Alternatives to the Mouse LD_{50} Assay For Botulinum Toxin Testing: An ICCVAM/NICEATM/ECVAM Sponsored Workshop

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Introduction

Currently, the mouse LD_{50} assay, which determines the concentration of botulinum neurotoxin (BoNT) that kills 50% of the mice tested, is used both for detecting BoNT and assessing the potency of BoNT-containing products. This assay is the predominant method used for submission to U.S. and European regulatory agencies. However, a number of alternative methods have recently been developed that may eventually reduce, refine (less pain and distress), and replace animal use.

In response to a nomination from the Humane Society of the United States (HSUS)[1], a workshop was organized by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for Alternative Toxicological Methods (NICEATM), and the European Centre for the Validation of Alternative Methods (ECVAM), and was convened in Silver Spring, MD on November 13 and 14, 2006. This workshop involved leading BoNT research scientists, representatives from national and international regulatory authorities, and the animal protection community.

[1]The nomination can be viewed at: http://iccvam.niehs.nih.gov/methods/biologics/botdocs/HSUSnomLD50.pdf

Workshop Goals

1. To review the state-of-the-science and current knowledge of alternative methods that may reduce, refine, and replace the use of mice for BoNT testing.
2. Identify priorities for research, development, and validation efforts needed to advance their use.

Workshop Objectives

- Review public health needs for BoNT testing, including the necessity to determine the safety and efficacy of products containing BoNT.
- Review the current state-of-the-science and identify knowledge gaps for BoNT structural aspects, mechanisms, and modes of action important to development of alternative methods, and prioritize future research initiatives to address them.
- Review current development and validation status of alternative methods and their potential to reduce, refine, or replace the use of the mouse LD_{50} assay.
- Identify alternative methods that should have the highest priority for development and validation studies to assess BoNT potency/toxicity.

Summary Of Workshop Outcomes

The general consensus of the panel discussions was that some alternatives could be used now, in specific circumstances (e.g., lot release testing or potency confirmation by someone other than the manufacturer) or in tiered-testing, to refine or reduce the use of mice for BoNT testing. However, none of the reviewed methods have been adequately validated as complete replacements for the mouse LD_{50} assay, although panelists noted that some methods show promise for eventually achieving this goal. It was stressed that any validation study must be specific for the intended use of a method, and that validation against the LD_{50} assay is critical if the intended use is to replace it. Best practices to decrease the number of animals tested were also proposed and include: (a) use of appropriate reference standards (b) standardized methodology, and (c) fewer doses tested in confirmatory assays.

A detailed report on the outcomes of the workshop will be accessible at: http://iccvam.niehs.nih.gov/methods/biologics/bot_workshop.htm.

Session 1: Overview of Public Health Needs for Botulinum Toxin Testing and Regulatory Requirements

This session summarized public health needs for testing and regulatory requirements in the U.S. and Europe to determine safety and efficacy of BoNT-containing products.

Moderators: A. Jacobs, Ph.D., U.S. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) and J. Kulpa-Eddy, D.V.M., U.S. Department of Agriculture (USDA)

Presentations

Overview of Botulinum Toxin and the Incidence and Severity of Botulism - S. Maslanka, Ph.D., U.S. Centers for Disease Control and Prevention (CDC)

Current Testing and Practices for Botulinum Prevention in Foods - S. Sharma, Ph.D., FDA, Center for Food Safety and Applied Nutrition (CFSAN)

Medical Conditions Treated with Botulinum Toxin - M. Hallett, M.D., U.S. National Institutes of Health (NIH)

Current Potency Testing Requirements and Practices for Botulinum Toxin Products - E. Shores, Ph.D., FDA, CDER

Current Testing Requirements and Practices for Botulinum Toxin for Vaccine Potency Testing - J. Kulpa-Eddy, D.V.M., USDA

Current Animal Diagnostic Testing Requirements and Practices for Botulinum Toxin Potency and Detection - T. Rocke, Ph.D., United States Geological Survey (USGS), National Wildlife Health Center (NWHC)

Session 2: Current Understanding and Knowledge Gaps for Botulinum Toxin

This session summarized the current understanding of structural aspects, mechanisms, and modes of action of BoNT; discussed the aspects of the endopeptidase (EP) function that must be modeled by alternative test methods; and prioritized research needs to address gaps needed to facilitate the development of alternative test methods.

