoNTs. The purpose of the meeting was to engage expert opinion on issues related to the development of the next generation of countermeasures against the BoNTs. A summary of this meeting is available at: http://www.niaid.nih.gov/dmid/pdf/bot_toxins.pdf.

These meetings pointed to the need for improved treatments for individuals exposed to BoNTs. Current therapy for botulism consists of supportive care and passive immunization with equine antitoxin, or in cases of infant botulism, the recently licensed BabyBIG[®]. The equine antitoxins include a licensed bivalent and monovalent antitoxin that contains neutralizing antibodies against BoNT types A/B and E, respectively and an investigational heptavalent (ABCDEFG) antitoxin. BabyBIG[®] is derived from the blood of immunized human donors and was developed specifically to treat infant botulism. These therapies are thought to be effective only in binding and neutralizing BoNT that has not yet been endocytosed by peripheral cholinergic neuronal cells, and thus their usefulness is limited to a narrow window of time post-exposure (18–36 hours depending on exposure). In addition, the use of the equine-derived antibody therapy raises significant safety concerns with respect to the percent of the population in which its use is automatically contra-indicated and its potential to induce unexpected serious adverse reactions in others. The expert panel noted that safe and effective second generation therapies that bind neurotoxin in the blood are needed, their use, however, does not eliminate, the need for supportive care (mechanical ventilation). Most cities do not have the intensive care respiratory unit capacity to cope with any significant demand for this type of care.

The purpose of this meeting was to discuss in greater detail the technical opportunities as well as the constraints to the development of next generation therapeutics for botulism. The meeting included the following sessions:

- Antibody-based therapies,

- Small molecule inhibitors,
- Clinically relevant targets,
 - The major steps in BoNT intoxication
- Identification and characterization of BoNT targets,
- Biophysical/biochemical characterization of BoNT targets,
- Genetic analysis of *C. botulinum* and its BoNTs,
- Basic mechanistic biology,
- Stability and pharmacokinetics of BoNT and inhibitors, and
- Research resources.

Antibody-based Therapy

Moderator: Dr. James Marks, UCSF

Monoclonal Antibodies: The development of monoclonal antibody treatments is currently focused on serotype A. Antibody libraries derived from immunized humans or mice, or immunized mice transgenic for the human immunoglobulin locus have been used as the source of immunoglobulin genes. Single chain variable fragments that bind BoNT are selected and evolved to increase their binding affinity and then engineered to whole IgG₁ molecules that can be evaluated *in vivo* in a mouse potency assay.

Dr. Marks described his progress in the discovery of therapeutic monoclonal antibodies for BoNT serotype A. While much progress has been made in the area of high throughput screening for high affinity antibodies the following obstacles remain:

1. The significant sequence variability between and within serotypes has not been thoroughly characterized. The majority of neutralizing antibodies recognize the binding domain of the toxin heavy chain, the site that represents the greatest sequence variation. Dr. Marks noted that there are 46 full-length or partial toxin sequences published, which can be classified into 17 subtypes based on sequence homology.
2. The potency of the neurotoxin and the large number of doses required dictates the need for highly potent therapies. While it is not possible to predict the maximum exposure levels that may be achieved as a result of intentional poisoning, based on a number of assumptions/predictions Dr. Marks has calculated a minimum potency of 10 IU/mg would be required for a commercially viable product.
3. No single antibody has been found to achieve this target potency. Dr. Marks has found that increasing the affinity of single antibodies increases their potency, but only up to a point. To date, only a cocktail of high affinity antibodies has demonstrated the required neutralization capacity in the mouse potency assay.

Antibodies that neutralize multiple subtypes must be selected by screening against the different subtypes. This emphasizes the need to have a complete characterization of the BoNT subtypes at an early stage of antibody discovery. In this regard, it is also important to consider the types and subtypes of the neurotoxin that animals from which immunoglobulin gene repertoires will be derived are immunized with. Technology to produce very high affinity monoclonal antibodies derived from immunized sheep exists. This technology has not been evaluated for its potential to produce higher potency antibodies than what are currently being developed through molecular evolution. It is essential to better understand the mechanisms of antibody-mediated toxin neutralization and clearance to determine whether emphasis should be placed on the discovery/creation of antibodies with exceedingly high affinities or whether other factors (e.g. Fc receptor-mediated clearance) also need to be considered and selected for.

