Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)/National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/European Centre for the Validation of Alternative Methods (ECVAM) Scientific Workshop on Alternative Methods to Refine, Reduce and Replace the Mouse LD$_{50}$ Assay For Botulinum Toxin Testing

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Introduction

Botulism, a potentially deadly illness, can be acquired by humans from consumption of food contaminated with a toxin excreted by the bacterium *Clostridium botulinum*. The toxin causes muscle paralysis due to its action on the nervous system and is the most poisonous substance known. Botulinum neurotoxin (BoNT), recognized as a public health hazard for centuries and today emerging as a significant bioterrorism threat, has been recently developed into a drug to treat many serious and painful medical conditions that affect the human nervous system.

Currently, the method for detecting BoNT in foods or in the environment, or for assessing the potency of the therapeutic product, is the mouse LD$_{50}$ assay. This assay involves dosing mice with dilutions of the sample being tested and determining the dilution at which 50% of the mice die. The LD$_{50}$ assay has been in use for many years and is currently accepted as the method-of-choice by all U.S. and European regulatory agencies. However, recent scientific and technological advances are providing opportunities for new alternative methods that may be faster and more accurate, and also may refine (less pain and distress), replace, and reduce animal use.

In October 2005, the Humane Society of the United States (HSUS) submitted a nomination to the ICCVAM to organize a workshop to evaluate the state-of-the-science for potential alternatives to the mouse LD$_{50}$ assay for BoNT potency testing. ICCVAM considered the nomination, and expressed support with a high priority for a workshop to discuss alternative methods and approaches that might reduce, refine, or replace the use of animals for BoNT testing with a high priority. Subsequently, the Scientific Advisory Committee on Alternative Toxicological Methods advised ICCVAM and NICEATM that it considered the workshop a high priority.

The workshop was organized by ICCVAM, NICEATM, and ECVAM. The workshop was held in Silver Spring, MD on November 13 and 14, 2006 and involved scientists from leading governmental and academic institutions, national and global regulatory authorities, and the animal protection community.

Workshop Goals

To review the state-of-the-science and current knowledge of alternative methods that may reduce, replace, and refine the use of mice for botulinum toxin testing and identify priorities for research, development, and validation efforts needed to advance the use of alternative methods.

Workshop Objectives

- Review the public health needs for botulinum toxin testing, including the necessity to determine the safety and efficacy of products containing botulinum toxin.
- Review the current state-of-the-science and identify knowledge gaps regarding botulinum toxin structural aspects, mechanisms, and modes of action that are important to the development of alternative methods for *in vivo* botulinum toxin tests, and prioritize future research initiatives that would address these knowledge gaps.
- Review current development and/or validation status of alternative test methods for *in vivo* botulinum toxin tests 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 future development and validation studies to assess potency/toxicity of botulinum toxin.

Workshop Structure and Content

The workshop was comprised of six sessions. Session 1 provided an overview of public health needs for BoNT testing and regulatory requirements, which summarized the public health needs for testing and the regulatory requirements in the United States, and Europe to determine safety and efficacy of products containing BoNTs. Sessions 2 - 5 were comprised of presentations concerning potential replacements, refinements, and reductions for the mouse LD$_{50}$ assay, followed by a panel discussion to evaluate and expand upon the information presented in the first part of each session. Panels consisted of the speakers for each session, plus additional members with expertise in the subjects under discussion. Session 6 consisted of summaries of each of the panel discussions.

A poster session was also held at the workshop.

An agenda, PowerPoint presentations shown at the workshop, poster abstracts, and a complete list of registered participants, is posted on the NICEATM website at: http://iccvam.niehs.nih.gov/methods/biolodocs/biolowkshp/wkshpinfo.htm

Summary Of Workshop Outcomes

In general, the consensus of the panel discussions was that some of the methods considered during this workshop could be used, in specific circumstances or in a tiered-testing strategy, to reduce or refine the use of mice in BoNT test protocols currently in use. However, none of the reviewed methods can serve as a complete replacement for the mouse LD$_{50}$ assay at the present time, either for detection of BoNT or for potency determination. The panel discussions noted that, with additional development and validation efforts, some of the methods might be useful as a replacement for the mouse LD$_{50}$ assay in the future. It was stressed, however, that any validation study must be specific to the intended use of a particular test method and that validation against the mouse LD$_{50}$ assay is critical if the intended use of a test method is as a replacement for the mouse LD$_{50}$ assay. Specific direction from international regulatory authorities about the development of alternatives to the LD$_{50}$ potency assay would enhance these efforts. Finally, some best practices to decrease the number of animals tested for studies that were discussed include, (a) the use of reference standards to minimize the number of replicate animals needed, (b) the use of standardized methodology, and (c) reduction in the number of doses tested for assays where potency is being confirmed (e.g., lot release testing or potency confirmation by someone other than the manufacturer).