Moderators: J. Keller, Ph.D., FDA, Center for Biologics Evaluation and Research (CBER), and R. Ramabhadran, Ph.D., U.S. Environmental Protection Agency (EPA)

Presentations

Overview of the Modes and Mechanisms of Action of Botulinum Toxin - D. Dressler, M.D., Ph.D., Rostock University, Germany

Pharmacokinetics of Botulinum Toxin - L. Simpson, Ph.D., Thomas Jefferson University, U.S.A.

Key Outcomes from Panel Discussion

Panelists: D. Dressler, M.D., Ph.D., Rostock University, Germany; L. Simpson, Ph.D., Thomas Jefferson University, U.S.A.; E. Johnson, Sc.D., University of Wisconsin, U.S.A.; A. Rummel, Medical School of Hannover, Germany; M. Hallett, M.D., NIH; and S. Sharma, Ph.D., EPA

- Knowledge gaps in understanding of the mechanism of action of BoNT that must be addressed to develop alternative methods for BoNT testing include:
  - Characterization of receptors for all serotypes, the roles of other proteins in the BoNT complex and effects on potency.
  - The extent that potency depends on the intended use of the method.
- Future research should focus on:
  - Development of a *functional* assay; currently, no single alternative assesses all functions of the BoNT molecule.
  - Development of cell-based assays that mimic presynaptic function.
  - Characterization of mechanism(s) involved in recognition of receptors and substrates, and light chain internalization/translocation.
- Regulatory agencies should express expectations and provide internationally harmonized guidance about alternatives development.
- To bridge from the LD_{50} test to alternative tests:
  - Calibrate alternative test results in terms of mouse LD_{50} units.
  - Develop reference standards that were tested in the LD_{50} test for use in validation studies.
- If an alternative is comparable to the LD_{50} test for a particular application, the LD_{50} test can be eliminated for that application and results from the new test expressed in LD_{50} equivalent units.

Session 3: Potential Replacement of Animal Use for BoNT Potency Testing

This session provided an overview of alternative *in vitro* models that, if sufficiently validated, could replace the current *in vivo* test.

Session 3A: Endopeptidase (EP) Assays

Moderators: S. Maslanka, Ph.D., CDC, and S. Sharma, Ph.D., FDA, CFSAN

Presentation

Overview of Endopeptidase Assays - D. Sesardic, Ph.D., U.K. National Institute for Biological Standards and Control (NIBSC)

Key Outcomes from the Panel Discussion

Panelists: D. Sesardic, Ph.D., NIBSC; J. Barr, Ph.D., CDC; A. Pickett, Ph.D., Ipsen, Ltd., U.K.; J. Schmidt, Ph.D., United States Army Medical Research Institute of Infectious Diseases (USAMRIID); C. Shone, Ph.D., Health Protection Agency, Centre for Emergency Preparedness and Response, U.K.; F. Gessler, Ph.D., University of Goettingen, Germany; E. Johnson, Sc.D., University of Wisconsin, U.S.A.; B. R. Singh, Ph.D., University of Massachusetts, U.S.A.; and R. Ramabhadran, Ph.D., EPA

- The EP assay cannot currently replace animal testing for BoNT detection or potency assessments
- In principle, an EP assay could be used to estimate BoNT concentration in a drug product, allowing for reduction in animal use.
  - An immediate reduction could be achieved if an EP assay was run in parallel with an LD_{50} assay, to eliminate neutralization tests.
- For an EP assay to replace the mouse bioassay:
  - It must be at least as sensitive as the mouse LD_{50} test.
  - It should detect all BoNT subtypes with the desired sensitivity.
  - The sensitivity must be unaffected by sample matrix.
  - Results should be obtainable within 5 hours.
  - Cost must not be prohibitive.
  - The results must be reproducible.