Studies to define the mechanisms of antibody-mediated toxin neutralization and clearance were discussed: mapping of neutralizing epitopes and structural studies of neutralizing antibody bound to neurotoxin are needed. There is a need to better define and understand the role of multiple antibodies in the observed incremental increase in potency with each additional antibody. For example, are multiple receptor/binding sites being blocked or is increased potency related to a

sheer stoichiometric effect of molecules of antibody bound per molecule of neurotoxin? What is the impact of Fc-mediated clearance of neurotoxin-antibody complexes on efficacy?

More data on the pharmacokinetics of BoNTs alone and in the presence of neutralizing antibodies after oral or inhalational exposure is required to make better estimates of likely blood toxin levels and thus requirements for potency and dose of neutralizing antibody products.

The need for antibodies with the longest possible *in vivo* half-life was discussed. These are likely to be antibodies of the IgG₁, IgG₂ or IgG₄ subclass. It was suggested that the half-life of antibodies could be improved through the addition of larger polymers, which may also increase the size domain in terms of blocking receptor-binding sites.

The need to manufacture multiple antibodies to neutralize any single serotype has significant cost implications. It is important to explore new technologies that would reduce the cost of manufacturing multiple monoclonal antibodies. These technologies include, but are not limited to, production of multiple monoclonal antibodies from a single working cell bank, transgenic animals, transgenic plants, and algae.

Polyclonal Antibodies: The advantages of a polyclonal product that neutralizes multiple serotypes of neurotoxin are clearly outlined in the previous report (http://www.niaid.nih.gov/dmid/pdf/bot_toxins.pdf). The current equine anti-sera and BabyBIG[®] are both polyclonal. BabyBIG[®] is derived from the blood of human donors vaccinated with a pentavalent (ABCDE) toxoid vaccine. Although this provides an excellent treatment for the small number of cases of infant botulism, this approach would not support production of the number of doses needed for a massive bioterrorist event.

The safety concerns associated with the use of non-human antibodies are clear. Several efforts are underway to produce animals transgenic for essential parts of the human antibody locus and which also lack the ability to utilize their own antibody locus. Significant progress is being made in the development of these transgenic animals; however, final proof of principle has yet to be achieved.

Finally, it was noted that in the event of a large-scale exposure to BoNTs, and given the current diagnostic capability, individuals are likely to be treated without knowing their actual exposure status. Given that presymptomatic treatment will include some individuals who have not been exposed, such treatments need to have an exceptional safety profile.

The development and provision of a panel of reference toxins that represent the intra-serotype diversity is needed. Also, a clearinghouse for the head-to-head comparison of antibodies as they are discovered would be the most efficient method for antibody candidate selection and moving forward to a final product.

The expedient development of antibody-based therapies will require:

- sufficient resources;
- access to and characterization of representative *Clostridium* isolates and neurotoxin complexes of each serotype, which requires import permits to obtain strains;
- reference reagents: pure and complexed toxin;
- definition of reference animal models;
- a well thought out development plan, with effective implementation;
- 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 in surrogate animals.

Small Molecule Inhibitors

Moderator: Dr. Charles Millard, USAMRIID

The DoD has a significant number of research activities in the BoNT inhibitor field, which were summarized by Dr. Millard. These activities include the development of the research resources required to pursue an efficient inhibitor discovery program. The underpinning research resources are:

- high-throughput screening assays,
- access to large libraries of putative therapeutics,
- toxin therapy compound database and repository,
- standard reagents,
- structural biology data to enhance rational design, and
- access to high performance computing.

A relevant target for small molecule inhibitors is the toxin active site, although, the large size of the active site may make this a more challenging target for small molecule inhibitors such as peptide analogs. In addition, there are two potential active sites on the light chain: the zinc binding site and the substrate recognition site. It was thought that the zinc-binding site is more likely to be conserved between serotypes and thus held some promise of identifying cross-protective inhibitors. On the other hand, the substrate recognition sites remain poorly characterized and more basic research is needed to characterize these targets.