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 the public health needs for testing and the regulatory requirements in the U.S. and Europe to determine safety and efficacy of products containing the toxin.

Moderators: Drs. Jacobs and Kulpa-Eddy

Presentations

Overview of Botulinum Toxin and the Incidence and Severity of Botulism - Dr. Maslanka

Current Testing and Practices for Botulinum Prevention in Foods - Dr. Sharma

Medical Conditions Treated with Botulinum Toxin - Dr. Hallett

Current Potency Testing Requirements and Practices for Botulinum Toxin Products - Dr. Shores

Current Testing Requirements and Practices for Botulinum Toxin for Vaccine Potency Testing – Dr. Kulpa-Eddy

Current Animal Diagnostic Testing Requirements and Practices for Botulinum Toxin Potency and Detection - Dr. Rocke

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 the botulinum toxin, 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: Drs. Keller and Ramabhadran

Presentations

Overview of the Modes and Mechanisms of Action of Botulinum Toxin - Dr. Dressler

Pharmacokinetics of Botulinum Toxin - Dr. Simpson

Key Outcomes from the Panel Discussion

Panelists: Drs. Dressler, Simpson, Johnson, Rummel, Hallett, and Sharma

- Knowledge gaps in the current understanding of the mechanism of action of BoNT that must be addressed to develop non-animal replacement methods for BoNT potency testing or detection include:
  - Characterization of the receptors for all serotypes and the roles of other proteins in the BoNT complex and their 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 receptor recognition and various substrates, and internalization/translocation of light chain.
- Regulatory agencies should express expectations and provide internationally harmonized guidance about the development of alternatives to the LD$_{50}$ potency assay.
- To bridge from alternative tests to the LD$_{50}$ test:
  - 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 for alternative test methods.
- If an alternative is deemed 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. However, for diphtheria and tetanus toxoids, this approach has not been universally adopted.

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* botulinum toxin test.

Session 3A: Endopeptidase (EP) Assays

Moderators: Drs. Maslanka and Sharma

Presentation

Overview of Endopeptidase Assays - Dr. Sesardic

Key Outcomes from the Panel Discussion

Panelists: Drs. Sesardic, Barr, Pickett, Schmidt, Shone, Gessler, Johnson, Singh, and Ramabhadran

- The EP assay cannot currently replace animal testing to determine BoNT potency or for the detection of BoNT in environmental or biological samples
  - Since the EP assay does not measure all biological activities of BoNT, it is debatable whether it could completely replace the mouse LD$_{50}$ test unless done in conjunction with another *in vitro* assay (e.g., receptor binding)
- In principle, an EP assay could be used to estimate BoNT concentration in a drug product, allowing for a potential reduction in animal use by using a narrower dose range in the LD$_{50}$ test, or to screen large numbers of a previously known and/or validated food matrix to identify a BoNT type/subtype.
  - An immediate reduction in animal use could be achieved by running an EP assay in parallel with an LD$_{50}$ assay in pre-identified BoNT-contaminated matrices, to eliminate the neutralization tests currently conducted.
- 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 toxin subtypes (e.g., for BoNT A, it must detect all 4 subtypes with the desired sensitivity).
  - The sensitivity must be unaffected by sample matrix.
  - Results should be obtainable in 5 hours or less.
  - Cost must not be prohibitive.
  - The results must be reproducible.
- Pros of the different EP assays reviewed include:
  - One-step fluorescence assays are likely more attractive to industry because they are potentially more robust and of higher precision.
  - The mass spectrometry (MS) platform has high throughput and enhanced specificity, based on mass of cleavage products of substrate.
- Cons include:
  - Sensitivity, specificity, robustness, and transferability vary with assay format.
  - May be susceptible to endogenous proteases in complex matrices.
  - Cost and reagent availability are critical - assays using monoclonal or site directed antibodies will have problems if a long-term supply of high-quality reagents is not guaranteed.
  - Sample preparation is difficult. The need for an antibody to remove toxin from the sample matrix limits detection to known toxin types and subtypes.
  - BoNT binding and translocation is not assessed.
  - The MS platform is expensive.
- Knowledge gaps regarding the reviewed EP methods that must be addressed to further their use in BoNT potency testing or detection include:
  - Identification of new BoNT subtypes/serotypes.
  - Optimization of substrate production methods.