Session 3B: Cell-Based Assays

Presentation

Overview of Cell-Based Assays - K. R. Aoki, Ph.D., Allergan

Key Outcomes from the Panel Discussion

Panelists: K. R. Aoki, Ph.D., Allergan; A. Rummel, Medical School of Hannover, Germany; M. Adler, Ph.D., United States Army Medical Research Institute of Chemical Defense (USAMRICD); J. O. Dolly, Ph.D., Dublin City University, Ireland; G. Gross, Ph.D., University of North Texas, U.S.A.; L. Smith, Ph.D., USAMRIID; J. Keller, Ph.D., FDA, CBER; and F. Gessler, Ph.D., University of Goettingen, Germany

- No cell-based method can now replace or reduce animal use, but potential exists.
- An advantage of the cell-based assay format is that it may be the best *in vitro* option to assess all three BoNT intoxication mechanisms (i.e., binding, translocation, and catalysis).
- Disadvantages of cell-based methods include:
  - Limiting factors include: ease of use, sensitivity, robustness, transferability, precision, reproducibility, cell line variability, and shelf life.
  - Cell-based assays are an order of magnitude less sensitive than LD_{50} test.
  - The assays are very unpredictable.
  - Sensitivity is poor - most methods work only with purified BoNT.
  - Variability relative to the LD_{50} assay is unknown.
  - The use of multiple cell lines, which may more closely mimic the *in vivo* mouse model, may be too complex for uniform adoption.
- Knowledge gaps that must be addressed to further the use of cell-based assays in BoNT potency testing or detection include:
  - Which cell lines are optimal.
  - Better understanding of the process of differentiation in motor neurons.
  - Better characterization of binding effects, receptors, expression, and sensitivity to environmental effects.
- For a cell-based assay to be considered a replacement for the mouse bioassay, it must:
  - Measure both the inhibition of neurotransmitter release and substrate cleavage.
  - Be standardized, rapid, easy to maintain, easily transferable, reproducible across laboratories, and not exhibit matrix effects.
  - Be as sensitive as the mouse bioassay and show reproducible correlation between the activity it measures and mouse LD_{50} units.
  - For practical reasons, an ideal assay would use an immortalized cell line instead of primary cultures.

Session 4: Refinement (Less Pain and Distress) of Animal Use for BoNT Potency Testing

This session provided an overview of alternative methods that, if adequately validated, could reduce or eliminate pain and distress associated with the current *in vivo* test. Three different approaches were discussed:

- *Ex vivo* test models prepared from humanely killed animals (i.e., the mouse phrenic nerve [MPN] assay).
- Alternate *in vivo* models to measure BoNT activity with non-lethal endpoints (i.e., the mouse hind limb and the mouse abdominal ptosis assays).
- The use of earlier non-lethal humane endpoints for the mouse LD_{50} assay.

Moderators: A. Rosenberg, Ph.D. FDA, CDER; L. Smith, Ph.D., USAMRIID; and W. Stokes, D.V.M., D.A.C.L.A.M., U.S. National Institute of Environmental Health Sciences (NIEHS)

Session 4A: *Ex Vivo* Methods

Presentation

Mouse Phrenic Nerve-Hemidiaphragm Assay - A. Rummel, Medical School of Hannover, Germany

Key Outcomes from the Panel Discussion

Panelists: D. Sesardic, Ph.D., NIBSC; E. Johnson, Sc.D., University of Wisconsin, U.S.A.; J. Calver, Ph.D., Calver Biologics Consulting, Canada; A. Rummel, Medical School of Hannover, Germany; C. Hendriksen, M.Vet.Sc., D.V.M., Ph.D., Netherlands Vaccine Institute; M. Stephens, Ph.D., HSUS; J. Keller, Ph.D., FDA, CBER; J. Schmidt, Ph.D., USAMRIID; and M. Adler, Ph.D., USAMRICD