Alternatively, small molecules that bind directly to neuronal cell receptors or receptor mimics might be considered as targets for small molecule inhibitors. One caveat of this approach is that blocking normal receptor function may have deleterious side effects. Low and high affinity receptors have been described. The low affinity receptor may be a lipid and accessible prior to binding the high affinity receptor, but the actual mechanism is unknown. Not only are receptors not well characterized but their normal physiological functions and the effects of blocking are also not known. In addition, blocking toxin action at the level of receptor binding does not improve the limited therapeutic window of currently available antibody-based therapies.

The potential to combine small molecule therapeutics and immunotherapy was also discussed as an opportunity to increase the overall effectiveness of treatment.

Several classes of compounds to reverse the effects of the toxin after exposure have been studied in experimental systems, including 3,4-diaminopyridine (3,4-DAP) and toosendanin. To date, these approaches have been shown to be either minimally effective or potentially unsafe. However, it was suggested that slow release formulation of these indirect treatments or other potassium blockers might improve safety. This approach was considered to be high risk (low probability of success) but with a potentially high payoff.

Additionally, the use of lectins to compete with toxin for binding to carbohydrate receptors on neurons has been explored in preliminary experimental systems. Lectins were criticized as a potential therapeutic, however, because they lack specificity of binding.

Emphasis should be placed on therapies that will lengthen the therapeutic window and reverse symptomatology; the long-range goal is to target toxin within the neuronal cell. This will require programs not just to identify inhibitors but also drug delivery into the cytoplasm of the peripheral cholinergic nerve cells. More focus on understanding the mechanisms associated with the persistence of toxin and thus the long-term clinical effect is needed. New data from Allergan Inc. reported at the 2003 Interagency Botulism Research Coordinating Committee (IBRCC) meeting demonstrated localization of the BoNT A light chain to the plasma membrane of affected cells, suggesting some type of sequestration that may be responsible for protecting the light chain from degradation.

The natural biology and ability of the BoNT heavy chain to be endocytosed and translocated may represent an opportunity to use it as a delivery vehicle for inhibitors. One drawback of this approach is that anti-heavy chain antibodies may be induced, thus preventing future medical use of BoNTs in that individual.

Resources that would enhance small molecule inhibitor discovery programs include:

- availability of assays: cell-free and cell-based (DoD assays published),
- reference reagents (e.g., substrates),
- bioinformatics (also to share negative results),
- medicinal chemistry,
- access to quality-controlled compound libraries,
- decision-tree network for drug discovery, and
- human resources.

Clinically Relevant Targets

Moderator: Dr. Lance Simpson, Thomas Jefferson University

Dr. Simpson outlined possible targets, which led to a discussion of strategies that are the most relevant based on:

- Is the strategy clinically appropriate?
- Is there a viable way to achieve the strategy?
- What are the major knowledge gaps?
- What are the major technical gaps?
- Are there promising opportunities that should be explored?

The discussion of the potential strategies was placed in the context of some fundamental principals of intervention based on various levels of exposure and varying times that patients might present for treatment following exposure. The need for minimum triage, and minimum utilization of human resources and medical facilities was also considered. In addition, the risk-to-benefit ratio should be acceptable for non-exposed persons and desirable for exposed persons.

The following strategies were discussed:

1. neutralization prior to absorption (gut and airway),
2. block absorption (gut or airway),
3. neutralization in the general circulation or extracellular space,
4. inactivation in the general circulation or extracellular space,
5. blockade of escape from vasculature,
6. antagonism of neuronal binding (one receptor vs two receptor models),
7. antagonists of productive internalization (endocytosis and/or translocation),
8. inhibition of endoprotease activity (inhibition of binding; inhibition of catalytic activity), and
9. promotion of metabolism (proteosome vs lysosome).

In summary, the post-exposure therapeutic window for neutralization prior to absorption or blocking absorption in the gastrointestinal tract or lung is too short. Individuals are unlikely to be aware that they have been exposed until they become symptomatic. Moreover, current diagnostics do not allow for the rapid detection of BoNTs in clinical samples, so even those patients that suspect exposure cannot be definitively diagnosed presymptomatically. It should be noted, however, that in the context of prophylactic protection, blocking toxin absorption from the gastrointestinal tract or lung is likely to be an effective strategy.

Neutralization or inactivation in the general circulation was discussed in the context of antibody-based inhibitors, as well as small molecule inhibitors (see previous sections). The post-exposure therapeutic window for these targets is slightly longer but still less than optimal. Once a patient is symptomatic these treatments have limited value, as most toxin will already have been

internalized and be protected in the intracellular environment. Again the value of passive or active immunity as a prophylactic strategy has been demonstrated in small animal models.

Nothing is known regarding the processes involved in the escape of toxin from the vasculature, therefore basic knowledge of this area does not currently allow rational design of inhibitors.

Strategies that target blocking BoNT binding to peripheral cholinergic neuronal cells will require a clearer understanding of the receptors than is currently available. The two-receptor model proposes the involvement of a low and a high affinity receptor. The low affinity receptor is thought to be a ganglioside, possibly sialic acid, and the high affinity receptor a synaptotagmin (14 isoforms have been described). This model does not explain how the BoNTs specifically target peripheral cholinergic cells, as both gangliosides and synaptotagmins are present on other cell types. Whether the receptors are the same for all BoNT serotypes is also still in question. Furthermore, targeting either gangliosides or synaptotagmins and inhibiting or blocking their normal function is likely to cause unacceptable side effects.

The precise mechanism of neuronal endocytosis is also not fully understood. Workshop participants discussed the evidence for the endosome as a recycling synaptic vesicle. As endocytosis is a global cellular mechanism how this mechanism could be exclusively targeted to peripheral cholinergic cells was not obvious, given current technology and knowledge.

Blocking translocation of the BoNT to the cytosol, although conceptually feasible, all channel blockers assessed to date were shown to be very toxic (e.g., a lethal dose of chloroquin was required to slow BoNT activity). Inhibition of chaperone proteins such as HSP70 was also thought to not be a good strategy because of the potential to inhibit overall protein synthesis. More information is needed on the critical elements of the BoNT structure that are required for translocation in order to consider designing compounds to disrupt that function.

Inhibition of the endoprotease activity of the BoNTs was seen to be a clinically relevant strategy, and the only strategy with the potential to reverse the clinical effects of intoxication: paralysis. This strategy is particularly attractive for treatment of BoNT A, as its persistence and clinical effect may last months. It is technically feasible to identify inhibitors for each BoNT serotype. A recent publication by has described good candidate inhibitors for serotype B. The most technically challenging aspect of this strategy may be the delivery of the inhibitor specifically into cholinergic nerve cells. There has been research interest in utilizing the BoNT heavy chain biological activity to bind, endocytose and translocate as a delivery vehicle for endopeptidase inhibitors. The inhibitors may be high affinity molecules with a high on:off rate ratio that compete with the enzyme's interaction with normal substrate, molecules that bind irreversibly and inhibit, or suicide substrates. Inhibitors may take the form of a pro-drug that is activated only when it reaches the appropriate cellular compartment. The discovery of such inhibitors requires a more focused and concentrated effort than is currently underway. A high throughput screening, medicinal chemistry structure-based iterative process is needed.

Understanding the mechanism by which serotype A is able to persist for such long periods may introduce new targets for accelerating the catabolism of the BoNT light chain and thus shortening the period of paralysis.

One other strategy that did not involve direct inhibition of the endoprotease was to replace the substrate at a rate that would allow for normal function of the cells (e.g., SNARE mimetic).

Identification and Characterization of BoNT Targets: Peptide Domains **Moderator: Bal Ram Singh, University of Massachusetts, Dartmouth**

This session focused on identifying domains on the BoNT molecule that are likely to represent targets for inhibitors. These domains may be required for binding to peripheral cholinergic

neuronal cells, endocytosis, translocation, or endopeptidase activity. Once again, general lack of knowledge of many of these mechanisms and specifically the essential BoNT domains involved in these processes does not allow the rationale design of inhibitors. New technologies and approaches are required to define the essential domains of the BoNT molecules involved in these processes.

Isolation of receptors on peripheral cholinergic cells, and characterizing affinities and binding sites were identified as high priorities. Competitive binding experiments with synthetic peptides or recombinant holotoxin with site specific mutations may be useful in delineating BoNT sites involved in receptor binding. In addition FRET, NMR, x-ray and electron crystallography, microcalorimetry, and atomic force microscopy were described as potentially useful tools for mapping receptor domains of BoNT.

Translocation of BoNT across the endocytic membrane is one of the least understood steps. Although a long hydrophobic segment within the heavy chain has been shown to be involved in the translocation process, it is possible that other domains of the toxin, or proteins in the nerve cell membrane are also involved. Improved cell culture systems are needed to further explore the mechanisms of endocytosis and translocation. Once again, holotoxin mutants are likely to be a useful tool in understanding the critical toxin domains for these processes.

Progress is being made in identifying the toxin domains involved in substrate recognition and cleavage. High throughput screening assays, synthetic peptides and recombinant light chain mutants are being employed. Such information will be critical for designing specific inhibitors.

Recommendations to further progress in identifying functional peptide domains of BoNT:

- Additional biochemical and biophysical characterization of BoNT in order to establish quantitative parameters for recombinant or mutant BoNT with altered peptide domains (see next section).
- Application of molecular mapping techniques such as FRET, x-ray crystallography, molecular modeling, and NMR to understand the structure and role of specific peptide domains.

Biophysical and Biochemical Characterization of BoNT

Moderator: Bal Ram Singh, University of Massachusetts Dartmouth

This session focused on the areas in which better biophysical and biochemical characterization of the BoNTs is needed. A better understanding of the biochemical features of BoNT is critical for developing assays, identifying therapeutic targets, and understanding the mechanisms by which the light chain persists and remains active within neuronal cells. Biophysical characterization of the BoNTs is important to understand the biologically active states and model structures for designing inhibitors.

Biophysical features such as molecular size, isoelectric points, secondary, tertiary, and quaternary structures, are known for some serotypes, either through x-ray crystal structures or through spectroscopic, cryomicroscopic, and hydrodynamic techniques. More comprehensive studies are, however, needed to better define the biophysical nature of BoNTs under physiological conditions.

Defining the structure of the receptor in the membrane, the structure of the toxin-receptor complex, and the effect of pH on the receptor structure were identified as high priorities. High-resolution spectroscopic techniques, electron microscopy, molecular modeling and x-ray crystallography may be applied to these problems. The availability of non-toxic but otherwise biologically active holotoxin would advance this field.

More information is needed to better understand the structural/conformational changes associated with binding to receptors on peripheral cholinergic cells, endocytosis and translocation. Electron crystallography holds particular promise in helping solve these questions. The role of pH and membrane channels in endocytosis and translocation processes needs to be better defined. Proof of the pore and the structure of the toxin in the membrane are still needed.

More detailed knowledge of active site topography of the light chain in the cytosol is also required for the rational design of inhibitors.

Genetic Analysis

Moderator: Eric Johnson, University of Wisconsin

This session focused on the knowledge base of genetic variability and gene arrangements in different serotypes and sub-serotypes of *C. botulinum* toxin gene clusters. Dr. Johnson presented data that aptly demonstrated the issues related to not only significant variation within the neurotoxin gene sequences but also gene arrangements for the accessory proteins.

Further development of clostridial genetics requires:

- ❖ improved expression systems for clostridial genes;
- ❖ gene replacement systems to enable manipulation of genes in the genome;
- ❖ understanding the mechanisms governing interspecies transfer of toxin genes; and
- ❖ better understanding the role of transposons, IS elements, conjugative plasmids (i.e. plasmids that can be used to transfer genes into the same or different species).

Basic Mechanistic Biology

Moderator: Subramanyam Swaminathan, Brookhaven National Laboratory

The purpose of this session was to focus on the outstanding questions related to the basic biology of the BoNTs transport and activity *in vivo*.

The general lack of detailed knowledge on transport across gut and lung, receptor mediated endocytosis, and translocation, were again highlighted.

The role of auxiliary proteins, particularly in transport across the gut and lung, was discussed. Although there are still gaps in our knowledge concerning the role of these proteins, this is not considered a constraint to the development of new therapeutics.

Whether BoNT binds or interacts with normal serum components was also discussed, as a largely unexplored area.

Translocation was considered to be the least studied process. Outstanding questions were discussed regarding:

- what induces channel formation,
- is oligomerization a pre-requisite for channel formation,
- does the catalytic domain separate from the heavy chain,
- does the catalytic domain unfold and refold in the cytosol, and
- could channel blockers be developed as therapeutics?

Other questions, as to how inhibitors might be targeted to neurotoxin-containing vesicles arose. The kinetics of the endocytosis and translocation process remains largely unknown. Whether targeting the translocation process would significantly lengthen the therapeutic window compared to targeting circulating toxin is still in question.

In the cytosol, the role of the heavy chain is unknown. The substrate recognition sites remain largely unsolved. Serotypes A and C cleave the same substrate (SNAP-25) but at peptide bonds differing by one residue. The same is true for serotypes D and F whose substrate is VAMP. Although enzymatic cleavage sites may be shifted by just residue, the essential elements of the substrate recognition sites and to what extent they differ between serotypes with common substrates is not known. This information is important in considering the feasibility of identifying cross-protective inhibitors.

Stability and Pharmacokinetics of BoNT and Inhibitors

Moderators: Lance Simpson, Thomas Jefferson University and Jim Marks, UCSF

This session focused on needs for basic data on the pharmacokinetics of the BoNTs in the presence and absence of putative inhibitors. Data on the rate of absorption following oral or inhalational exposure, plasma half-life, and extracellular fluid half-life are needed. These studies will be required to understand the most appropriate use and therapeutic window for both existing and new therapies. Availability of a full-length non-toxic holotoxin would greatly advance this area.

Issues related to the intracellular stability and possible compartmentalization of the BoNTs were discussed. Could metabolism of BoNTs be promoted in the proteasome or the lysosome?

Research Resources

Moderators: Saul Tzipor, Tufts University

This session focused on the outstanding needs for research tools to advance the discovery and development of new therapeutics.

In vitro Assays: Current antigen detection *in vitro* assays are relatively sensitive (pg range) but not useful for screening for inhibitors.

More cell-based (relatively high throughput) assays are needed. A cell-based assay that utilizes an immortalized cell line and bears authentic receptors and can internalize toxin is needed. Additionally, a channel membrane assay to determine whether translocation is blocked would benefit the field.

In vivo Assays: Currently the mouse protection assay detects the equivalent of 1 MLD₅₀ (aprox. 7 pg) of BoNT. The rat hind limb denervation assay in which standardized doses of toxin are used to induce paralysis of the extensor digitorum longus muscle is used to evaluate the effects potential inhibitors or therapeutics. This assay requires relatively pure toxin preparations to avoid infections and nonspecific reactions, and it also has a very qualitative endpoint (i.e., when does the animal regain limb function).

The classical hemidiaphragm assay gives insights into *in vivo* efficacy of therapeutics and inhibitors at the neuromuscular junction.

It has been recognized that, in addition to mice and rabbits that are commonly used, guinea pigs and other animals may need to be used for some small animal studies, as rats are rather insensitive to the BoNTs.

Reagents: Toxin reagents need to be very well quality-controlled. There is a need for reliable expression and purification systems for the production of recombinant toxin fragments and inactive holotoxin. Many of the recombinant light and heavy chain products have proven to be unstable. Methods for their stable production in high yields are needed.

Information management systems are needed to support high throughput assays for the development of therapeutics, as well as, bioinformatics systems for genomic and proteomic analysis.

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