Session 3B: Cell-Based Assays

Moderators: Drs. Maslanka and Sharma

Presentation

Overview of Cell-Based Assays - Dr. Aoki

Key Outcomes from the Panel Discussion

Panelists: Drs. Aoki, Rummel, Adler, Dolly, Gross, Smith, Keller, and Gessler

- No cell-based method can currently replace or reduce animal use, but potential exists.
- 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.
- An advantage of the cell-based assay format is that it may be best option to assess all three BoNT intoxication mechanisms *in vitro*: binding, translocation, and catalysis.
- Cons of cell-based methods include:
  - The assays are very unpredictable.
  - Sensitivity is poor - most methods work only with purified toxin.
  - Variability relative to the LD$_{50}$ assay is unknown.
  - The use of multiple cell lines, which may more closely mimic the mouse, may be too complex for uniform adoption.
- Knowledge gaps regarding the reviewed cell-based methods that must be addressed to further their use in BoNT potency testing or detection include:
  - Which cell lines work best.
  - Better understanding of motor neuron differentiation.
  - Better characterization of binding effects, receptors, expression, and sensitivity to environmental effects.
- A single cell line method may be the easiest to develop, standardize, and validate, but in order to strive toward total replacement, a multiculture approach should also be pursued.
- For an cell-based assay to replace 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.
  - An ideal assay would use an immortalized cell line, not primary cultures.

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

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

- The use of *ex vivo* test models prepared from humanely euthanized animals (i.e., the mouse phrenic nerve [MPN] assay).
- The use of alternate *in vivo* models to measure botulinum activity without lethality (i.e., the mouse hind limb assay and the mouse abdominal ptosis assay).
- The use of earlier non-lethal humane endpoints for the current *in vivo* botulinum assay.

Session 4A: *Ex Vivo* Methods

Moderators: Drs. Rosenberg and Smith

Presentation

Mouse Phrenic Nerve-Hemidiaphragm Assay - Dr. Rummel

Key Outcomes from the Panel Discussion

Panelists: Drs. Sesardic, Johnson, Calver, Rummel, Hendriksen, Stephens, Keller, Schmidt, and Adler

- The MPN assay is currently undergoing validation in Germany; it may be adequate for batch release testing.
- Intercostal neuromuscular junction (INMJ) assay is also currently in validation.
- Limitations for both assays include:
  - Animals are necessary for tissue donation - assays are not replacements, but refinements/reductions.
  - Assays are technically challenging, with set-up difficulties and complex equipment requirements.
  - Matrix effects are not completely resolved. However, sample preparation for environmental or biological samples (e.g., dialysis) may improve performance.
- The MPN assay is promising to reduce the number of animals used for potency testing. Validation will define the achievable reduction; at least 50% anticipated.
- Pros of the MPN assay include:
  - It captures all mechanisms of intoxication - binding, translocation, and catalysis.
  - Results are obtained within 2 hours.
  - Experimental conditions are easily varied.
  - The assay can quantify neutralizing antibodies.
  - The assay has quantitative endpoints.
- A disadvantage of the MPN assay is low throughput.

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

Moderators: Drs. Rosenberg and Smith

Presentation

Mouse Hind Limb Assay - Dr. Aoki

Mouse Abdominal Ptosis Assay - Dr. Sesardic

Key Outcomes from the Panel Discussion

Panelists: Drs. Sesardic, Johnson, Calver, Rummel, Hendriksen, Stephens, Keller, Schmidt, and Adler

- The potential exists to replace the severe LD$_{50}$ endpoint with a less severe procedure, from which mice typically recover.
- Limitations of these assays include:
  - Calibration of these assays vs. the LD$_{50}$ assay is required, since BoNT potency is defined in mouse LD$_{50}$ units.
  - These assays are labor intensive and there are associated transferability and training issues. Reference photographs and a training video would help. Photodocumentation of results may support findings.
  - In order to determine a non-lethal dose, the level of BoNT in the sample must be known.
- Pros of the Mouse Hind Limb Assay
  - The non-lethal endpoint (i.e., local weakness) is a clinically relevant measure of activity. It measures local, not distal, effects.
  - The assay shows good dose response and repeatability.
- Pros of the Abdominal Ptosis Assay
  - It is a fully functional, robust and easily transferable assay with dosing similar to clinical use.
  - Animals normally exhibit no signs of stress or pain, so minimal monitoring is required.
  - It is rapid (results in 48 hrs) compared to the LD$_{50}$ assay (results in 72 - 96 hrs) and requires no specialized equipment or reagents.
- Cons of Both Assays
  - Animals are used, so 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.
  - A published method is needed as a test guideline.