- The MPN assay is currently undergoing validation in Germany. It may already be considered adequate for batch release testing
- The MPN assay may reduce animal use for potency testing by 50% or more.
- Advantages of the MPN assay include:
  - It captures all mechanisms of intoxication.
  - Results are obtained within 2 hours.
  - Experimental conditions are easily varied.
  - The assay can quantify neutralizing antibodies.
  - The assay has quantitative endpoints.
- Disadvantages of the MPN assay include:
- Low throughput.
- Assays are technically challenging.
- Matrix effects are not completely resolved.
- The Intercostal Neuromuscular Junction (INJ) assay, is also undergoing validation in the U.K. Like the MPN assay, it is also technically challenging and matrix effects are problematic.

Session 4: Refinement (Less Pain and Distress) of Animal Use for BoNT Potency Testing

Session 4B: Non-lethal *In Vivo* Methods

Presentations

Mouse Hind Limb Assay - K. R. Aoki, Ph.D., Allergan

Mouse Abdominal Ptosis Assay - D. Sesardic, Ph.D., NIBSC

Key Outcomes from the Panel Discussion

Panelists: D. Sesardic, Ph.D., NIBSC; E. Johnson, Sc.D., University of Wisconsin, U.S.A.; J. Calver, Ph.D., Calver Biologics Consulting, Canada; A. Rummel, Medical School of Hannover, Germany; C. Hendriksen, M.Vet.Sc., D.V.M., Ph.D., Netherlands Vaccine Institute; M. Stephens, Ph.D., HSUS; J. Keller, Ph.D., FDA, CBER; J. Schmidt, Ph.D., USAMRIID; and M. Adler, Ph.D., USAMRICD

- The death endpoint might be replaced with one from which mice recover.
- Advantages of the Mouse Hind Limb Assay
  - The endpoint, local weakness, is a clinically relevant measure of activity.
  - The assay shows good dose response and repeatability.
- Advantages of the Abdominal Ptosis Assay
  - It is robust and easily transferable with dosing similar to clinical use and measures all biological functions of BoNT.
  - Animals normally exhibit no signs of stress or pain; requires minimal monitoring.
  - Rapid (results in 48 hrs) and requires no specialized equipment or reagents.
- Disadvantages of both Mouse Hind Limb Assay and Abdominal Ptosis Assay
  - Calibration vs. the LD_{50} assay is required.
  - These assays are labor intensive and there are associated transferability and training issues.
  - In order to determine a non-lethal dose, the level of BoNT in the sample must be known.
  - These assays are refinements, not replacements or reductions.
  - Scoring is subjective and qualitative.
- Knowledge gaps include:
  - Knowledge about correlation with LD_{50}; selection of suitable samples to be included in studies will be critical.
  - Knowledge about transferability and robustness.
  - Standardization of test method protocols is needed.

Session 4C: Humane Endpoints

Presentations

Overview of the Physiological Progression of Botulism in Mice - E. Johnson, Sc.D., University of Wisconsin, U.S.A.

Potential Behavioral and Pharmacological Endpoints Predictive of Mouse Lethality - J. Calver, Ph.D., Calver Biologics Consulting, Canada

Key Outcomes from the Panel Discussion

Panelists: D. Sesardic, Ph.D., NIBSC; E. Johnson, Sc.D., University of Wisconsin, U.S.A.; J. Calver, Ph.D., Calver Biologics Consulting, Canada; A. Rummel, Medical School of Hannover, Germany; C. Hendriksen, M.Vet.Sc., D.V.M., Ph.D., Netherlands Vaccine Institute; M. Stephens, Ph.D., HSUS; J. Keller, Ph.D., FDA, CBER; J. Schmidt, Ph.D., USAMRIID; and M. Adler, Ph.D., USAMRICD