Session 4C: Humane Endpoints

Moderator: Dr. Stokes

Presentation

Overview of the Physiological Progression of Botulism in Mice - Dr. Johnson

Potential Behavioral and Pharmacological Endpoints Predictive of Mouse Lethality - Dr. Calver

Key Outcomes from the Panel Discussion

Panelists: Drs. Sesardic, Johnson, Calver, Rummel, Hendriksen, Stephens, Keller, Schmidt, and Adler

- Moribund animals (i.e., those in a pre-death condition) should be euthanized. Caution: some animals that become moribund near the end of the study could potentially still be alive at study termination.
- 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 for other potential non-lethal endpoints to demonstrate the predictivity for death during the observation period should also be conducted.
- 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* botulinum toxin test.

Moderators: Drs. Halder and McFarland

Presentations

Impact of Sample Size and Toxin Reference Standards on LD$_{50}$ Results - Dr. Gaines-Das

Proposed Testing Strategies that Would Reduce Animal Use in Botulinum Toxin Testing - Dr. Clarke

Key Outcomes from the Panel Discussion

Panelists: Drs. Sesardic, Terrell, Pickett, Gaines-Das, Jacobs, Maslanka, Rocke, and Mr. Bishop

- 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 United Kingdom (at the National Institute for Biological Standards and Control), manufacturer's potency (as measured by LD$_{50}$) is currently confirmed with an alternative test.
- Comparability acceptance criteria are not well defined; assigning prospective criteria for acceptance is currently very subjective. A statistical approach is needed.
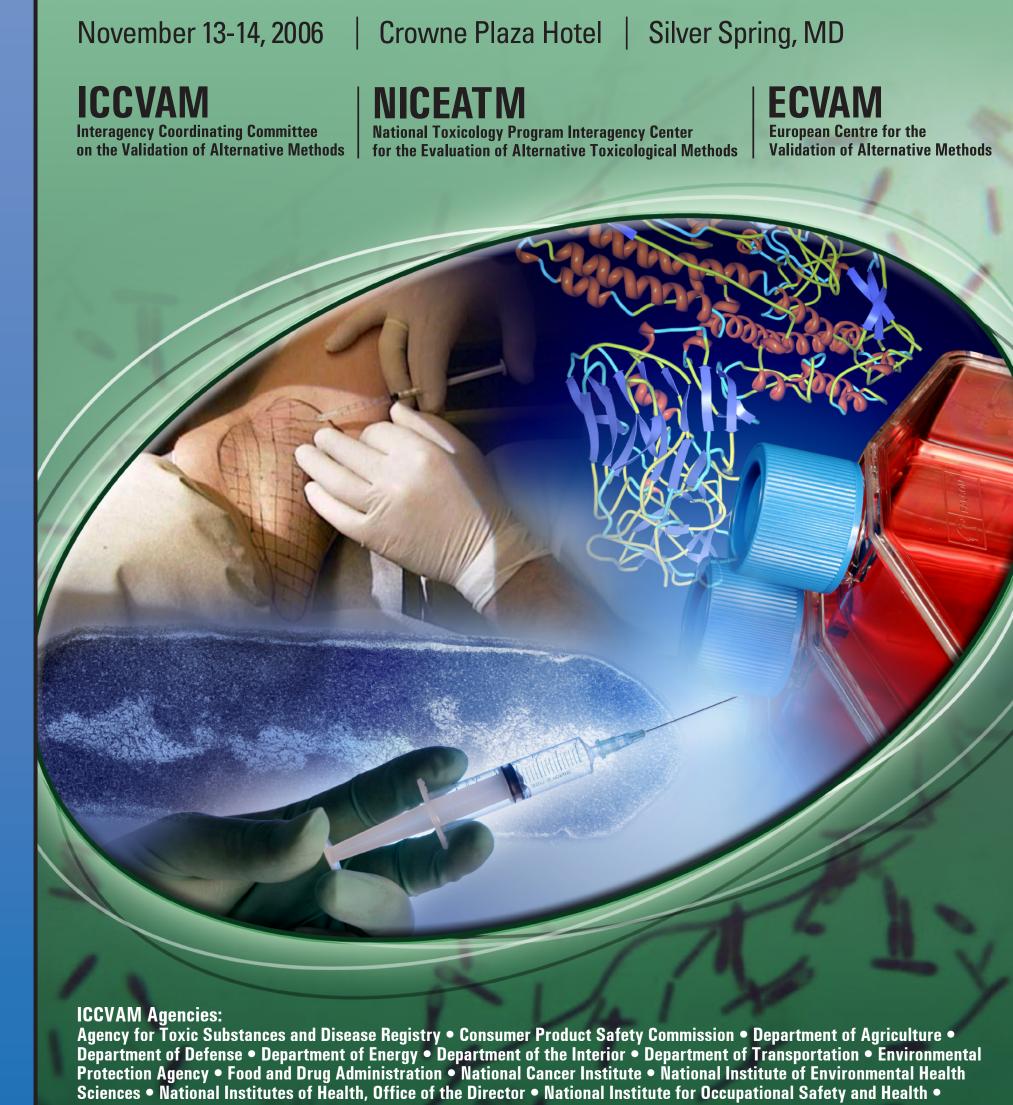- A modified lot release assay could reduce animal use by allowing for testing of fewer animals at doses far from the dose response in confirmatory tests.
- A potency reference standard program reduces *in vivo* testing by refinements to:
  - Extend the shelf life of the working reference standard.
  - 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.

List of Speakers and Panelists

Name	Affiliation	Country
Michael Adler, Ph.D.	United States Army Medical Research Institute of Chemical Defense	United States
K. Roger Aoki, Ph.D.	Allergan	United States
John Barr, Ph.D.	Centers for Disease Control	United States
Christopher Bishop, B.Sc., C.Chem., F.R.S.C.	Wickham Laboratories, Inc.	United Kingdom
Jerry Calver, Ph.D.	Calver Biologics Consulting	Canada
Kenneth Clarke, Ph.D.	Allergan	United States
J. Oliver Dolly, Ph.D.	Dublin City University	Ireland
Dirk Dressler, M.D., Ph.D.	Rostock University	Germany
Rose Gaines-Das, Ph.D.	National Institute for Biological Standards and Control	United Kingdom
Frank Gessler, Ph.D.	University of Goettingen	Germany
Guenter Gross, Ph.D.	University of North Texas	United States
Mark Hallett, M.D.	National Institutes of Health	United States
Marlies Halder, Ph.D.	European Centre for the Validation of Alternative Methods	Italy
Coenraad Hendriksen, M.Vet.Sc., D.V.M., Ph.D.	Netherlands Vaccine Institute	Netherlands
Abby Jacobs, Ph.D.	U.S. Food and Drug Administration, Center for Drug Evaluation and Research	United States
Eric Johnson, Sc.D.	University of Wisconsin	United States
James Keller, Ph.D.	U.S. Food and Drug Administration, Center for Biologics Evaluation and Research	United States
Jodie Kulpa-Eddy, D.V.M.	U.S. Department of Agriculture	United States
Susan Maslanka, Ph.D.	Centers for Disease Control	United States
Richard McFarland, M.D. Ph.D.	U.S. Food and Drug Administration, Center for Biologics Evaluation and Research	United States
Andy Pickett, Ph.D.	Ipsen, Ltd.	United Kingdom
Ram Ramabhadran, Ph.D.	U.S. Environmental Protection Agency	United States
Tonie Rocke, Ph.D.	United States Geological Survey, National Wildlife Health Center	United States
Amy Rosenberg, Ph.D.	U.S. Food and Drug Administration, Center for Drug Evaluation and Research	United States
Andreas Rummel	Medical School of Hannover	Germany
James Schmidt, Ph.D.	United States Army Medical Research Institute of Infectious Diseases	United States
Dorothea Sesardic, Ph.D.	National Institute for Biological Standards and Control	United Kingdom
Shashi Sharma, Ph.D.	U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition	United States
Clifford Shone, Ph.D.	Health Protection Agency, Centre for Emergency Preparedness and Response	United Kingdom
Elizabeth Shores, Ph.D.	U.S. Food and Drug Administration, Center for Drug Evaluation and Research	United States
Lance Simpson, Ph.D.	Thomas Jefferson University	United States
Bal Ram Singh, Ph.D.	University of Massachusetts	United States
Leonard Smith, Ph.D.	United States Army Medical Research Institute of Infectious Diseases	United States
Martin Stephens, Ph.D.	Humane Society of the United States	United States
William Stokes, D.V.M., D.A.C.L.A.M.	National Institute of Environmental Health Sciences, NIH	United States
Timothy Terrell, Ph.D.	Allergan	United States
Lillian Van de Verg, Ph.D.	National Institute of Allergies and Infectious Diseases, NIH	United States

Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD$_{50}$ Assay for Botulinum Toxin Testing

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