- Moribund animals (i.e., those in a pre-death condition) should be euthanized, even though some animals that become moribund near the end of the study could survive until the end of the study period.
- Health Canada has validated and has been using an earlier non-lethal endpoint (i.e., severely raised scaphoid in conjunction with hiccough and eyes wide open). A collaborative study using Health Canada endpoint observations should be conducted.
- Studies should also be conducted to determine other potential non-lethal endpoints obtained during the observation period that are predictive of death.
- Increased observation frequency may identify moribund animals and decrease spontaneous deaths.
- Other clinical signs that occur during botulism, their severity, and reversibility (essential to accurately predict death) must be documented.
- Evaluate each clinical sign, or a battery of clinical signs, and severity for use as a predictive humane endpoint.
- Collect complete clinical signs and other objective data during routine LD_{50} studies to identify predictive early endpoints.

Session 5: Reduction of Animal Use for *In Vivo* Botulinum Testing

This session discussed strategies to reduce the number of animals used in the current *in vivo* test.

Moderators: M. Halder, Ph.D., ECVAM and R. McFarland, M.D., Ph.D., FDA, CBER

Presentations

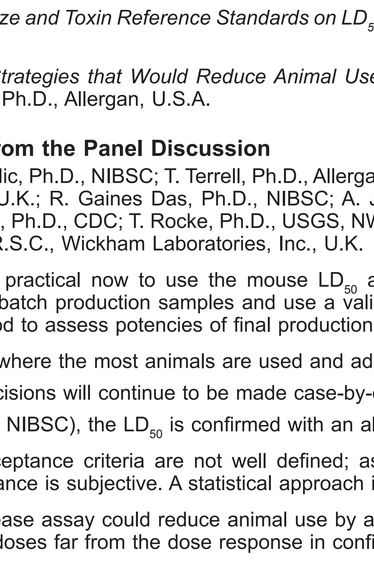
Impact of Sample Size and Toxin Reference Standards on LD_{50} Results - R. Gaines Das, Ph.D., NIBSC

Proposed Testing Strategies that Would Reduce Animal Use in Botulinum Toxin Testing - K. Clarke, Ph.D., Allergan, U.S.A.

Key Outcomes from the Panel Discussion

Panelists: D. Sesardic, Ph.D., NIBSC; T. Terrell, Ph.D., Allergan, U.S.A.; A. Pickett, Ph.D., Ipsen, Ltd., U.K.; R. Gaines Das, Ph.D., NIBSC; A. Jacobs, Ph.D., FDA, CDER; S. Maslanka, Ph.D., CDC; T. Rocke, Ph.D., USGS, NWHC; and C. Bishop, B.Sc., C.Chem., F.R.S.C., Wickham Laboratories, Inc., U.K.

- It is feasible and practical now to use the mouse LD_{50} assay to assess the potency of BoNT batch production samples and use a validated *in vitro* and/or *ex vivo* test method to assess potencies of final production lots.
  - Identify areas where the most animals are used and address these first.
  - Regulatory decisions will continue to be made case-by-case.
- In the U.K. (at the NIBSC), the LD_{50} is confirmed with an alternative test.
- Comparability acceptance criteria are not well defined; assigning prospective criteria for acceptance is subjective. A statistical approach is needed.
- A modified lot release assay could reduce animal use by allowing for testing of fewer animals at doses far from the dose response in confirmatory tests.
- A potency reference standard program reduces *in vivo* testing by refinements to:
  - Extend the shelf life of the working reference standard.
  - Improve the efficiency of the qualification program.
- In validation studies, it is essential to use a common set of suitable samples. Inclusion of a set of common samples with known long-term stability and in sufficient quantity for multiple uses is therefore desirable.
- Use and establishment of an international standard would contribute towards harmonization; however, this would be very difficult to implement.
- At present, each manufacturer uses its own, product specific standard for potency testing.



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More information on ICCVAM and NICEATM can be accessed at
http://iccvam.niehs.nih.gov/

NIEHS National Institute of Environmental Health Sciences

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods