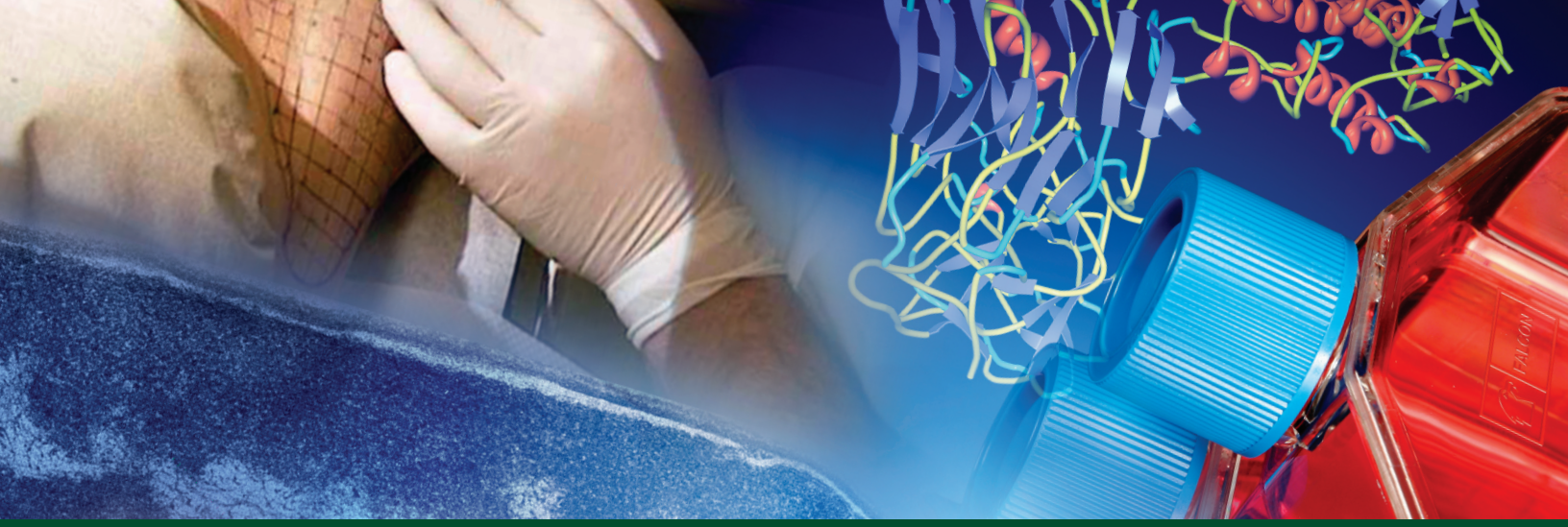


Report on the ICCVAM-NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce or Replace the Mouse LD_{50} Assay for Botulinum Toxin Testing

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

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

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

February 2008
NIH Publication Number 08-6416

About the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
and
The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

In 1997, the National Institute of Environmental Health Sciences (NIEHS), one of the National Institutes of Health (NIH), established ICCVAM to:

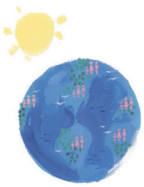
- Coordinate interagency technical reviews of new and revised toxicological test methods, including alternative test methods that reduce, refine, or replace the use of animals
- Coordinate cross-agency issues relating to validation, acceptance, and national and international harmonization of new, modified, and alternative toxicological test methods

On December 19, 2000, the ICCVAM Authorization Act (Public Law 106-545, 42 U.S.C. 285*l*-3) established ICCVAM as a permanent interagency committee of NIEHS under NICEATM.

ICCVAM is comprised of representatives from 15 U.S. Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability. ICCVAM promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety or hazards of chemicals and products and that refine (decrease or eliminate pain and distress), reduce, and/or replace animal use. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. More information about ICCVAM and NICEATM can be found on the NICEATM-ICCVAM web site (http://iccvam.niehs.nih.gov) or obtained by contacting NICEATM (telephone: [919] 541-2384, e-mail: niceatm@niehs.nih.gov).

The following Federal regulatory and research agencies are ICCVAM members:

- Consumer Product Safety Commission
- Department of Agriculture
- Department of Defense
- Department of Energy
- Department of Health and Human Services
  - Centers for Disease Control and Prevention
    - Agency for Toxic Substances and Disease Registry
    - National Institute of Occupational Safety and Health
  - Food and Drug Administration
  - National Institutes of Health
    - Office of the Director
    - National Cancer Institute
    - National Institute of Environmental Health Sciences
    - National Library of Medicine
- Department of the Interior
- Department of Labor
  - Occupational Safety and Health Administration
- Department of Transportation
- Environmental Protection Agency



The NICEATM-ICCVAM graphic symbolizes the important role of new and alternative toxicological methods in protecting and advancing the health of people, animals, and our environment.

Report on the ICCVAM-NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce or Replace the Mouse LD_{50} Assay for Botulinum Toxin Testing

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LIST OF ABBREVIATIONS AND ACRONYMS

BBR	Bureau of Biologics and Radiopharmaceuticals, Health Canada
BoNT	Botulinum neurotoxin
BWG	ICCVAM Biologics Working Group
C. barati	*Clostridium barati*
C. botulinum	*Clostridium botulinum*
C. butyricum	*Clostridium butyricum*
CBER	Center for Biologics Evaluation and Research, U.S. FDA
CDC	U.S. Centers for Disease Control and Prevention
CDER	Center for Drug Evaluation and Research, U.S. FDA
CFR	U.S. Code of Federal Regulations
CFSAN	Center for Food Safety and Nutrition, U.S. FDA
CMAP	Compound muscle action potential
CNS	Central nervous system
CVB	Center for Veterinary Biologics, U.S. FDA
DAS	Digital abduction score
DIG-ELISA	ELISA using digoxigenin-labeled antibodies
DOI	U.S. Department of the Interior
ECVAM	European Centre for the Validation of Alternative Methods - Institute for Health and Consumer Protection, Joint Research Centre, European Commission
ED_{50}	Effective dose in 50% of test animals
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopeia
EPA	U.S. Environmental Protection Agency
EU	European Union
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
HPLC	High-performance liquid chromatography
HSUS	Humane Society of the United States
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems, Inc.
IM	Intramuscular
KDa	Kilodalton
LD_{50}	Dose causing lethality in 50% of test animals
mL	Milliliter
MPN	Mouse phrenic nerve assay
MS	Mass spectrometry
NIAID	National Institute of Allergy and Infectious Disease, U.S. NIH
NIBSC	U.K. National Institute for Biological Standards and Control
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences, U.S. NIH
NIH	U.S. National Institutes of Health
NMJ	Rat intercostal neuromuscular junction assay

NVSL	National Veterinary Services Lab, USDA
NWHC	National Wildlife Health Center, U.S. DOI
PCR	Polymerase chain reaction
pg	Picograms
RT-PCR	Real-time polymerase chain reaction
SBIR	Small Business Innovation Research
STTR	Small Business Technology Transfer
U.K.	United Kingdom
U.S.	United States
USAMRICD	United States Army Medical Research Institute of Chemical Defense
USAMRIID	United States Army Medical Research Institute of Infectious Diseases
USC	United States Code
USDA	U.S. Department of Agriculture
USGS	U.S. Geological Survey

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PREFACE

In October 2005, the Humane Society of the United States submitted a nomination to the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) (http://iccvam.niehs.nih.gov/methods/biologics/botdocs/hsus.pdf) to organize a workshop to evaluate the state-of-the-science for potential alternatives to the mouse LD_{50} assay for botulinum neurotoxin (BoNT) potency testing. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) endorsed the nomination as a high priority activity. The Scientific Advisory Committee on Alternative Toxicological Methods, which advises ICCVAM and NICEATM, also considered the development and validation of alternatives to the mouse LD_{50} assay for BoNT potency testing a high priority.

NICEATM subsequently published a *Federal Register* (*FR*) notice in January 2006 (http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E6_1019.pdf), requesting (1) information on any activities directed towards the development and/or validation of alternatives to the mouse LD_{50} assay for BoNT potency testing, (2) input from the public on the nominated activity, (3) nominations of scientific experts who might participate in the workshop, and (4) data from mouse LD_{50} BoNT potency testing and *ex vivo* and *in vitro* test methods used for BoNT potency testing.

The workshop was held on November 13 and 14, 2006 in Silver Spring, MD, and included scientists from leading governmental and academic institutions, national and global regulatory authorities, private industry, and the animal protection community (the *FR* announcing the meeting can be viewed at: (http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E6_13525.pdf).

The workshop was co-sponsored by the Joint Research Centre, European Commission. We would like to thank Dr. Marlies Halder, European Centre for the Validation of Alternative Methods (Institute for Health and Consumer Protection, Joint Research Centre, European Commission), liaison to the ICCVAM Biologics Working Group (BWG), for her invaluable assistance in making this workshop an international effort.

In addition, the efforts of many other individuals who contributed to the organization of this workshop and the preparation, review, and revision of this report are gratefully acknowledged. We especially recognize all of the individuals who served as speakers and panelists at the workshop for their generous contributions of time and effort.

The BWG, under the leadership of co-chairs Drs. Jodie Kulpa-Eddy (U.S. Department of Agriculture) and Abigail Jacobs (U.S. Food and Drug Administration), was instrumental in both organizing and participating in the workshop.

The efforts of the NICEATM staff in organizing and preparing the workshop materials, administering the workshop and preparing this final report are greatly appreciated. We especially acknowledge Dr. Neepa Choksi, Mr. Thomas A. Burns, Jr., Dr. David Allen, Ms. Linda Litchfield, Dr. Elizabeth Lipscomb, Ms. Catherine Sprankle, and Mr. Douglas Winters of Integrated Laboratory Systems, Inc., the NICEATM Support Contractor, for their efforts. We also want to thank Ms. Debbie McCarley, Special Assistant to the Director, and Dr. Raymond Tice, Deputy Director of NICEATM, for their contributions to this project.

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EXECUTIVE SUMMARY

Workshop Goals

The goals of the workshop were to review the state-of-the-science and current knowledge of alternative methods with the potential to reduce, refine (cause less pain and distress), or replace the use of animals for botulinum neurotoxin (BoNT) testing, and to identify priorities for research, development, and validation efforts needed to advance their use. Specific workshop objectives included:

- Reviewing the public health needs for BoNT testing, including the necessity to determine the safety and efficacy of products containing botulinum toxin
- Reviewing the current state-of-the-science and identifying knowledge gaps regarding botulinum toxin structural aspects, mechanisms, and modes of action that are important to the development of alternative methods for *in vivo* BoNT tests, and prioritizing future research initiatives that address these knowledge gaps
- Reviewing the current development and validation status of alternative test methods for *in vivo* BoNT tests, and their potential to reduce, refine, or replace the use of the mouse LD_{50} assay
- Identifying alternative methods that should have the highest priority for future development and validation studies to assess potency or toxicity of BoNT

The panel discussions, held after meeting presentations, addressed a list of questions posed by the workshop organizers, taking into account the information presented by the speakers.

The Mouse LD_{50} Assay and Potential Alternatives

BoNT testing is currently performed using the mouse LD_{50} assay, which is accepted for BoNT potency testing by United States and European regulatory agencies. BoNT testing is conducted to (1) detect or serotype toxin in environmental or biological samples, (2) detect and titer BoNT-specific antibodies in patients who have been treated with BoNT, and (3) determine the potency of antitoxin or of therapeutic drug product. Due to the continually expanding list of off-label clinical indications for BoNT, along with its potential use as a biological weapon, the demand for testing is expected to increase.

Efforts to develop alternative methods for BoNT testing to reduce, refine, and ultimately replace animal use are ongoing. Alternative BoNT test methods can be separated into four categories: *in vitro* biochemical assays, cell-based methods, *ex vivo* methods, and *in vivo* models with alternative, non-lethal endpoints.

Knowledge Gaps and Characteristics of an Ideal Replacement Assay

The panel identified knowledge gaps in the understanding of the mechanism of action of BoNTs that must be addressed to facilitate development of non-animal replacement methods for potency testing or toxin detection. These include:

- More complete characterization of the receptors for all BoNT serotypes, the roles for other proteins in the holotoxin complex, and their effects on potency
- Improved understanding of the relationship between BoNT potency and its intended use

- Improved understanding of the roles of other proteins in the complex and how they affect BoNT potency

The panel also agreed that methods to demonstrate comparability between products and assays must be identified, and that standardized test method protocols and a list of reference materials must be developed.

The panel stated that an ideal replacement assay for the determination of BoNT potency should measure each step of the *in vivo* intoxication process, which includes:

- Binding of the BoNT protein to receptors on the surface of the target cell
- Endocytosis of the molecule
- Entry of the catalytic light chain into the cytosol
- Cleavage of the protein substrates

The assay should be at least as sensitive as the mouse LD_{50} and be capable of determining all known serotypes and subtypes. The assay should be able to detect BoNT in complex substrate matrices and have a relatively simple platform that is adaptable to a wide range of laboratory and field situations. It is important that each potential replacement assay be validated for the specific application for which it is intended, and that the most appropriate way to validate each alternative test as a replacement for the LD_{50} test be determined.

Potential *In Vitro* Alternatives

Endopeptidase Assays

The various serotypes of BoNT catalytic light chains cleave specific cellular proteins associated with the release of neurotransmitters from the cell. Most biochemical assays targeted as potential replacements for the LD_{50} assay measure BoNT endopeptidase activity. Endopeptidase assay formats vary according to the detection method for substrate cleavage products; immunological methods (e.g. Enzyme-linked immunosorbent assay [ELISA]), high-performance liquid chromatography (HPLC), and mass spectroscopy have all been employed. In addition to measuring endopeptidase activity, an assay intended for use in BoNT potency determination must be able to accurately quantify small amounts of active toxin in finished product. It must also be able to detect toxin in the presence of high concentrations of bulking and stabilizing material of known quantity.

The panel agreed that endopeptidase assays cannot currently be used to replace animals for BoNT potency testing or for detecting BoNT in environmental or biological samples. Furthermore, since endopeptidase assays only measure one component of BoNT biological activity, these methods may not be able to completely replace the mouse LD_{50} test for potency determination, unless they are performed in conjunction with another *in vitro* assay (e.g., a receptor binding assay). However, the panel stated that it should be possible in principle to use an endopeptidase assay for estimating BoNT concentration in a pharmaceutical preparation. This could reduce the number of animals needed for subsequent LD_{50} testing by narrowing the requisite dose range. An endopeptidase assay could also be used for screening large numbers of samples in a previously validated food matrix to detect a specific BoNT type or subtype, with a subsequent LD_{50} assay required to test only for endopeptidase-positive samples. This same approach could also be used to eliminate the need for neutralization studies in pre-identified, toxin-contaminated matrices, resulting in an immediate reduction in animal use. Because BoNT endopeptidases require a controlled

buffer environment for optimal expression of enzymatic activity, sample matrix effects in environmental or biological samples impede accurate and sensitive measurements of activity, limiting the use of these techniques to known toxin types or subtypes in pre-identified sample matrices. A sample clean-up/antibody capture step could help in this regard.

Cell-based Methods

The panel agreed that cell-based methods provide a model that more closely approximates the *in vivo* model. Immortalized cell lines would be preferred over primary cultures for routine use in a product release assay.

Endpoints that have been used in cell-based assays include:

- Measurement of loss of intact substrate or appearance of cleavage product by various means (e.g., western blot, fluorescence labeling)
- Assessment of exocytosis by measuring the appearance of a product from inside of the cell (e.g., ^{3}H-glycine, various neurotransmitters)
- Measurement of neuronal network activity (e.g., spontaneous spiking and bursting)

However, the panel also concurred that no cell-based method could currently be used to reduce or replace animals for BoNT potency testing or for detecting botulinum toxin in environmental or biological samples. In their current state of development, cell-based methods are not sufficiently sensitive. Which cell types and/or cell lines are the most appropriate is also not known.

Potential *Ex Vivo* Alternatives

Promising *ex vivo* methods discussed at the workshop include the mouse phrenic nerve assay and the rat intercostal neuromuscular junction (NMJ) assay. Both assays use NMJ preparations isolated from animals and maintained *in vitro* in the laboratory. The assays measure the amplitude of a twitch response to electrical stimulation of the nerve. Toxin potency can be directly determined as the decrease in the amplitude of the twitch response after toxin is applied to the medium. The endpoint is the time until a 50% decrease in amplitude is observed. Both assays are currently undergoing validation. Since these assays still require animals for donation of tissues, they are not considered replacements, but rather refinement/reduction alternatives, with an anticipated reduction in animal use of at least 50%.

Potential Alternatives Using Non-lethal Endpoints

Alternative *in vivo* assays that use non-lethal endpoints reviewed at the workshop include the mouse hind limb assay and the mouse abdominal ptosis/flaccid paralysis assay. Both assays assess localized paralysis caused by BoNT intoxication. Neither assay is a potential replacement for the LD_{50} because animals are still required. However, both are promising as refinement/reduction alternatives, because they have the potential to replace the severe LD_{50} endpoint with a considerably less severe endpoint, from which mice typically recover.

The use of earlier humane endpoints to end animal studies before animals die was discussed. Health Canada has validated and has been using a non-lethal clinical endpoint in the mouse LD_{50} assay protocol (i.e., severely raised scaphoid in conjunction with hiccough and eyes wide open) for a number of years. The panel recommended that an international study using

the same non-lethal endpoint be conducted. Other, earlier humane endpoints for the mouse LD_{50} assay have not yet been identified.

Conclusions

The panel agreed that it is currently feasible and practical to use the mouse LD_{50} assay to assess the potency of BoNT batch production samples, and then use a validated *in vitro* and/or *ex vivo* test method to assess potencies of final production lots. The National Institute for Biological Standards and Control (United Kingdom) currently conducts confirmatory potency testing by means of the mouse flaccid paralysis assay for new pharmaceutical products and for calibration of reference materials, and also assesses potencies of final production lots using validated endopeptidase assays. For confirmatory assays, it is also currently feasible to use a modified lot release assay to reduce the number of animals used, by testing fewer animals at doses that are farther from the estimated LD_{50}. Finally, the panel agreed that the proper and routine use of reference standards for BoNT potency testing could significantly reduce animal use.

In conclusion, the panel's consensus 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 current *in vivo* BoNT testing protocols. However, at the present time, none of the reviewed methods can serve as a complete replacement for the mouse LD_{50} assay, either for detection of BoNT or for potency determination. The panel 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 national and international regulatory authorities would assist these efforts. Finally, some best practices discussed that could decrease the number of animals required for testing included:

- The use of reference standards to minimize the number of replicate animals needed
- The use of standardized methodology
- A 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)

REPORT ON THE ICCVAM-NICEATM/ECVAM SCIENTIFIC WORKSHOP ON ALTERNATIVE METHODS TO REFINE, REDUCE OR REPLACE THE MOUSE LD_{50} ASSAY FOR BOTULINUM TOXIN TESTING

INTRODUCTION

Botulism poisoning is a potentially deadly illness that can be acquired by humans from consumption of food contaminated with botulinum neurotoxin (BoNT), a toxin excreted by the bacterium *Clostridium botulinum*. The toxin causes muscle paralysis due to its action on the nervous system and is among the most poisonous substances known. Recognized as a public health hazard for centuries, BoNT is also an emerging bioterrorism threat. However, BoNT also has therapeutic uses, and is an ingredient in drug products both for cosmetic indications and for more serious and painful conditions that affect the human nervous system (Dressler et al. 2005).

The current method for detecting BoNT in foods or in the environment, or for assessing the potency of the therapeutic drug product, is the mouse LD_{50} assay. This assay involves dosing mice with dilutions of the sample being tested and calculating the dilution at which 50% of the mice would be expected to die. The LD_{50} assay has been in use for many years and is accepted as the method of choice for BoNT potency testing by international regulatory agencies. However, advances have been made in the development of alternative methods that may be faster and more accurate, and also may reduce, refine (cause less pain and distress), or replace animal use.

In October 2005, the Humane Society of the United States (HSUS) submitted a nomination to the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) (http://iccvam.niehs.nih.gov/methods/biologics/botdocs/hsus.pdf) to organize a workshop to evaluate the state-of-the-science for potential alternatives to the mouse LD_{50} assay for BoNT potency testing. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) endorsed the nomination as a high priority activity. The Scientific Advisory Committee on Alternative Toxicological Methods, which advises ICCVAM and NICEATM, also considered the development and validation of alternatives to the mouse LD_{50} assay for BoNT potency testing a high priority.

The workshop, which was held on November 13 and 14, 2006, in Silver Spring, MD, included leading scientists from governmental and academic institutions, national and global regulatory authorities, private industry, and the animal protection community.

BACKGROUND ON BoNT AND ITS MECHANISM OF ACTION

BoNT is a proteinaceous neurotoxin produced by bacteria of the genus *Clostridium* (*C. botulinum, C. barati, and C. butyricum*). Seven serotypes of BoNT occur naturally and there are an unknown number of subtypes. Serotypes A, B, E, and F are responsible for the majority of human cases of botulism, while serotypes B, C, D, and E cause disease in animals. Serotype G has yet to be associated with disease in any species. Three types of botulism occur naturally. In order of prevalence in the United States, they are: (1) infant botulism, in which the intestinal tract of an infant is infected and colonized by BoNT-producing bacteria, (2) wound botulism, which results from an anaerobic wound infection

and occurs mainly in intravenous drug-users, and (3) food-borne botulism, caused by ingestion of BoNT-contaminated food.

All BoNT serotypes are 150 kilodalton (KDa) polypeptides that are cleaved into two chains after protein translation. The active protein is composed of three functional domains of ~ 50 KDa each; a carboxyl-terminal binding domain, an adjacent translocation domain, and an amino-terminal catalytic domain that has Zn^{2+}-dependent protease activity. The binding and translocation domains are continuous and comprise the molecule's heavy chain. The catalytic domain comprises the molecule's light chain and is linked to the heavy chain via a disulfide bond.

The toxin binds to cholinergic receptors at the motor nerve ending via the heavy chain. The light chain crosses the plasma membrane via receptor-mediated endocytosis and is released into the cytoplasm by a pH-dependent translocation process. Once in the cytosol, the light chain protease cleaves transport proteins necessary for exocytosis, which prevents the release of acetylcholine from the cell. The cleaved transport proteins are serotype-specific (i.e., SNAP-25 is cleaved by BoNT A, C and E; VAMP by BoNT B, D, and F; and syntaxin by BoNT C).

Clinical botulism in humans is characterized by descending flaccid paralysis,[1] which can cause death in severe cases by involvement of the muscles essential for respiration. The disease is rarely fatal if prompt medical attention is received (Simpson 2004).

WORKSHOP GOALS

The goals of the workshop were to review the state-of-the-science and current knowledge of alternative methods that may reduce, refine, or replace the use of mice for BoNT testing, and to identify priorities for research, development, and validation efforts needed to advance the use of alternative methods.

WORKSHOP OBJECTIVES

The objectives of the workshop were to:

- Review the public health needs for BoNT testing, including the need 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* BoNT tests, and prioritize future research initiatives that would address these knowledge gaps
- Review the current development and/or validation status of alternative test methods for *in vivo* BoNT tests, and their potential to reduce, refine, and 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 BoNT

[1] Descending flaccid paralysis refers to a lack of muscle control that progresses in a symmetric descending manner in the body.

WORKSHOP STRUCTURE

The workshop was comprised of six sessions:

- Session 1 - Overview of Public Health Needs for BoNT Testing and Regulatory Requirements
- Session 2 - Current Understanding and Knowledge Gaps for BoNT
- Session 3 - Potential Replacement of Animal Use for BoNT Potency Testing
- Session 4 - Refinement of Animal Use for BoNT Potency Testing
- Session 5 - Reduction of Animal Use For *In Vivo* BoNT Testing
- Session 6 - Summary of Panel Discussions

Session 1 consisted entirely of presentations by invited speakers on subjects related to the session topic. During sessions 2 through 5, speakers gave presentations, after which panel discussions were held and summarized by rappateurs for each session. These summaries were presented by the chairperson of each session during the final session (Session 6). Participants had the opportunity to review each summary and provide further comments during the final session of the workshop.

This report summarizes the content of each session, the responses to the panel discussion questions and the overall conclusions from the workshop. The individual panel discussions are summarized under the sessions during which they occurred and are edited to take into account any relevant comments provided during Session 6 of the workshop.

All presentations are viewable on the NICEATM-ICCVAM website at http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/wkshp_pres.htm.

SESSION 1 - OVERVIEW OF PUBLIC HEALTH NEEDS FOR BOTULINUM TOXIN TESTING AND REGULATORY REQUIREMENTS

Co-Chairs: Abigail Jacobs, Ph.D. (Center for Drug Evaluation and Research [CDER], United States (U.S.) Food and Drug Administration [FDA]) and Jodie Kulpa-Eddy, D.V.M. (U.S. Department of Agriculture [USDA])
This session summarized the public health needs for testing and the regulatory requirements in the United States to determine the safety and efficacy of products containing BoNT.

Laboratory Confirmation of Human Cases of Botulism

Presenter: Susan Maslanka, Ph.D. (U.S. Centers for Disease Control and Prevention [CDC])
Botulism poisoning is a rare disease, with approximately 150 cases reported annually in the United States. Botulism poisoning is also a global disease, occurring worldwide, with disease caused by different serotypes in different locales (e.g., BoNTs A and E in North and South America and Asia, BoNT B in Europe).

Samples to be tested for the presence of BoNT come from diverse sources, including serum, gastric fluid, vomitus, sterile water enemas, rectal swabs and stool samples, food samples, and tissue samples. The mouse bioassay, capable of detecting as little as 10 picograms (pg) of BoNT, is the method of choice for diagnosis of botulism and for strain identification. However, conducting the bioassay can be hazardous to laboratory workers due to the risk of accidental injection of materials containing BoNT. The bioassay also requires extensive animal use (testing of a single sample can require up to 100 mice) and up to four days to

generate results. In addition, the number of animal facilities in many countries, including the United States and European countries, are inadequate to accommodate the number of mouse bioassays that would need to be conducted during botulism outbreaks. A reliable *in vitro* test would be very beneficial in this regard.

Clinical laboratories need to determine toxin potency in addition to whether or not BoNT is present. The level of BoNT in contaminated food sources and in the circulation of exposed patients needs to be ascertained in order to determine an effective antitoxin dose. It is also of interest to measure BoNT levels produced by different strains during outbreaks in order to compare production levels.

The CDC has developed *in vitro* assays to detect BoNT for screening purposes. An enzyme-linked immunosorbent assay (ELISA) for BoNT serotypes A, B, E, and F has increased national testing capacity by 85% and sample throughput by 67%, while detection time is reduced by 44%. However, the ELISA is currently limited to four serotypes and may be insensitive to BoNT subtypes due to differences in epitopes among subtypes. The sample matrix can also interfere with the assay.

Real-time polymerase chain reaction (RT-PCR) has been used for identification of BoNT DNA for serotypes A through G. A result can be obtained within 24 hours. This assay has reduced the time required to identify the strain responsible for a particular outbreak by 5 to 15 days. A disadvantage of this method is identification of false negatives, which occur because some *Clostridium* strains have non-functional BoNT genes with sufficient sequence homology to functional genes that they interact with the PCR primer. As with the ELISA, the sample matrix may interfere with the assay.

CDC has developed a sensitive and rapid test using mass spectrometry (MS) to detect specific BoNT cleavage products. This test yields results in less than 24 hours and can be automated to increase throughput. However, the required equipment is expensive, the assay is limited to known toxin types and may be insensitive to subtypes, and sample matrix effects are unknown.

In summary, there are significant challenges to the development of a valid *in vitro* test for human cases of botulism. A satisfactory test must differentiate between the seven known BoNT serotypes and possibly among as yet unknown subtypes. It must function reliably with diverse sample matrices, be capable of detecting low levels (1 – 2 mouse LD_{50} units) in clinical specimens, and be easily transferable to be useful during an outbreak.

Current Testing and Practices for Botulinum Prevention in Foods

Presenter: Shashi Sharma, Ph.D. (Center for Food Safety and Nutrition [CFSAN], FDA)
The FDA is concerned with foodborne biological hazards. Requirements for registration, manufacturing, and process filing of low-acid canned foods and acidified foods are detailed in Title 21 Code of Federal Regulations (CFR), Parts 108, 113 and 114 (accessible at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm). The purpose of these sections of the CFR is to ensure safety from harmful bacteria and their toxins, especially *C. botulinum*.

The six serotypes of *C. botulinum* responsible for causing foodborne illness contain proteolytic (serotypes A, B, and F) and non-proteolytic (serotypes B, E, and F) strains.

Proteolytic strains exhibit growth at temperatures as low as 10°C, while the non-proteolytic strains can grow at even cooler temperatures (*ca.* 3°C). According to 21 CFR 114, growth of *C. botulinum* can be controlled by adequate food processing, including manufacturing controls, proper cooking temperatures and durations, adequate acidification (≤ pH 4.6) or by controlling water activity. Compliance with these CFRs is essential, as there are currently no regulatory requirements for finished product testing for *C. botulinum* except as a follow-up to reports of clinical symptoms.

The mouse bioassay is currently used to screen food samples for the presence of BoNTs, to determine BoNT titer, and to determine serotype by demonstrating BoNT neutralization with specific antisera. Supernatants of enrichment cultures and single colony isolates are tested for the presence of toxin, and serotyping is conducted if toxin is detected. A single sample tested for both toxin presence and serotype requires 48 mice.

An ELISA test that uses digoxigenin-labeled IgGs (DIG-ELISA) can be used to reduce the number of mice required for this type of testing. Positive results are then confirmed with a mouse bioassay, but negative results do not require additional testing. The DIG-ELISA is used by both CFSAN and CDC, and has been validated for BoNTs A, B, E, and F in food, for strains isolated in outbreaks, and for clinical and culture specimens. AOAC International, a not-for profit organization that is currently focusing on providing new methods in areas of increasing international interest, has submitted the method for validation. If validated, it will become the official screening method. More information on the DIG-ELISA can be found at http://www.cfsan.fda.gov/~ebam/bam-17.html.

Other alternate methods for BoNT detection include:

- The BioVeris M1M analyzer (see http://www.bioveris.com/homelandsecurity/instruments/m1m.htm)[2]
- Biosensors developed for the U.S. Department of Homeland Security
- An endo-ELISA from BBtech, Inc (see the abstract of the poster #7 presented at this workshop in **Appendix C** or at http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/Abstracts.pdf)
- RT-PCR techniques as described above

Indications for BoNT Therapy

Presenter: Mark Hallett, M.D. (U.S. National Institutes of Health [NIH])
Commercial therapeutic products have been made only from BoNT serotypes A and B. The type A products include Botox® (Allergan, Inc., Irvine, CA), Dysport® (Ipsen, Ltd., Berkshire, United Kingdom [U.K.]), and Xeomin® (Merz Pharma, Frankfurt, Germany). The only type B product is Myobloc® (known as Neurobloc® in Europe) (Solstice Neurosciences, Malvern, PA).

[2] By providing this link, ICCVAM/NICEATM/ECVAM are not endorsing the information presented or products discussed. The link is only being provided for informational purposes.

The FDA approved Myobloc® for cervical dystonia in 2000. The FDA has approved Botox® for the following indications (date approved in parentheses):

- Strabismus (1989)
- Blepharospasm and hemifacial spasm (1989)
- Cervical dystonia (2000)
- Glabellar rhytides (2002)
- Primary axillary hyperhidrosis (2004)

However, there have been many off-label clinical uses reported for botulinum toxin drugs. Some of these include cosmetic indications (e.g., removal of wrinkles, improved wound healing), movement disorders (e.g., focal dystonias, spasticity caused by stroke, multiple sclerosis, head injury, cerebral palsy), bladder dysfunction, and pain syndromes.

Side effects of botulinum toxin therapy include both local and systemic muscle weakness, effects on the autonomic nervous system (e.g., dry mouth, constipation, gall bladder dysfuction), pain, rash, hematoma, and infection. Patients can develop resistance to the toxin due to antibody production.

Potency Assays for Botulinum Products

Presenter: Elizabeth Shores, Ph.D. (CDER, FDA)
The FDA requires potency testing for biological products, but the type of potency test is not specified. Federal regulations regarding potency testing for biological products can be found in 21 CFR 610.10 and 21 CFR 600.3 (accessible at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm) and in the Public Health Services Act Title 42 United States Code (USC) 262 (accessible at http://www.law.cornell.edu/uscode/42/usc_sec_42_00000262----000-.html). An acceptable potency assay must be suitable for the intended purpose of the product in question and must be validated for sensitivity, specificity, reproducibility and robustness.

For BoNTs, a suitable potency assay must be used to determine the dose of the final product or to compare the relative activities of different lots. Because BoNT activity is dependent upon three functional domains within the protein molecule, an acceptable potency assay must account for the activity of all domains, or a battery of assays that account for the activity of each domain would be required.

The mouse LD_{50} has been used for many years to determine clinical dose levels. This assay measures the activity of all of the functional domains and is sufficiently sensitive. It also may be used for immunogenicity testing, data from which are included on the package insert for biologics. The weak points of the assay include variability and sensitivity to variations in animal physiology, and that death of the mouse is the endpoint.

***C. Botulinum* Testing - U.S. Department of Agriculture**

Presenter: Jodie Kulpa-Eddy, D.V.M. (USDA)
The USDA operates the National Veterinary Services Laboratory (NVSL), which has requirements for both diagnostic and potency testing for BoNTs. Diagnostic testing is usually

done to confirm botulinum intoxication in horses and to determine the toxin type via a mouse protection assay. The USDA also regulates a botulinum type C toxoid product (see 9 CFR 113.110, accessible at http://a257.g.akamaitech.net/7/257/2422/14mar20010800/edocket.access.gpo.gov/cfr_2003/pdf/9cfr113.110.pdf). This product requires several different potency tests.

The potency of the type C toxoid is determined via a bioassay in mink. The assay uses eight animals, five vaccinated with the toxoid and three unvaccinated controls. The animals are challenged at 21 - 28 days with a BoNT type C standard. For a positive test, all controls must die of botulism and 80% of the vaccinates must remain asymptomatic. The BoNT C standard is maintained by USDA's Center for Veterinary Biologics (CVB), which uses a mouse bioassay to standardize the toxin preparation for the mink bioassay. The mouse bioassay uses 40 animals; 9 of 10 replicates typically die of botulism at a median toxin concentration while 9 of 10 survive for 7 days at the lowest toxin concentration.

A type B antitoxin product is made for treatment of horses suspected of exposure to BoNT, but none was produced in 2005. This product's potency is assessed with a mouse bioassay that uses 48 mice per run.

A type B toxoid is made to immunize healthy horses against botulism. It is given to pregnant mares and immunity is passed on to the foal via the colostrum. The potency of this product is assessed by vaccination of guinea pigs and the presence or absence of a protective level of antibodies is assessed via a mouse protection assay.

Product testing is required by the manufacturer for product release, but is optional at the U.S. Federal level, allowing for some reduction of animal use. CVB also encourages refinement by permitting the use of humane endpoints, including the euthanasia of moribund animals (see CVB Public Notice 04-09, accessible at http://www.aphis.usda.gov/vs/cvb/notices/2004/04-09.pdf).

Botulism Diagnostics for Animals

Presenter: Tonie Rocke, Ph.D. (U.S. Department of the Interior [DOI])
The U.S. Geological Survey's (USGS) National Wildlife Health Center (NWHC) is primarily concerned with the diagnosis of botulism and the identification of causative strains in waterfowl, fish, and other wild and domestic animals. The disease also occurs in ranched fish and mink. *C. botulinum* serotype C is most often the etiologic agent in wildlife outbreaks, although serotypes A, B, D, and E have also been identified in animals. Serotype C botulism outbreaks in wildlife can be severe, resulting in the deaths of millions of animals.

On average, the NWHC runs about 300 tests per year. The mouse bioassay is the primary diagnostic tool, although alternatives currently used include RT-PCR (Nol et al. 2004; Steinman et al. 2006) and a serotype C-specific Immunostick ELISA (Rocke et al. 1998). The RT-PCR assays require that DNA be extracted from both spores and cells, as spores commonly occur in animals. The Immunostick ELISA method was developed because non-specific interference occurs in traditional ELISA assays run on microtiter plates. An advantage of the assay is that it can test larger sample volumes. Examples of outbreaks investigated by NWHC using the Immunostick ELISA include one that killed 427 of 441 adult Holstein dairy cattle in a 1,200-cow dairy (Galey et al. 2000) and another that killed 45 wild bighorn sheep (Swift et al. 2000).

An ideal diagnostic assay for BoNT in animals must be:

- Able to detect active toxin
- Adaptable to both field and laboratory use
- Inexpensive, rapid, and unaffected by matrix effects, since it must be effective for environmental samples, carcasses and live animals

SESSION 2 - CURRENT UNDERSTANDING AND KNOWLEDGE GAPS FOR BOTULINUM TOXIN

Co-Chairs: James Keller, Ph.D. (Center for Biologics Evaluation and Research [CBER], FDA) and Ram Ramabhadran, Ph.D. (U.S. Environmental Protection Agency [EPA])
This session summarized the current understanding of structural aspects, mechanisms, and modes of action of the botulinum toxin, discussed the aspects of the endopeptidase function that would need to be modelled by alternative test methods, and prioritized research needs to address gaps needed to facilitate the development of alternative test methods.

Botulinum Toxin Mechanisms of Action

Presenter: Dirk Dressler, M.D., Ph.D. (Rostock University, Germany)
BoNT drugs contain a highly complex mixture of various proteins and excipients (Dressler and Bigalke, in press). The botulinum toxin component contains BoNT and non-toxic (complexing) proteins, which may be hemagglutinating or non-hemagglutinating. Excipients are added by the manufacturer for stabilizing purposes. Xeomin®, a recently introduced BoNT type A drug does not contain complexing proteins and, therefore, has a substantially reduced molecular size.

BoNT can be used to treat disorders of the motor system and disorders of the autonomic nervous system. When BoNT is used in the motor system, the therapeutic effect occurs within a few days and starts to gradually decline after approximately two months. Degree and duration of effect is dose-dependent. BoNT may produce target muscle hypotrophy or reduction of target muscle hypertrophy. Trophic effects, however, are not compulsory. If they occur, they are fully reversible.

BoNT is used to treat various autonomic disorders using smooth muscles as target tissues as in achalasia, anal fissures, bladder dysfunction, and gastroparesis or exocrine glands as in hyperhidrosis, hyperlacrimation, and drooling.

BoNT affects the muscle spindle organ by paralyzing its intrafusal muscle fibers. Reduced Ia/II input to the spinal cord - in return - reduces the spinal stretch reflex and thus produces a 'reflex inhibition' which is believed to contribute to BoNTs antidytonic and antispastic effects (Ludlow 1990; Kaji et al. 1995a, 1995b; Yoshida et al. 1998).

BoNT has no direct effects on the central nervous system (CNS). Systemic spread is minimal for BoNT type A, but higher for BoNT B (Takamizawa et al. 1986; Dressler and Benecke 2003). Because of its molecular size, BoNT cannot penetrate the blood brain barrier. Retrograde axonal transport into the spinal cord is slow and not transsynaptic (Wiegand et al. 1976). BoNT, however, can exert indirect effects on the CNS including normalization of abnormal reciprocal inhibition in upper limb dystonia (Priori et al. 1995), normalization of

abnormal intracortical inhibition in dystonia (Gilio et al. 2000), and normalization of abnormal median nerve sematosensory evoked potential in writer's cramps (Dressler et al. 1995). Recently, BoNT has been used to treat various pain conditions, possibly by affecting non-cholinergic transmitter systems.

The effects of BoNT upon the human neuromuscular junction has been used to construct dose-effect curves which can be applied to monitor the potency of BoNT drugs and to detect antibodies against BoNT (Dressler and Rothwell 2000). BoNT antibodies can be detected with the sternocleidmastoid test (Dressler et al. 2000) and the extensor digitorum brevis test (Kessler and Benecke 1997). The frontalis test, which monitors the effect of BoNT upon the frontalis muscle, may be used as a screening test. Other body functions including sweating or salivation can also be used to determine BoNT potency and to detect BoNT antibodies. These human clinical tests can be used to avoid animal-consuming BoNT tests for certain special tasks.

Pharmacokinetics of Botulinum Toxin

Presenter: Lance Simpson, Ph.D. (Thomas Jefferson University, U.S.)
Understanding the absorption, distribution, metabolism, and elimination of BoNT is important for developing both *in vitro* and *in vivo* assays for characterizing the mechanisms of action of BoNT antagonists. Such knowledge is also critical for determining the window of opportunity within which medical countermeasures can be effectively administered to block BoNT action.

BoNT exposure typically occurs via the oral or inhalation route. BoNT binds to the apical surface of airway or gut epithelial cells and is transported into the general circulation via endocytosis and transcytosis. BoNT is then transported to cholinergic cells, where it binds to specific cell surface receptors via the carboxy-terminal portion of the heavy chain. The amino-terminal portion of the heavy chain facilitates internalization. Once in the cytosol, the light chain endoprotease cleaves polypeptides essential for the release of acetylcholine, resulting in a long-lasting flaccid paralysis.

BoNT is synthesized as a single chain of approximately 150 KDa, and activated when nicked by specific proteases either in the bacterium or in the gut, depending on the species in which it is produced. Unnicked dichain, nicked dichain, and both single chains can all reach the systemic circulation, but only the nicked dichain form is toxic.

In the circulation, BoNT does not enter blood cells and is only minimally protein bound, facilitating efficient distribution to the target cells and organs. Labelling studies have shown that BoNT is stable in blood and minimally metabolized in the circulation (half-lives of BoNT A, B, and C are approximately 200 hours).

The window of opportunity for effective treatment with antiserum is very short because of the extreme toxicity of BoNT. Studies have shown that BoNT is cleared from the circulation rapidly (i.e., within 2 minutes) after antiserum is administered. The rate of BoNT clearance from the circulation is independent of the time that the antiserum is administered.

Currently, the mechanisms for systemic metabolism of BoNT and the elimination of BoNT metabolites are not well understood and thus research is needed in this area.

Essential Characteristics of Potential Test Methods to Replace the Mouse LD_{50} for Botulinum Toxin Potency Testing

Presenter: Eric Johnson, D.Sc. (University of Wisconsin, U.S.)
The mechanism of toxicity of BoNT is complex and is directly related to its three-domain protein structure. An ideal potency assay accounts for the activity of all three domains and an ideal alternative assay should evaluate each step of the *in vivo* intoxication process. However, most *in vitro* methods currently measure only the activity of individual steps.

Additionally, an ideal *in vitro* assay should be at least as sensitive as the mouse bioassay (1-2 LD_{50} units per milliliter (mL) = about 7-15 pg/mL). It should distinguish between, and be capable of, identifying all toxin serotypes and multiple subtypes. The desired false positive rate should be no more than 1 in 1,000,000, and the desired false negative rate no more than 1 in 1000. Depending on intended use (e.g., diagnostic, environmental screening), a rapid assay may be required. The assay should be robust (i.e., capable of detecting BoNT in complex food and clinical matrices) and have a relatively simple platform that is adaptable to a wide range of laboratory and field situations.

Botulism Research NIAID, NIH

Presenter: Lillian Van De Verg, Ph.D. (U.S. National Institute of Allergy and Infectious Diseases [NIAID], NIH)
Approximately one-third of the 100 botulism research grants at the NIH are from the NIAID. Most of these are directed towards biodefense research. The National Institute of Neurological Disorders and Stroke, the National Institute of General Medical Sciences, the National Eye Institute, and the National Institute of Child Health and Human Development fund the additional NIH research on botulism.

Immediate and long-term research goals of BoNT biodefense research include:

- Development and testing of human monoclonal antibodies to the seven serotypes (A-G)
- Development of a recombinant vaccine against all of the serotypes
- Identification of the toxin receptors
- Characterization of mechanisms of action for the all of the serotypes
- Determination of the molecular basis of proteolytic specificity for all of the serotypes
- Development of small molecule inhibitors of light chain endoprotease activity for use as possible treatments following intoxication

At the NIAID, basic botulism research is funded through research project grants, small grants, and exploratory/developmental NIH grants (R01, R03, and R21, respectively). Basic research efforts currently being funded under these programs include an investigation of BoNT receptors for neurotoxins and an investigation of the crystal structure of the BoNT catalytic domain-substrate complex.

Research is also funded through Research Projects Cooperative Agreements (U01). Ongoing Cooperative Agreement research includes efforts directed towards antibody-based therapies

(e.g., human monoclonal antibodies, humanized rabbit polyclonal antibodies), vaccines, small molecule inhibitors, and improved diagnostics.

Small Business Technology Transfer (STTR) Grants (R41 and R42) and Small Business Innovation Research (SBIR) Grants (R43 and R44) are also available. Ongoing STTRs and SBIRs are funding initiatives directed towards vaccines and vaccine delivery systems, small molecule inhibitors, plant-derived human monoclonal antibodies, and protein arrays for antibody detection.

Botulism research is also funded through contracts generated by the NIAID in response to specific needs and announced via Requests for Proposal. For example, current research associated with the Food and Waterborne Integrated Research Network program includes studies that are investigating animal models, high throughput drug screening, small molecule inhibitors, x-ray crystallography, and *in vitro* assays, as well as a project focused on the pharmacokinetics of BoNT.

Botulinum Neurotoxin: Research Activities on Alternative Activity Tests in Europe

Presenter: Andreas Rummel (Medizinische Hochschule Institut für Toxikologie, Hannover, Germany)
Ongoing research efforts in Europe are focused on three types of *in vitro* assays for BoNTs:

- Assays for protein and/or DNA detection
- Assays for light chain activity
- Assays to assess biological activity of the protein

Assays for protein and/or DNA detection include immunological methods and assays to detect DNA encoding BoNT. These assays may not provide information on biological activity. ELISAs are the assay format most often used for immunological detection of BoNT protein, while DNA is detected predominantly through polymerase chain reaction (PCR) methods. Assays for the light chain endopeptidase activity, of either the single chain or dichain form, measure light chain activity by detecting specific cleavage products by immunofluorescence or mass spectroscopy. Assays appropriate to determine the full biological activity or potency include *ex vivo* methods that are discussed under **Session 4A**.

Panel Discussion on the Current Understanding and Knowledge Gaps for Botulinum Toxin (Session 2 Panel)

Moderators: James Keller, Ph.D. (CBER, FDA) and Ram Ramabhadran, Ph.D. (EPA)

Panelists: Dirk Dressler, M.D., Ph.D. (Rostock University, Germany), Lance Simpson, Ph.D. (Thomas Jefferson University, U.S.), Eric Johnson, Sc.D. (University of Wisconsin, U.S.), Lillian Van De Verg, Ph.D. (NIAID, U.S.), Andreas Rummel (Medical School of Hannover, Germany), Mark Hallett, M.D. (NIH, U.S.), Shashi Sharma, Ph.D. (FDA, U.S.)
The panel was presented with questions regarding knowledge gaps that must be addressed to develop non-animal replacement methods for BoNT potency testing or detection, the extent to which current research addresses them, and research initiatives necessary to further characterize mechanisms and modes of action of BoNT. A list of the questions presented to the panel can be found in **Appendix B** and at

http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/PanelQuestions.pdf.

The panel identified the following knowledge gaps in the understanding of the mechanism of action of BoNTs that must be addressed to facilitate development of non-animal replacement methods for potency testing or toxin detection:

- Characterization of the receptors for all BoNT serotypes, the roles for other proteins in the holotoxin complex, and their effects on potency
- The roles for other proteins in the complex and how they affect potency

The panel also agreed that methods to demonstrate comparability between products and assays must be identified, and that test method protocols and reference materials must be standardized. This includes a need to establish an internationally recognized "standard references" facility or facilities. Regulatory agencies should also describe their expectations in regard to alternatives to the LD_{50} potency assay. In this regard, regulators should provide internationally harmonized guidance. The panel also suggested that mechanisms for predicting variability in human responsiveness would be useful.

A consensus emerged within the panel that little basic research is currently being funded in the United States, where the majority of funding related to BoNT testing is devoted to product development for biodefense. The panel also suggested that European Union (EU) funding for research on botulinum toxin should be coordinated among its member states. The panel recommended funding a consortium of laboratories to evaluate the different ways of measuring BoNT potency.

The panel agreed that there is a need to determine the most appropriate way to validate alternative tests as replacements for the LD_{50} test. For example, *in vitro* results must be calibrated in terms of mouse LD_{50} units, and reference standards, tested in the mouse LD_{50} test, must be developed. Alternatively, once it has been established that an alternative method is comparable to the LD_{50} test, the LD_{50} test could be dropped and LD_{50} equivalent units used instead. A functional assay is the critical standard but thus far, no single alternative *in vitro* assay addresses all biological functions of BoNT.

Other suggested research initiatives necessary to advance the development of non-animal replacement methods for potency testing or detection of botulinum toxin include:

- The use of phylogenetically lower species (e.g., daphnia)
- Cell-based assays that mimic presynaptic function
- Characterization of the mechanism(s) involved in receptor recognition and the various enzyme substrates
- Internalization and translocation as targets for therapeutic intervention

SESSION 3 - POTENTIAL REPLACEMENT OF ANIMAL USE FOR BOTULINUM TOXIN POTENCY TESTING

Co-Chairs: Susan Maslanka, Ph.D. (CDC) and Shashi Sharma, Ph.D. (CFSAN, FDA)
This session provided an overview of alternative *in vitro* models that, if adequately validated, could potentially replace the current *in vivo* botulinum toxin test (i.e., the mouse LD_{50} test).

Endopeptidase Assays for Botulinum Toxins

Presenter: Dorothea Sesardic, Ph.D. (National Institute for Biological Standards and Control [NIBSC], U.K.)

BoNT endopeptidase activity is serotype-specific, cleaving its target polypeptide between two different amino acid residues in each case. Determining the specific cleavage products generated by the endopeptidase can identify serotypes. The endopeptidase assay offers several advantages because:

- It reflects an important mode of action for BoNT.
- It can provide sensitivity comparable to *in vivo* mouse models depending on the detection system chosen.
- It can be toxin serotype-specific.
- It can provide quantitative responses within a day.

However, the requirements for the assay can differ depending on whether it is used to detect toxin or to measure the potency of a biological product. For example, matrix effects are not an issue when measuring the potency of a highly purified drug product.

BoNT endopeptidase assays are based on the *in vivo* intracellular mode of action and require as a substrate a synthetic or recombinant peptide that contains the endopeptidase-binding site and is greater than 30 amino acids in length. Generally, the various assays differ according to the detection systems, which also impacts on their sensitivity. Currently used detection systems range from immunochemical to analytical chemistry platforms (Hallis et al. 1996; Ekong et al. 1997; Sesardic et al. 1997; Wictome et al. 1999; Anne et al. 2001; Liu et al. 2003; Schmidt and Stafford 2003; Dong et al. 2004; Barr et al. 2005; Boyer et al. 2005; Ferracci et al. 2005; Gilmore et al. 2005; Parpura and Chapman 2005; Kalb et al. 2006). The endopep-MS assay has been successfully used to measure the activity of and differentiate between all seven BoNT serotypes (Barr et al. 2005; Boyer et al. 2005; Kalb et al. 2006). However, the sensitivity of this assay is affected by the sample matrix, which can cause it to vary by several orders of magnitude (Barr et al. 2005; Boyer et al. 2005; Kalb et al. 2006). Endopeptidase assays using antibody capture techniques are currently being considered to replace the use of animals for detection of toxins in biological samples.

The SNAP-25 assay has been validated (Gaines Das et al. 1999) and used at NIBSC since 1999 to verify potency as a measure of consistency of clinical samples containing BoNT type A (Sesardic 1999), leading to an in-house reduction in animal use of 90%. The assay has also been set up for serotypes A, B, C, E, and F (Sesardic et al. 2004).

Overview of Cell-Based Assays for Botulinum Neurotoxin Product Release

Presenter: K. Roger Aoki, Ph.D. (Allergan, Inc.)

Cell-based assays are being developed as potential replacements for the mouse bioassay for BoNT product-release potency testing. Several challenges exist when attempting *in vitro* potency measurements with formulated products. With concentrations of BoNT in the picomolar range, losses due to surface adsorption can markedly affect results, as can formulation excipients, which can interfere with analytical methods.

Primary cells and cell lines have been used, with the most commonly used cell lines derived from rodents. However, for routine use in a product release assay, a stably transfected cell line would be the most appropriate in order to minimize variability.

Different endpoints have been explored for cell-based assays. Loss of intact substrate or appearance of cleavage product, as measured via western blot, directly measures substrate cleavage. However, this type of assay requires appropriate antibodies for use and has limited throughput. Measurement of exocytosis directly quantifies a cellular target that reflects the activity of all three functional domains of the BoNT molecule, and this may be amenable for use as a screening assay. However, the assay usually requires a radioactive or fluorescent tracer and equipment associated with tissue culture. Assessment of neuronal network activity, using primary cells, measures changes in spontaneous spiking and bursting in neuronal networks formed from embryonic murine neurons, or measures exocytosis directly. Both the neuronal network activity and the exocytosis readouts measure an average of activity in all cells. The sensitivity of the assay is therefore directly proportional to cell uptake. However, neither measurement is as sensitive as the mouse bioassay.

Fluorescent sensors are being used in both PC12 cells and Neuro-2A cells (Dong et al. 2004; Steward et al. 2005). A fluorescence resonant energy transfer-based readout enables increased sensitivity and throughput. Another advantage of this measurement system is that only positive (transfected) cells respond. However, extensive cloning or cell modification may be required, making it more difficult to create a stable cell line.

Summaries of Panel Discussions on Potential Replacement of Animal Use for Botulinum Toxin Potency Testing (Session 3A and 3B Panels)

The panel was asked whether any of the alternative methods discussed in this session could be used now to reduce, refine, or replace the mouse LD_{50} assay. The panel was also asked to discuss the pros and cons of each reviewed method, identify knowledge and research gaps related to them, and prioritize the methods for further development and validation studies. A list of the questions presented to each panel can be found in **Appendix B** and at http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/PanelQuestions.pdf.

Panel Discussion on Endopeptidase Assays (Session 3A Panel)

Moderators: Susan Maslanka, Ph.D. (CDC) and Shashi Sharma, Ph.D. (CFSAN, FDA)

Panelists: Dorothea Sesardic, Ph.D. (NIBSC, U.K.), John Barr, Ph.D. (CDC, U.S.), Andy Pickett, Ph.D. (Ipsen, U.K.), James Schmidt, Ph.D. (United States Army Medical Research Institute of Infectious Disease [USAMRIID], U.S.), Clifford Shone, Ph.D. (Health Protection Agency, Centre for Emergency Preparedness and Response, U.K.), Frank Gessler, Dr. med. vet. (University of Goettingen, Germany), Eric Johnson, Sc.D. (University of Wisconsin, U.S.), Bal Ram Singh, Ph.D. (University of Massachusetts, U.S.), Ram Ramabhadran, Ph.D. (EPA, U.S.)

The panel reached a consensus that the currently available endopeptidase assay cannot be used to replace animal testing for BoNT potency or for detecting BoNT in environmental or biological samples. Given that endopeptidase assays do not completely account for all BoNT biological activities, it is debatable whether these assays could completely replace the mouse

LD_{50} test unless performed in conjunction with another *in vitro* assay (e.g., receptor binding). The panelists also agreed that, if there is no endopeptidase activity, then there is almost certainly no LD_{50} activity. However, the converse may not be true (i.e., preparations may show endopeptidase activity without LD_{50} activity).

The panelists agreed also that, for an endopeptidase assay to be useful in estimating potency, several criteria would have to be met:

- The assay would have to be sensitive (at least as sensitive as the mouse test).
- The assay should detect all toxin sub-types (e.g., for BoNT/A, the assay should detect all four subtypes A1 – A4 with the desired sensitivity).
- The sample media should not affect assay sensitivity.
- The assay should take less than 5 hours to conduct.
- The cost should not be prohibitive.
- The results should be reproducible.

However, the panel agreed that, in principle, it should be possible to use an endopeptidase assay to estimate BoNT concentration in a preparation intended for pharmaceutical use. This would reduce the number of animals needed in the LD_{50} test by identifying a relatively narrow dose range, based on the toxin concentration indicated by the endopeptidase assay. Reduction might also be achieved by screening large numbers of previously characterized food matrices or by the use of a specific assay validated to identify a botulinum toxin type or subtype. LD_{50} assays could then be used to test only endopeptidase-positive samples. An endopeptidase assay, run in parallel with an LD_{50} assay, could eliminate the need for neutralization studies in predetermined toxin-contaminated matrices, which would result in an immediate reduction in the number of animals used. However, because of the need for an antibody capture clean-up step, this method is limited to the detection of known toxin types and subtypes in pre-identified sample matrices.

The panel identified the following advantages of endopeptidase methods:

- Fluorescence-based assays are one-step assays. Use of fluorescent substrates is likely to be more attractive to manufacturers, because these methods provide potentially more robust and high precision assays.
- The MS platform has high-throughput capacity and enhanced specificity based on mass of substrate cleavage products.
- Endopeptidase assays do not require large specialized equipment, which would allow them to be easily deployed in the field.

Disadvantages that were identified include:

- Endopeptidase assays do not account for BoNT binding and translocation.
- BoNT endopeptidases require a controlled buffer environment for optimal enzymatic activity.
- Results are very sensitive to variations in assay conditions (e.g., pH, sample matrix).

- Endopeptidase assays would not be useful for product stability studies because changes to the heavy chain do not affect light chain activity.
- Endogenous proteases, which may differ between samples, may cause problems when assaying samples in complex matrices. Exacting sample preparation using antibodies may be needed to remove the toxin from the matrix. This would limit detection to known toxin types and possibly subtypes.
- Availability of reagents is a critical factor. Assay formats relying on antibodies will encounter problems if long-term supply of high quality reagents cannot be guaranteed.
- Some analytical platforms (e.g., mass spectrometry) are expensive.

The panel did not set priorities for validation of specific assays. They did recommend that validation criteria, reflecting the intended use of the assay, be established within the developing laboratory, followed by multi-laboratory (i.e., round robin) testing to determine robustness.

Panel Discussion on Cell-Based Assays (Session 3B Panel)

Moderators: Susan Maslanka, Ph.D. (CDC) and Shashi Sharma, Ph.D. (CFSAN, FDA)

Panelists: K. Roger Aoki, Ph.D. (Allergan, U.S.), Andreas Rummel (Medical School of Hannover, Germany), Michael Adler, Ph.D. (United States Army Medical Research Institute of Chemical Defense [USAMRICD], U.S.), J. Oliver Dolly, Ph.D. (Dublin City University, Ireland), Guenter Gross, Ph.D. (University of North Texas, U.S.), Leonard Smith, Ph.D. (USAMRIID, U.S.), James Keller, Ph.D. (FDA, U.S.), Frank Gessler, Dr. med. vet. (University of Goettingen, Germany)

The panel reached consensus that, while potential exists for their utility, no cell-based methods could currently replace or reduce animal use for potency testing. Limitations to the usefulness of cell-based assays include:

- Different assays do not give similar results with the same cell line.
- Cell line shelf lives might be limited.
- Matrix effects could give inaccurate results.
- The assays may not be as sensitive as the LD_{50} test.

The panel agreed, however, that cell-based methods might be the best *in vitro* option for capturing all mechanisms of intoxication - binding, translocation, and enzymatic activity. However, cell-based methods lack the sensitivity of the LD_{50} test, and most methods work only when conducted using purified toxin. Multiple cell lines might more closely mimic the mouse, but might be too complex for uniform adoption in many laboratories. The panel indicated that co-culture methods should be explored.

For a cell-based assay to replace the mouse bioassay, the panel agreed that several criteria would have to be met:

- It must account for both inhibition of release and cleavage of the substrate.
- It must be standardized, easy to maintain, and easily transferable.

- It must be rapid, especially in the context of responding to bioterrorism or naturally occurring foodborne outbreaks.
- It must be as sensitive as the mouse bioassay and show a reproducible correlation between activity and mouse LD_{50} units.
- It must not be susceptible to matrix effects.
- It must be reproducible within and among laboratories.

To minimize variability and to eliminate potential shelf-life problems, the ideal cell-based assay would use an immortalized cell line instead of primary cultures.

Knowledge gaps of cell-based methods identified by the panel include identifying the optimum cell types and/or cell lines, and how closely a cell-based method mimics the physiological situation of an intact organism. Better characterization of factors such as binding effects, how receptors are expressed, and sensitivity to environmental effects is needed.

The panel expressed interest in cell-based alternative methods using neuronal network biosensors. These methods, which were not specifically discussed in this session, provide cellular responses using the sensitive electrophysiological mechanisms that are directly affected by BoNT action.

The panel's consensus was that methods that use a single cell line might initially be the easiest to develop, standardize, and validate. However, a multicellular approach should also be pursued, in order to strive toward total replacement. When validating a particular method, the toxins and subtypes to be used must be specified, and each particular method must be validated for the specific purpose for which it is intended (i.e., detection, potency, antitoxin, etc.).

SESSION 4 - REFINEMENT (LESS PAIN AND DISTRESS) OF ANIMAL USE FOR BOTULINUM TOXIN POTENCY TESTING

Co-Chairs: Elizabeth Shores, Ph.D. (CDER, FDA), Leonard Smith, Ph.D. (USAMRIID) and William Stokes, D.V.M., D.A.C.L.A.M. (NICEATM, U.S. National Institute of Environmental Health Sciences [NIEHS])

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
- The use of alternate *in vivo* models to measure botulinum activity without lethality
- The use of earlier non-lethal humane endpoints for the current *in vivo* botulinum assay

Botulinum Neurotoxin: Determination of Potency Using a Mouse *Ex Vivo* Test

Presenter: Andreas Rummel (Medical School of Hannover, Germany)

The mouse phrenic nerve assay (MPN) is a possible alternative to the mouse LD_{50} bioassay for the determination of BoNT potency. It uses a phrenic nerve preparation isolated from the mouse diaphragm. The nerve preparation is maintained in an organ bath at a controlled temperature (37 °C), pH, and oxygen tension, which allows it to remain viable for hours in the laboratory. The assay measures the amplitude of a twitch response to electrical stimulation of the nerve. Toxin potency can be directly determined as the decrease in the amplitude of the twitch response after toxin is applied to the medium. The usual endpoint of the assay is the time until a 50% decrease in amplitude is observed (Göschel et al. 1997).

Because the MPN assay requires mice, it cannot fully replace the use of animals for BoNT potency testing. However, it is a more humane alternative because the mice do not slowly die from the distressing effects of botulism. As a potency assay, it assesses all three functional domains of BoNT, provides results within two hours, and allows experimental conditions to be easily varied. It also can be used to quantify neutralizing antibodies. However, it requires laboratory personnel trained in the use of sophisticated and expensive equipment. Additional comparability studies are needed to fully characterize the usefulness and limitations of the MPN assay.

Mouse Hind Limb Assay

Presenter: K. Roger Aoki, Ph.D. (Allergan, U.S.)
Starting in 2001, Allergan investigated an assay that used a digital abduction score (DAS) as an endpoint for determining safety margins for several BoNT serotypes. The DAS assay is a clinically relevant measure of BoNT activity that does not use lethality as an endpoint (Aoki 2001). The DAS depends on the effect of local muscular weakness induced by BoNT on the toe-spread reflex in mice. The varying degrees of abduction are scored on a five-point scale. Studies indicate that the DAS assay is dose responsive, reproducible, and repeatable. It also provides a means to quantitatively compare local vs. distal effects of BoNT. Peak DAS values can be used to determine an intramuscular (IM) ED_{50} value, which is useful for comparison of the potencies of different serotypes and products. Additionally, an IM LD_{50} value can be determined. The ratio of the IM LD_{50} to the IM ED_{50} is defined as the safety margin for a product or preparation (Aoki 2001). The DAS assay is applicable to multiple serotypes and products, and has potential as a potency assay, if used relative to a reference standard.

Mouse Abdominal Ptosis Assay or Flaccid Paralysis: Non-lethal Mouse Model for Botulinum Toxin Potency Testing

Presenter: Dorothea Sesardic, Ph.D. (NIBSC, U.K.)
The mouse flaccid paralysis assay (also known as the mouse abdominal ptosis assay) relates the activity of BoNT to the degree of abdominal bulging seen after toxin is subcutaneously injected in the left inguinocrural region of a mouse (Sesardic et al. 1996, 2004; Jones et al. 2006). It has been proposed as a refinement to the mouse LD_{50} test because it relies on a humane endpoint (only a sub-lethal dose of BoNT is injected). This endpoint is more relevant to clinical use than death as an endpoint, since it evaluates localized muscle paralysis rather than systemic toxicity. The flaccid paralysis assay is more rapid than a lethality test, yielding

results in 24 to 48 hours. It measures the activity of all three functional domains of the BoNT protein, and it is relatively easy to conduct, with no specialized equipment required.

Two or more independent observers score animals at 24 and 48 hours on a five-point scale according to the size of the local abdominal bulge. The magnitude of the flaccid paralysis endpoint is dose-dependent for all serotypes except serotype D (Sesardic et al. 2004). This assay has been validated at NIBSC for potency testing of BoNT products (Sesardic et al. 1996). Since sub-lethal doses are used (maximum dose is 0.2 mouse LD_{50} units), the ED_{50} is 10-fold more sensitive than the LD_{50}.

The flaccid paralysis assay has been used to verify activity in product-specific reference samples. Relative potency estimates determined by flaccid paralysis and LD_{50} assays are comparable, with precision depending on numbers of mice used in each case. A flaccid paralysis assay using 68 mice yields results with precision comparable to an LD_{50} assay using more than 380 mice.

The flaccid paralysis assay has been included in the most recent European Pharmacopeia (EP) monograph for BoNT type A for injection, effective from 2005, and a transferability exercise with a U.K. testing laboratory was initiated in 2006 (EP 2005). The flaccid paralysis assay has also been adopted for use with potency testing of botulinum antitoxins (Jones et al. 2006).

Overview of the Physiological Progression of Botulinal Symptoms in Mice

Presenter: Eric Johnson, D.Sc. (University of Wisconsin, U.S.)
The first symptom of botulism toxicity in the mouse is usually ruffled fur, which starts in the shoulders and progresses along the back. This symptom does not always occur, and may be short-lived if it does occur. The first classic botulism toxicity symptom is wasp-waist, which is visible as a constriction of the diaphragm. Wasp-waist generally occurs early, may be subtle, and is best evaluated when compared to a non-injected mouse. Hind limb paralysis and decreased overall movement with difficulty in walking occurs next, followed by labored breathing. Labored breathing is considered a severe symptom and is a classic symptom of botulism toxicity if it follows wasp-waist. The decision to euthanize can be considered at this point, although mice do occasionally recover after displaying these symptoms.

A rapid lethality assay has been developed in which mice are injected intravenously in the tail vein with high does of BoNT. Time to death is dose-dependent and results may be obtained in approximately one day if a sufficiently high dose of BoNT (10^6 mouse LD_{50} units) is used, but the assay is not as sensitive as the classic lethality assay.

Non-lethal Endpoints in Botulinum Toxin Potency Assay

Presenter: Jerry Calver, Ph.D. (Calver Biologics Consulting, Canada)
BoNT products are subject to the lot release system, which is a system of approval for release of a specific lot onto the Canadian market. Animal testing is an integral part of the lot release system at the Bureau of Biologics and Radiopharmaceuticals (BBR), Health Canada. Approval is based upon certification that the lot meets appropriate in-process controls, and control tests on the final product.

Research done at the BBR and Animal Resources Division at Health Canada has significantly reduced pain and distress in test animals involved in botulinum toxin potency testing (Calver et al. 2000). Clinical endpoints for euthanasia (i.e., stage 3 symptoms as described below) were chosen in lieu of death. The limits and criteria of acceptance for the assay remained the same as when death was used as an endpoint.

Mice exhibit three successive symptomatic stages following injection of adequate concentrations of BoNT:

- Stage 1: slightly indrawn scaphoid abdomen (lightly raised rib)
- Stage 2: severe indrawn scaphoid abdomen (highly raised rib)
- Stage 3: severe indrawn scaphoid abdomen with respiratory distress in the form of hiccough & pupillatory dilation (eyes wide open, bulging)

In a number of different experiments using lethality as the endpoint, it was noted that mice that exhibited stage 3 symptoms died within the 72-hour monitoring period. In separate experiments, for a reference toxin lot, LD_{50} determinations made by using euthanasia at stage 3 as an endpoint were similar to those determined by using lethality as an endpoint.

Dr. Calver recommended that a collaborative study should be undertaken to verify the use of euthanasia at stage 3 symptoms as an appropriate non-lethal endpoint for BoNT testing.

Panel Discussion on Refinement (Less Pain and Distress) of Animal Use for Botulinum Toxin Potency Testing (Sessions 4A, 4B and 4C Panels)

The panel was asked whether any of the alternative methods discussed in this session could be implemented now to reduce, refine, or replace the mouse LD_{50} assay. The panel were also asked to discuss the pros and cons of each reviewed method, identify knowledge and research gaps related to them and prioritize the methods for further development and validation studies. A list of the questions presented to each panel can be found in **Appendix B** and at http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/PanelQuestions.pdf.

Panel Discussion on *Ex Vivo* Methods (Session 4A Panel)

Moderator: Elizabeth Shores, Ph.D. (CDER, FDA)

Panelists: Dorothea Sesardic, Ph.D. (NIBSC, U.K.), Eric Johnson, Sc.D. (University of Wisconsin, U.S.), Jerry Calver, Ph.D. (Calver Biologics Consulting, Canada), K. Roger Aoki, Ph.D. (Allergan, U.S.), Andreas Rummel (Medical School of Hannover, Germany), Coenraad Hendriksen, M.Vet.Sc, D.V.M., Ph.D. (Netherlands Vaccine Institute, The Netherlands), Martin Stephens, Ph.D. (HSUS), James Keller, Ph.D. (FDA, U.S.), James Schmidt, Ph.D. (USAMRIID, U.S.), Michael Adler, Ph.D. (USAMRICD, U.S.)

The panel noted that the MPN assay has undergone validation and is considered adequate for batch release testing in Germany. Similarly, the panel noted that the rat intercostal neuromuscular junction (NMJ) assay, which was not specifically discussed in this session, but which was the subject of two poster presentations, is currently undergoing validation. Comparability studies are ongoing for both assays. These assays still require animals for

donation of tissues and therefore cannot be considered complete replacement alternatives to the mouse LD_{50} assay, but rather as refinement/reduction alternatives. The MPN assay is promising as a reduction alternative with estimates of at least a 50% reduction in animal use reported.

The panel identified several advantages of the MPN assay. It is not an *in vivo* animal experiment but uses animals as tissue donors, and therefore is an *ex vivo* test. The assay has quantitative endpoints and experimental conditions can easily be varied, with results available within two hours. The assay can also be used to quantify neutralizing antibodies.

Limitations of the MPN include lack of throughput, difficulty preparing the equipment to run the assay, the complexity of equipment required, and the fact that these assays are technically challenging to run. There may also be matrix effects, although sample preparation techniques such as dialysis may improve performance. Human serum albumin, a common constituent of commercial BoNT preparations, does not appear to interfere with the assay, provided the albumin concentration remains constant. Given the lack of available information, particular attention should be devoted to the possible effects of excipients in products on the activity of BoNT in *ex vivo* muscle preparations.

The panel also considered that smooth muscle preparations from larger animals could be used, which would yield a greater number of preparations from a single animal. However, they did not consider slaughterhouse animals to be useful or appropriate for this use.

Panel Discussion on Non-lethal *In Vivo* Methods (Session 4B Panel)

Moderator: Len Smith, Ph.D. (USAMRIID)
The panel noted that neither the hind limb assay nor the abdominal ptosis assay is a complete replacement alternative to the mouse LD_{50} assay since both require animals. However, these assays do have the potential to replace the severe LD_{50} endpoint with a considerably less severe procedure from which mice typically recover. The panel suggested that qualitative endpoints (e.g., photodocumentation) may support assay results, and that reference photographs and a training video would help resolve any transferability or training issues. Drawbacks relevant to detecting BoNT in environmental or biological samples include that the effect of sample matrices is often not well defined and that prior knowledge of approximate level of toxin in the sample is needed to determine a non-lethal dose range. Moreover, the tolerated dose is often unknown. These methods are also labor-intensive.

The panel also noted that several variations on *in vivo* models based on muscular paralysis exist, in addition to flaccid paralysis or abdominal ptosis in the mouse. Some of these approaches were developed primarily to study toxin duration of action and muscle weakness. Other approaches have focused on monitoring changes in membrane potential associated with postsynaptic action such as compound muscle action potential (CMAP). All show important dose-dependent changes in response to toxin, which is essential for potency testing. However, at present, most of these assays are considered only as research tools.

The panel identified several advantages of the hind limb assay. For example, it provides a clinically relevant measure of BoNT activity by measuring local weakness using a non-lethal endpoint, it is applicable to wide range of products, it shows a robust dose response, and it is amenable to repeated measurement.

Advantages of the abdominal ptosis assay noted by panelists include that it is a fully functional assay with dosing more similar to clinical use than that of the LD_{50} assay. Animals normally exhibit no signs of stress or pain, therefore minimal monitoring of animals is required. The assay is more humane, ethical, and economical. The assay duration is relatively short compared to LD_{50} (48 hours vs. 72 or 96 hours), and no specialized equipment or reagents are required. The assay is robust and easily transferred to other laboratories.

One disadvantage of both assays indicated by the panel is that they use animals. No reduction in total numbers is achieved, and some non-lethal *in vivo* assays may actually use more animals. Another disadvantage to both assays is that they use subjective/qualitative scoring systems.

The panel agreed that both methods need to be validated and that a correlation with LD_{50} must be demonstrated.

Panel Discussion on Humane Endpoints (Session 4C Panel)

Moderator: William Stokes, D.V.M., D.A.C.L.A.M. (NICEATM, NIEHS)
The panel noted that Health Canada has validated and has been using a non-lethal endpoint in the mouse LD_{50} assay for a number of years. The panel suggested that a multi-laboratory study should be conducted using the same endpoint as used at Health Canada (i.e., severely raised scaphoid in conjunction with hiccough and eyes wide open).

The panel did not identify any other clinical signs or any objective endpoints (e.g., temperature, heart rate, blood pressure, pO_2) that are sufficiently predictive of mouse lethality that they could be used as humane endpoints in the mouse LD_{50} assay. The panel agreed that clear documentation of the clinical signs and their severities, which occur throughout the progression of toxicity, is necessary during routine studies. Additional studies could demonstrate the predictivity of each potential non-lethal endpoint (or a battery of endpoints) for death within the observation period. Increasing the frequency of observations may identify moribund animals and decrease the number of spontaneous deaths. Identifying the clinical signs that indicate the lack of reversibility is essential to accurately predict death. The panel also recommended that clinical signs be evaluated in the dark cycle when behaviors are typically exhibited, and suggested that differences in clinical symptoms (in both progression and time of appearance) among serotypes might exist.

For measuring body temperature, a subcutaneously implanted temperature transmitter can be used with stress-free external monitoring. This approach has been used in several animal models for vaccine potency testing, such as whole cell pertussis potency testing. Telemetry could be used to collect objective data, but this approach may be cost prohibitive. Videography/photography should be used to record observations. For all LD_{50} assays, all available scores, objective measures (such as temperature), mouse characteristics (such as weight, sex, etc.), and other recommended endpoints (e.g., respiratory changes, daily body weights) should be collected and appropriately linked in databases.

Finally, the panel reached a consensus that multi-laboratory validation studies to identify endpoints that are predictive of eventual death should be conducted.

SESSION 5 - REDUCTION OF ANIMAL USE FOR *IN VIVO* BOTULINUM TESTING

Co-Chairs: Marlies Halder, Ph.D. (European Centre for the Validation of Alternative Methods [ECVAM]) and Richard McFarland M.D., Ph.D. (CBER, FDA)
This session discussed strategies to reduce the number of animals used in the current *in vivo* botulinum toxin test.

Statistical Considerations for the Mouse LD_{50} Assay and the Impact of Toxin Reference Standards

Presenter: Rose Gaines Das, Ph.D. (NIBSC, U.K.)
The appropriate use of animals for estimates of LD_{50} or relative potency values in individual experiments requires an optimal experimental design and selection of a suitable statistical model. Some assumptions are inherent in the estimation of an LD_{50} parameter. The simplest model used is that the proportion of animals responding increases with increasing concentration of a toxin. Typically, stronger assumptions are made about the dose-response relationship and the tolerance distribution in the population under consideration.

Important considerations for the optimal experimental design for LD_{50} estimation include the selection of the number and concentration of dose levels, the total number of animals tested, and their distribution among the selected dose levels. Two broad principles apply: 1) doses with response levels closer to 50% are more informative than doses with more extreme responses, and 2) the greater the number of independent replicates, the greater the precision of the LD_{50} estimate.

Various numerical methods to analyze data obtained from an LD_{50} determination exist. A numerical method can fail (i.e., provide a poor estimate) because of incorrect assumptions made when designing the experiment or by choosing the wrong numerical method for data analysis.

An LD_{50} value estimated from a single experiment is of limited utility. When interpreting results, it is important to realize that absolute measures such as LD_{50} values are not biological constants and that proper interpretation is dependent on assay conditions and their effect on the endpoint (McLellan et al. 1996, Sesardic et al. 2003).

For the assay of BoNT A, the EP (EP 2005) now requires that a suitable reference preparation be assayed in parallel. If a reference standard is incorporated into a bioassay for potency, the unit of measurement becomes relative potency instead of potency. A relative potency value is expressed as unit of activity defined in terms of the activity of the reference standard. Relative potency reflects relative biological activity as does the LD_{50}, but unlike the LD_{50}, may be system-independent to the extent that the biological systems used to determine the absolute and relative potencies are specific for the same essential activity (Sesardic et al. 1994, 2003). If a meaningful comparison between relative potency values for different preparations is to be made, functional similarity of the dose–response curves for the reference standard and samples is a fundamental condition for assay validity.

The comparative nature of biological methods makes suitable controls or reference standards essential. Their use allows greater independence from assay conditions, which leads to

improved reproducibility and consistency between and within laboratories. The proper use of such methodology will provide wider opportunities for the development and use of alternative endpoints to lethality for refinement of animal methods and may lead to eventual replacement.

Refinement and Reduction in Botulinum Toxin Testing

Presenter: Kenneth Clarke, Ph.D. (Allergan, U.S.)
Drug product lot release and testing of potency reference standards represent two areas of opportunity for reduction or refinement of the mouse LD_{50} assay which would have immediate impact. The dose-response curve for BoNTs is very steep; typically, in mice, no response is seen with doses less than 0.5 units, and a full response (i.e., death) occurs with doses above 2 units. However, doses outside of this range are routinely used in the mouse LD_{50} assay. Testing over a four-fold range (i.e., 0.5 to 2.0 units) could achieve reduction in animal use and eliminate obtaining unnecessary information. Lot release testing is confirmatory testing (i.e., it confirms results already obtained during batch testing) that is done on a finished product of known potency produced via a validated manufacturing process. Therefore, a significant reduction in animal use could be achieved at this stage by limiting the dose range used and decreasing the number of animals used per dose. Further reduction could be obtained by averaging reportable values (combining replicates into the overall analysis) and tightening specification limits for the average.

Reduction of animal use by approximately 33% has also been achieved by increasing the shelf life of required potency reference standards used during testing, and decreasing the need for *in vivo* qualification testing during the development of new references standards. Fine-tuning of the *in vivo* qualification test methodology for lot release testing has yielded a further 25% reduction in animal use.

Another approach under consideration for further reduction of animal use is to increase the volume of drug product lots for batch release, stability testing and supplemental regulatory filings. This would effectively reduce the amount of *in vivo* testing on a per vial basis.

Panel Discussion on Reduction of Animal Use for *In Vivo* Botulinum Testing (Session 5 Panel)

The panel was asked about the feasibility of using a validated *in vitro* or *ex vivo* test method to assess potencies of final production lots and about the use of reference standards and other best practices to minimize the number of animals used. A list of the questions presented to each panel can be found in **Appendix B** and at http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/PanelQuestions.pdf.

Moderators: Marlies Halder, Ph.D. (ECVAM) and Richard McFarland, M.D., Ph.D. (CBER, FDA)

Panelists: Dorothea Sesardic, Ph.D. (NIBSC, U.K.), Tim Terrell, Ph.D. (Allergan, U.S.), Kenneth Clarke, Ph.D. (Allergan, U.S.), Andy Pickett, Ph.D. (Ipsen, U.K.), Christopher Bishop, B.Sc., C.Chem., F.R.S.C. (Wickham Laboratories, Inc., U.K.), Rose Gaines Das, Ph.D. (NIBSC, U.K.), Abigail Jacobs, Ph.D. (FDA, U.S.), Susan Maslanka, Ph.D. (CDC, U.S.), Tonie Rocke, Ph.D. (USGS, U.S.)

The panel reached a consensus that it is feasible and practical to use the mouse LD_{50} assay to assess the potency of batch production samples of botulinum toxin and to use a validated *in vitro* or *ex vivo* test method to assess potencies of final production lots. In fact, this approach is currently being used at NIBSC for the purposes of confirmatory testing; manufacturers provide potency values determined via LD_{50} tests to NIBSC, which are confirmed with an endopeptidase assay. To further develop this strategy, areas where the most animals are used must be identified and evaluated first. Regulatory decisions will continue to be made on a case-by-case basis with the ultimate priority being protection of public health.

The panel noted that other practices that could be implemented to decrease the number of mice tested include:

- Use of a modified lot release assay would reduce the number of animals used by testing fewer animals at extreme ranges of the dose-response curve.
- A potency reference standard program could reduce *in vivo* testing by (1) extending the shelf-life of the working reference standard, and (2) improving the efficiency of the qualification program.
- It is essential to use a common set of suitable samples in any validation studies and in any studies comparing different assay methods. Inclusion of a set of common samples with known long-term stability and in sufficient quantity for multiple uses is therefore desirable. This allows for comparison between different studies at different times and for continuity/harmonization of assay methods.

The panel agreed that use and establishment of an international reference standard would contribute towards harmonization, but that this would be very difficult to implement. Product-specific standards are still likely to be needed, and at present, each manufacturer uses its own product-specific standard for potency testing. Therefore, a central repository with associated standardized methodology would be useful.

WORKSHOP OUTCOMES

In general, the consensus of the panel was that some of the alternative 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 these methods can currently be considered a complete replacement for the mouse LD_{50} assay, either for detection of BoNT or for potency determination. The panel noted that, with additional development and validation efforts, some of the methods might serve 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 needed if the intended use of a test method is as a replacement for the mouse LD_{50} assay. Specific direction from international regulatory authorities would assist these efforts. Finally, best practices for BoNT testing were discussed to decrease the number of animals used in routine testing, including:

- The use of reference standards to minimize the number of replicate animals needed
- The use of standardized methodology
- The reduction in the number of doses used in confirmatory testing for potency (e.g., lot release testing)

WORKSHOP PARTICIPANTS

A list of all participants at the workshop may be found in **Appendix E** and at: http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/ParticipantList.pdf

POSTER SESSION

In addition to the talks and panel discussions, ten posters were presented at the workshop. A list of these, accompanied by abstracts, is in **Appendix C**. Abstracts may also be viewed at: http://iccvam.niehs.nih.gov/methods/biologics/botdocs/biolowkshp/Notebooks/Abstracts.pdf

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APPENDIX A

Workshop Agenda

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ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD_{50} Assay For Botulinum Toxin Testing (November 13-14, 2006)

Agenda

Abstract

Botulism is a potentially deadly illness. It can be acquired by humans from eating 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. Botulism has been a public health hazard for centuries and today is emerging as a significant bioterrorism threat. Botulinum toxin also has been recently developed into a drug to treat many serious and painful medical conditions that affect the human nervous system.

Currently, the most frequently used method for detecting botulinum toxin in foods or in the environment, or for assessing the potency of the drug, is a test called the mouse LD_{50} assay. This test 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.

This workshop has been convened to bring together stakeholders and scientists from leading governmental and academic institutions, national and global regulatory authorities, and the animal protection community to review the current state-of-the-science for alternative methods that may reduce, replace, and refine (cause less pain and distress) the use of mice for botulinum toxin testing, and to identify high priority research, development, and validation studies.

Workshop Goals

To review the state-of-the-science and current knowledge of alternative methods that may reduce, replace, and refine (less pain and distress) 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 (less pain and distress), 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 Agenda and Topics

Day 1 **Monday, November 13, 2006**

0830 ***Welcome and Introduction of Workshop Goals and Objectives***
- William Stokes, Director, NICEATM
- Len Schechtman, Chair, ICCVAM
- Marlies Halder, ECVAM

Session 1 **Overview of Public Health Needs for Botulinum Toxin Testing and Regulatory Requirements**
This session will summarize 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.
Co-Chairs: Abby Jacobs and Jodie Kulpa-Eddy

Food Safety:

0840 ***Overview of Botulinum Toxin and the Incidence and Severity of Botulism***
- Susan Maslanka (U.S. Centers for Disease Control and Prevention)

This topic will provide a brief overview on botulinum toxin and provide information on the history and global outbreaks of botulism.

0905 ***Current Testing and Practices for Botulinum Prevention in Foods***
- Shashi Sharma (U.S. Food and Drug Administration, CFSAN)

This topic will provide a brief overview of regulations for food safety and testing in the U.S. and Europe.

Drug Safety:

0915 ***Medical Conditions Treated with Botulinum Toxin***
- Mark Hallett (U.S. National Institutes of Health, National Institute of Neurological Disorders and Stroke)

This topic will provide a brief overview of the clinical applications of botulinum toxin.

0940 ***Current Potency Testing Requirements and Practices for Botulinum Toxin Products***
- Elizabeth Shores (U.S. Food and Drug Administration, CDER)

This topic will provide a brief overview of regulations for drug safety testing in the U.S. and Europe.

Vaccine Potency Testing:

0950 ***Current Testing Requirements and Practices for Botulinum Toxin for Vaccine Potency Testing***
- Jodie Kulpa-Eddy (U.S. Department of Agriculture)

This topic will provide a brief overview of regulations for vaccine safety and potency testing in the U.S. and Europe.

Diagnostic Needs:

1000 ***Current Animal Diagnostic Testing Requirements and Practices for Botulinum Toxin Potency and Detection***

- Tonie Rocke (U.S. Geological Survey, National Wildlife Health Center)

This topic will provide a brief overview of requirements for botulinum toxin detection and testing of environmental samples in the U.S. and Europe.

1010 **Break**

Session 2 **Current Understanding and Knowledge Gaps for Botulinum Toxin**

This session will summarize the current understanding of structural aspects, mechanisms, and modes of action of the botulinum toxin; discuss the aspects of the endopeptidase function that would need to be modeled by alternative test methods; and prioritize research needs to address gaps needed to facilitate the development of alternative test methods.
Co-Chairs: James Keller and Ram Ramabhadran

1030 ***Overview of the Modes and Mechanisms of Action of Botulinum Toxin***

- Dirk Dressler (Rostock University, Germany)

This talk will discuss background information on botulinum toxin mechanisms of action to provide a basis for consideration of information provided in the remainder of this workshop.

1100 ***Pharmacokinetics of Botulinum Toxin***

- Lance Simpson (Thomas Jefferson University, U.S.)

This talk will discuss the absorption, distribution, metabolism and excretion of botulinum toxin *in vivo,* and aspects that will need to be modeled or measured in *in vitro* replacements for the current *in vivo* test.

1130 **Lunch**

1230 ***Essential Characteristics of Potential Test Methods to Replace the Mouse LD_{50} for Botulinum Toxin Potency Testing***

- Eric Johnson (University of Wisconsin, U.S.)

This talk will discuss the criteria for an acceptable replacement for the mouse LD_{50} test method for botulinum toxin potency testing.

1245 ***Overview of U.S. and European Research Initiatives on Botulinum Toxin***

- Lillian Van De Verg (U.S. National Institutes of Health, National Institute of Allergy and Infectious Diseases)
- Andreas Rummel (Medical School of Hannover, Germany)

This talk will describe current research on botulinum toxin being funded by NIH and in Europe.

1305 **Panel Discussion on the Current Understanding and Knowledge Gaps for Botulinum Toxin (Session 2 Panel)**

- *Moderators:* James Keller and Ram Ramabhadran
- *Panelists:* Dirk Dressler, Mark Hallett, Eric Johnson, Andreas Rummel, Shashi Sharma, Lance Simpson

Session 3 **Potential Replacement of Animal Use for Botulinum Toxin Potency Testing**

This session will provide an overview of alternative *in vitro* models that, if sufficiently validated, could replace the current *in vivo* botulinum toxin test.
Co-Chairs: Susan Maslanka and Shashi Sharma

Session 3A: **Potential Replacement: Endopeptidase Assays**

1405 ***Overview of Endopeptidase Assays***

- Dorothea Sesardic (National Institute for Biological Standards and Control, U.K.)

This talk will provide an overview of the endopeptidase assays for botulinum toxin detection and potency testing as a proposed alternative for the mouse LD_{50} assay and describe the advantages, limitations, and current validation status of the various detection methods.

1435 **Break**

Session 3B: **Potential Replacement: Cell-Based Assays**

1455 ***Overview of Cell-Based Assays***

- K. Roger Aoki (Allergan, Inc., US)

This talk will provide an overview of frequently used cell-based assays for botulinum toxin detection and potency testing as proposed alternatives for the mouse LD_{50} assay and describe their advantages, limitations, and current validation status.

1525 **Panel Discussion on Potential Replacement of Animal Use for Botulinum Toxin Potency Testing (Session 3 Panel)**

Panel for Session 3A

- *Moderators:* Susan Maslanka and Shashi Sharma
- *Panelists:* John Barr, Frank Gessler, Eric Johnson, Andy Pickett, Ram Ramabhadran, James Schmidt, Dorothea Sesardic, Clifford Shone, Bal Ram Singh

Panel for Session 3B

- *Moderators:* Susan Maslanka and Shashi Sharma
- *Panelists:* Michael Adler, K. Roger Aoki, J. Oliver Dolly, Frank Gessler, Guenter Gross, James Keller, Andreas Rummel, Leonard Smith

1655 **Close of Day 1**

1700 Poster Session

Day 2 **Tuesday, November 14, 2006**

Session 4 **Refinement (Less Pain and Distress) of Animal Use for Botulinum Toxin Potency Testing**

This session will provide 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 will be discussed:

- The use of *ex vivo* test models prepared from humanely euthanized animals
- The use of alternate *in vivo* models to measure botulinum activity without lethality
- The use of earlier non-lethal humane endpoints for the current *in vivo* botulinum assay

Co-Chairs: Elizabeth Shores, Leonard Smith, and William Stokes

Session 4A: Refinement: Using *Ex Vivo* Assays to Avoid Pain and Distress in Botulinum Testing

0830 ***Mouse Phrenic Nerve-Hemidiaphragm Assay***

- Andreas Rummel (Medical School of Hannover, Germany)

This talk will provide an overview of the mouse phrenic nerve-hemidiaphragm assay for botulinum toxin detection and potency testing as a proposed alternative for the mouse LD_{50} assay and describe its advantages, limitations, and current validation status.

Session 4B: Refinement: Alternative *In Vivo* Botulinum Assays that Do Not Require Death as an Endpoint

0845 ***Mouse Hind Limb Assay***

- K. Roger Aoki (Allergan, Inc., U.S.)

This talk will provide an overview of the mouse hind limb assay for botulinum toxin detection and potency testing as a proposed alternative for the mouse LD_{50} assay and describe its advantages, limitations, and current validation status.

0900 ***Mouse Abdominal Ptosis Assay***

- Dorothea Sesardic (National Institute for Biological Standards and Control, U.K.)

This talk will provide an overview of the mouse abdominal ptosis assay for botulinum toxin detection and potency testing as a proposed alternative for the mouse LD_{50} assay and describe its advantages, limitations, and current validation status.

Session 4C: Refinement: Potential Use of Non-Lethal Endpoints in Botulinum LD_{50} Testing to Minimize Pain and Distress

0915 ***Overview of the Physiological Progression of Botulism in Mice***

- Eric Johnson (University of Wisconsin, U.S.)

This talk will describe the progression of the disease of botulism in mice to provide a framework for a consideration of humane endpoints for the LD_{50} assay and alternative *ex vivo* assays.

0925 ***Potential Behavioral and Pharmacological Endpoints Predictive of Mouse Lethality***

- Jerry Calver (Calver Biologics Consulting, Canada)

This talk will describe humane endpoints that could be used to predict lethality in mice used for botulinum testing and the current validation status of each endpoint.

0940 **Break**

0955 **Panel Discussion on Refinement (Less Pain and Distress) of Animal Use for Botulinum Toxin Potency Testing (Sessions 4A, 4B, and 4C Panels)**

- *Moderators:* Leonard Smith and William Stokes
- *Panelists:* Michael Adler, K. Roger Aoki, Jerry Calver, Coenraad Hendriksen, Eric Johnson, James Keller, Andreas Rummel, James Schmidt, Dorothea Sesardic, Martin Stephens

1130 **Lunch**

Session 5 **Reduction of Animal Use For *In Vivo* Botulinum Testing**

This session will discuss strategies to reduce the number of animals used in the current *in vivo* botulinum toxin test.
Co-Chairs: Marlies Halder and Richard McFarland

1230 ***Impact of Sample Size and Toxin Reference Standards on LD_{50} Results***

- Rose Gaines Das (National Institute for Biological Standards and Control, U.K.)

This talk will provide a statistical consideration of the mouse LD_{50} assay and the effects of decreasing the number of animals tested. This talk also will describe the use of toxin reference standards the mouse LD_{50} assay and the effects on the accuracy, sensitivity, specificity, false positive rate, and false negative rate.

1255 ***Proposed Testing Strategies that Would Reduce Animal Use in Botulinum Toxin Testing***

- Kenneth Clarke (Allergan, Inc., U.S.)

This talk will outline areas that need to be addressed in order to reduce the amount of *in vivo* testing associated with botulinum toxin manufacturing prior to the availability of non-animal replacement assay. Areas include the current

protocol used for drug product lot release as well as the potency reference standard program.

1320 **Panel Discussion on Reduction of Animal Use for *In Vivo* Botulinum Testing (Session 5 Panel)**

- *Moderators:* Marlies Halder and Richard McFarland
- *Panelists:* Christopher Bishop, Kenneth Clarke, Rose Gaines Das, Abby Jacobs, Susan Maslanka, Andy Pickett, Tonie Rocke, Dorothea Sesardic, Timothy Terrell

1420 **Break (15 mins)**

Session 6 **Wrap-up of Panel Discussions**

This session will summarize the outcomes from each panel discussion.
Chair: Len Schechtman

1435 ***Summary of Session 2 Discussions: Knowledge Gaps and Research Needs***

- James Keller and Ram Ramabhadran

1450 ***Summary of Session 3 Discussions: Replacement Alternatives***

- Susan Maslanka and Shashi Sharma

1505 ***Summary of Session 4 Discussions: Refinement Alternatives***

- Elizabeth Shores, Leonard Smith and William Stokes

1520 ***Summary of Session 5 Discussions: Reduction Alternatives***

- Marlies Halder and Richard McFarland

1535 **Closing Comments**

1550 **End of Meeting**

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APPENDIX B

Panel Discussion Questions

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ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD_{50} Assay For Botulinum Toxin Testing

Panel Discussion Questions

The following panel questions have been reviewed and approved by the organizers. However, ICCVAM, NICEATM, and ECVAM would welcome additional issues, comments, suggestions, and questions that should be addressed or discussed during this workshop.

Session 2 Panel Questions: Current Understand and Knowledge Gaps for Botulinum Toxin[1] (Moderators: James Keller and Ram Ramabhadran)

- What knowledge gaps in the current understanding of the mechanism of action of botulinum toxin must be addressed to develop non-animal replacement methods for potency testing or detection of botulinum toxin?
- To what extent does current research address these knowledge gaps? Does additional effort need to be applied to these areas?
- What research initiatives are necessary to address these knowledge gaps and further characterize mechanisms and modes of action in order to advance the development of non-animal replacement methods for potency testing or detection of botulinum toxin?

Session 3A Panel Questions: Potential Replacement of Animal Use for Botulinum Toxin Potency Testing - Endopeptidase Assays (Moderators: Susan Maslanka and Shashi Sharma)

- Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current endopeptidase methods be used now to replace animals for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the endopeptidase assays discussed be used to **replace** animals for these kinds of samples? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Can any of the current endopeptidase methods be used now to **reduce** the number of animals used for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the endopeptidase assays discussed be used to **reduce** the number of animals used for these kinds of samples? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?

[1] There is no panel discussion in Session 1, so there are no panel discussion questions for Session 1.

- Should endopeptidase methods other than those discussed so far during this workshop be considered for development and validation for potency testing or detection of botulinum toxin?
- What are the pros and cons of the different endopeptidase methods reviewed?
- What current knowledge gaps with regard to the reviewed endopeptidase methods must be addressed to further their use in potency testing or detection (as discussed in Session 1) of botulinum toxin? What additional studies are needed?
- Of the endopeptidase methods discussed, which should have the highest priority for further development and validation studies?
- What are the essential characteristics of an endopeptidase method sufficient to replace or reduce the number of animals used for potency testing or detection (as discussed in Session 1) of botulinum toxin?
- What is the best way to assess the validation status of these endopeptidase methods?

Session 3B Panel Questions: Potential Replacement of Animal Use for Botulinum Toxin Potency Testing - Cell-Based Assays (Moderators: Susan Maslanka and Shashi Sharma)

- Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current cell-based methods be used now to **replace** animals for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the cell-based assays discussed be used to **replace** animals for these kinds of samples? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Can any of the current cell-based methods be used now to **reduce** the number of animals used for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the cell-based assays discussed be used to **reduce** the number of animals used for these kinds of samples? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Should cell-based methods other than those discussed so far during this workshop be considered for development and validation for potency testing or detection of botulinum toxin?
- What are the pros and cons of the different cell-based methods reviewed?
- What current knowledge gaps with regard to the reviewed cell-based methods must be addressed to further their use in potency testing or detection (as discussed in Session 1) of botulinum toxin? What additional studies are needed?

- Of the cell-based methods discussed, which should have the highest priority for further development and validation studies?
- What are the essential characteristics of a cell-based method sufficient to replace or reduce the number of animals used for potency testing or detection (as discussed in Session 1) of botulinum toxin?
- What is the best way to assess the validation status of these cell-based methods?

Session 4A Panel Questions: Refinement: Using *Ex Vivo* Assays to Avoid Pain and Distress in Botulinum Testing (Moderators: Leonard Smith and William Stokes)

- Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current *ex vivo* methods be used now to **replace** animals for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the *ex vivo* assays discussed be used to **replace** animals for these kinds of samples? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Can any of the current *ex vivo* methods be used now to **reduce** the number of animals used for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the *ex vivo* assays discussed be used to **reduce** the number of animals used for these kinds of samples? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Should *ex vivo* methods other than those discussed so far during this workshop be considered for development and validation for potency testing or detection of botulinum toxin?
- What are the pros and cons of the different *ex vivo* methods reviewed?
- What current knowledge gaps with regard to the reviewed *ex vivo* methods must be addressed to further their use in potency testing or detection (as discussed in Session 1) of botulinum toxin? What additional studies are needed?
- Of the *ex vivo* methods discussed, which should have the highest priority for further development and validation studies?
- What is the best way to assess the validation status of these *ex vivo* methods?

Session 4B Panel Questions: Refinement: Alternative *In Vivo* Botulinum Assays that Do Not Require Death as an Endpoint (Moderators: Leonard Smith and William Stokes)

- Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current non-lethal *in vivo* methods be used now to **replace** animals for potency testing of botulinum toxin? If no,

what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?

- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the non-lethal *in vivo* assays discussed be used to **replace** animals for these kinds of samples? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?
- Can any of the current non-lethal *in vivo* methods be used now to **reduce** the number of animals used for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the non-lethal *in vivo* assays discussed be used to **reduce** the number of animals used for these kinds of samples? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?
- Should non-lethal *in vivo* methods other than those discussed so far during this workshop be considered for development and validation for potency testing or detection of botulinum toxin?
- What are the pros and cons of the different non-lethal *in vivo* methods reviewed?
- What current knowledge gaps with regard to the reviewed non-lethal *in vivo* methods must be addressed to further their use in potency testing or detection (as discussed in Session 1) of botulinum toxin? What additional studies are needed?
- Of the non-lethal *in vivo* methods discussed, which should have the highest priority for further development and validation studies?
- What is the best way to assess the validation status of these non-lethal *in vivo* methods?

Session 4C Panel Questions: Refinement: Potential Use of Non-Lethal Endpoints in Botulinum LD_{50} Testing to Minimize Pain and Distress (Moderators: William Stokes and Leonard Smith)

- Is there sufficient data to support the use of moribund condition instead of death as an endpoint for the mouse LD_{50} assay? Can this change be implemented now? If not, what studies would be needed to evaluate this alternative endpoint?
- Based on what is known about the progression of botulism in mice, are any other clinical signs sufficiently predictive of mouse lethality that they should be used, or further investigated, as earlier humane endpoints in order to allow for humane euthanasia of mice used in LD_{50} botulinum testing once they are observed?
- Are there objective endpoints (e.g., temperature, heart rate, blood pressure, pO_2) that are sufficiently predictive of mouse lethality that they can be used, or should be further investigated, as humane endpoints to terminate early a mouse LD_{50} test once observed?

- What current knowledge gaps regarding predictive humane endpoints should be addressed in research, development, and validation studies? What additional studies are needed?
- Are there additional data recommended for collection during future animal studies that might aid in identifying and validating more humane, non-lethal endpoints for botulinum toxin testing?

Session 5 Panel Questions: Reduction of Animal Use for *In Vivo* Botulinum Testing (Moderators: Marlies Halder and Richard McFarland)

- Is it feasible to use the mouse LD_{50} assay to assess the potency of batch production samples of botulinum toxin and use a validated *in vitro* and/or *ex vivo* test method to assess potencies of final production lots? Why, or why not?
- Are there validated test method modifications (e.g., use of reference standards) that could be made to the current mouse LD_{50} test method protocol to decrease the number of mice tested?
- Should a reference standard always be used in a validation study conducted on the *in vitro* and *ex vivo* methods discussed at this workshop? Why, or why not? Should an international botulinum toxin reference standard be created for this purpose? If yes, how should it be maintained?
- What are the best practices for minimizing the number of animals used?

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APPENDIX C

Abstracts of Posters Presented at the Workshop

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ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD_{50} Assay for Botulinum Toxin Testing

(November 13-14, 2006)

List of Posters Presented at the Workshop

Page Number/ Poster Board Number	Author List	Abstract Title
1	De Medici D, Fenicia L, Anniballi F, Delibato E, Aureli P	Polymerase Chain Reaction (PCR) as Alternative Method to Mouse Bioassay for Typing of Botulinum Neurotoxin-Producing Clostridia
2	Fenicia L, Anniballi F, Bolle P, Evandri MG, Martinoli L, Aureli P	Detection of Botulinum Neurotoxins Using *Daphnia magna* Toxicity Test
3	France R, McLaren J, Cox H, Banks L, Quirk R, Shakesheff K, Thompson D, Panjwani N, Shipley S, Pickett A	A New *Ex Vivo* Assay for Determining the Potency of Botulinum Type A Toxin-Hemagglutinin Complex (Dysport®): The Intercostal Neuromuscular Junction (NMJ) Assay
4	France R, McLaren J, Cox H, Banks L, Quirk R, Shakesheff K, Thompson D, Panjwani N, Shipley S, Pickett A	Validation of *Ex Vivo* Assays for the Potency Determination of Botulinum Toxins: The Impact of Experimental Variables in the Intercostal NMJ Assay
5	Gross GW	Quantification of BoNT-A Activity Suppression in Neuronal Networks Growing on Microelectrode Arrays *In Vitro*
6	Huber A, Shakesheff KM, Pickett A	Rat Rib Cage-Derived Nerve-Muscle Preparations Provide a Reproducible Tissue Source for Use in Alternative *In Vitro* Potency Testing of Botulinum Toxin Preparations
7	Singh BR, Lindo P, Cai S	Endo-ELISA Bioassay of Live Botulinum Neurotoxins
8	Stanker LH, Merrill P	Development of High Affinity Monoclonal Antibodies Specific for Botulinum Neurotoxin Type A and a Sensitive Immunoassay with Detection Near that of the Mouse Bioassay
9	Torii Y, Takahashi M, Ishida S, Sakamoto T, Harakawa T, Ginnaga A, Kozaki S, Kaji R	Quantification of the Activity of Causing Flaccid Paralysis of Botulinum Neurotoxin by Measuring the Compound Muscle Action Potential (CMAP)

10	Xu J, Hang J, Lee JH, Zhu P, Amstutz P, Tang CM, Shelton D, Poli M, Rivera V, Shone CC	Assays to Detect Active Botulinum Neurotoxin

Polymerase Chain Reaction (PCR) as alternative method to mouse bioassay for typing of botulinum neurotoxin-producing clostridia.

De Medici D.*, Fenicia L., Anniballi F., Delibato E., and Aureli P.
National Reference Centre for Botulism, National Centre for Food Quality and Risk Assessment, Istituto Superiore di Sanità, Roma, Italy

Botulism is a severe neuroparaliytic disease characterized by flaccid paralysis. Seven antigenically distinct toxin types (A, B, C_1, D, E, F and G) of the botulinal neurotoxins (BoNTs) have been identified [1]. Although BoNTs are classically produced by *Clostridium botulinum*, since 1979 other BoNT-producing species have been isolated. In particular, *Clostridium baratii*, which produces BoNT type F, has been isolated in the United States and in Hungary *Clostridium butyricum,* which produces BoNT type E, has been isolated in Italy, in China and in India. [2, 3].

Since different species of Clostridia have the ability to produce BoNTs, conventional isolation and identification of BoNTs-producing clostridia cannot rely solely on the basis of biochemical characteristics.

Currently, microbiological methods take into consideration only *C. botulinum* species, and the identification procedure included the confirmation and typing of the BoNTs production of the strain by mouse bioassay [4]. This technique is highly sensitive and specific, but costly, time-consuming, laborious, raises ethical concern due to the use of experimental animals, and does not take in consideration other BoNTs-producing clostridia. Moreover, efforts have been made to develop alternative methods to animal testing, as international legislation recommends (Directive 86/609/EEC).

The International Organization for Standardisation (ISO) has underlined the need to harmonize and standardize PCR-based methods to detect BoNTs-producing clostridia (Resolution 259 taken during the 24th Meeting of ISO TC34 SC9 – Warsaw – June 2005).

The development of molecular biological methods based on the detection of BoNTs genes would be ideal. Different PCR methods have been described for detection of BoNTs-producing clostridia in food and clinical samples, and results obtained using PCR assays to detect neurotoxin gene fragments show a very high level of agreement with those from the mouse bioassay [5]. In our experience, where the typing of BoNTs-producing clostridia has been performed for two years using multiplex PCR and the confirmation of the production of the toxins using mouse bioassay halved the use of the animals in our laboratory. During this period we have analyzed about 400 samples (clinical and food) using a modified multiplex conventional PCR method and the all the results obtained were correctly confirmed by mouse bioassay.

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Detection of botulinum neurotoxins using *Daphnia magna* toxicity test

Fenicia L[1*]., Anniballi F[1]., Bolle P.,[2] Evandri M.G.[2], Martinoli L.[2], and Aureli P.[1]
[1]National Reference Centre for Botulism, National Centre for Food Quality and Risk Assessment, Istituto Superiore di Sanità, Roma, Italy
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Botulism is a rare disease naturally occurring or caused by accidental or intentional exposure to botulinum toxins (BoNTs).
BoNTs are a group of seven (A-G) antigenically distinct metalloproteinase classically produced by *Clostridium botulinum*, and by rare strains of *Clostridium baratii* and *Clostridium butyricum* [1]. All the toxins exert their action on the cholinergic system at the presynaptic motor-neuron terminal by blocking acetylcholine transmission across the neuromuscular junction, causing neuromuscular blockade, resulting in flaccid paralysis.
Since the 1970s, the therapeutic value of the paralytic potential of BoNTs for the treatment of muscular contraction was recognized and BoNT type A and B are now used in over 50 therapeutic and cosmetic preparations.
At present, the currently used method to detect and quantify the BoNTs is the Mouse Test that is complex, very expensive and entails the use of laboratory animals with ethical issues resulting.
The aim of this study was to assess whether *Daphnia magna* was a sensitive organism to detect BoNTs. The microcrustacean *Daphnia magna* Straus, besides being commonly used in standard toxicity tests as reference species, was reported to have a cholinergic system and contains cholinoreceptors identical (with respect to pharmacodynamic parameters) to those in human and animal organisms [2].
In this preliminary approach the *Daphnia magna* acute toxicity test was used. The experiments were conducted using cultures of *C. botulinum* type A, B and E, and were performed in accordance to the ISO Standard 6341 protocol [3] with minor modifications. In each test session, three replicates of treatment concentrations, including reference blank, and four replicates of control group, were applied. For each replicate, four *daphnids* less than 24-h old at the beginning of the test and coming from a third brood, were used.
Results showed that 300, 20, and 80 mouse MLD/ml of type A, B and E BoNTs respectively, gave a 100 % mortality at 48 h in *Daphnia magna* (reference blank group 0 % mortality). Specificity of the toxic effect was asserted exposing *daphnids* in medium containing botulinum culture and polyvalent antitoxin.
These results demonstrate that *Daphnia magna* is sensitive to BoNTs although less than the mouse. Further investigations using chronic toxicity test with end-points different from lethality (e.g. motility), could increase the test sensitivity.

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A New Ex-Vivo Assay for Determining the Potency of Botulinum Type A Toxin-Hemagglutinin Complex (Dysport®): The Intercostal Neuromuscular Junction (NMJ) Assay.

Richard France[1], Jane McLaren[1], Helen Cox[1], Lisa Banks[1], Robin Quirk[1], Kevin Shakesheff[1], David Thompson[2], Naveed Panjwani[3], Sarah Shipley[3], Andy Pickett[3].
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Dysport® is a highly purified botulinum type A toxin complex used in a wide variety of indications within neurology, rehabilitation medicine and dermatology since 1990. The accurate determination of potency in clinical application of botulinum toxins such as Dysport® is critical to ensure clinical efficacy and safety, and is currently achieved using a lethal dose (LD_{50}) assay in mice. The increasing desirability of alternative methods for potency testing of botulinum toxin therapeutics is well established. As one such alternative, we have developed the *ex-vivo* Intercostal NMJ assay, which uses substantially fewer animals and does not involve the exposure of live animals to toxin. The assay records the decay of force from isolated and innervated intercostal muscle tissue sections stimulated electrically, and thus combines the important mechanisms of receptor binding, translocation and enzymatic action of the toxin molecule. Here we present data from a recent pre-validation study undertaken to determine the performance of the assay (including accuracy, precision and the replicate numbers required for routine use). Application of toxin to intercostal muscle results in the reduction of tissue lifetime in a dose dependant manner over a 0-60 Ipsen LD_{50} Unit dose range. Statistical analysis has been used to describe the relationship between applied dose and tissue lifetime. This regression model allows the prediction of toxin potency from the measured area under force decay curves. We show initial data demonstrating the assay provides accurate and precise results when tested with samples of well characterised reference batch material. Under the current methodology and within acceptable limits of confidence, potency determination of an individual batch of Dysport® requires a minimum of 60 tissue section replicates, currently achieved using a total of 12 animals.

Ethical and Legal Statement: All animal work was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Validation of Ex-Vivo Assays for the Potency Determination of Botulinum Toxins: The Impact of Experimental Variables in the Intercostal NMJ Assay.

Richard France[1], Jane McLaren[1], Helen Cox[1], Lisa Banks[1], Robin Quirk[1], Kevin Shakesheff[1], David Thompson[2], Naveed Panjwani[3], Sarah Shipley[3], Andy Pickett[3].
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Ethical and Legal Statement: All animal work was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Quantification of BoNT-A Activity Suppression in Neuronal Networks Growing on Microelectrode Arrays *in vitro*.

Guenter W. Gross
Department of Biological Sciences and Center for Network Neuroscience, University of North Texas, Denton, TX.

Spontaneously active neuronal networks cultured on microelectrode arrays (MEAs) can provide quantitative, multifaceted data on cytotoxicity, functional neurotoxicity, and pharmacological efficacy [1,2,3], where functional neurotoxicity is defined as the loss of network action potential (AP) production in the absence of cytotoxicity. One pregnant mouse with ten embryos can seed over 1,000 MEAs if several regions of the central nervous system are used. Each network can support one BoNT assay, providing remarkable animal and tissue utilization efficiency.

The primary response of these systems to BoNT-A is a gradual but irreversible reduction in spontaneous activity. The minimum concentration yielding reliable responses was 2 ng/ml, (13 picoMolar, using 150kD as the BoNT-A MW). Above this level, network responses were assessed at 100 ng/ml (n= 9), 50 ng/ml (n=11), 25 ng/ml (n=9), and 5 ng/ml (n=7). Delay times required to reach 50% and 90% activity reductions were concentration-dependent and could be approximated by power functions. At 100 ng/ml, these values were 2.8 h and 3.1 h, respectively, and reached 5 and 9 h at 10 ng/ml. Antisera provided by JVAP in 2001 (stored at -80°C) were used in 15 experiments. Sera additions ranging from 0.5 to 5% and high concentrations of BoNT (50-250 ng/ml) were used to ascertain protection. Of these, nine serum samples (selected at random) provided protection over long monitoring periods (up to 50 hours).

To perform such tasks rapidly and efficiently, it is desirable to use multinetwork platforms for parallel recording using robotic maintenance. Such platforms have reached the "proof of concept" stage under a Texas Advanced Technology Program grant, but require further development in the domains of automated data analysis and effective user interface programs. A 16-network system using the industry-standard 24 well plate is in the design stage.

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Approved by the institutional animal care and use committee of the University of North Texas in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Institute of Laboratory Animal Resources.

Rat rib cage-derived nerve-muscle preparations provide a reproducible tissue source for use in alternative *in vitro* potency testing of botulinum toxin preparations

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Since their introduction in the early 1980s, therapeutic Botulinum neurotoxin type A preparations have found an increasing number of clinical uses in a large variety of neuromuscular disorders and dermatological conditions. These treatments exploit the ability of the toxin to prevent neurotransmitter release from mainly cholinergic motor neurons, thereby resulting in a weakened contractile response. The use of therapeutic preparations of accurate potency in the clinical environment is crucial for correct patient treatment. Currently, the potency of a preparation is determined by using the LD_{50} assay. However, operational and ethical concerns associated with this assay have prompted the development of alternative assays that could potentially become replacements. Several different *ex vivo* muscle-nerve preparations have been used to study botulinum intoxification, including isolated muscle preparations of the extensor digitorum longus (EDL), and phrenic nerve-hemidiaphram. These are models on which potential alternative assays may be based, however, incomplete evaluations of their quantitative performance and robustness have hindered their use as replacement test methods. Recently, Ipsen (Wrexham, UK) in collaboration with RegenTec (Nottingham, UK) have developed the use of isolated rat intercostal rib sections in an alternative *ex vivo* neuromuscular junction assay (the "Intercostal NMJ Assay"). This alternative assay addresses ethical and operational constraints of a live animal assay. Here, we demonstrate that the six individual rat intercostal nerve-muscle preparations isolated from a single animal's rib cage provide a valuable source of highly similar preparations for an *ex vivo* neurotoxicity assay. A high degree of structural similarity was observed in terms of the anatomy of sections, as highlighted by the patterns of innervating neuromuscular junctions identified by acetylcholinesterase staining. Similarly, the physiological nature of the intercostal muscle sections was determined by their level of succinic dehydrogenase activity. Again, no significant differences were observed between sections. In summary, our results confirm that independent intercostal nerve-muscle preparations are anatomically and physiologically similar in nature supporting their use in an *ex vivo* alternative potency test such as the Intercostal NMJ Assay.

Ethical and Legal Statement: All animal work was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Endo-ELISA Bioassay of Live Botulinum Neurotoxins

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We have developed antibodies against purified and complex forms of botulinum neurotoxins which can detect the toxin below 50 pg/ml concentration in a sandwich format, without any additional amplification. However, this approach only provides detection of the toxin without any distinction of denatured or live toxin state. To address this issue, we have designed an approach in which the toxin is captured on a microtiter plate by either monoclonal or polyclonal antibodies raised against the heavy chain of the type A botulinum neurotoxin (BoNT/A). The captured toxin is subsequently incubated with BoNT/A neuronal substrate, SNAP-25-GST fusion protein or His-tagged SNAP-25, and its cleavage is followed by subsequent determination of the cleaved product either by anti-SNAP-25 IgG directly or after capturing the product with anti-GST antibodies. In both cases the results suggested that antibody captured BoNT/A was functional and its endopeptidase activity was monitored in the enzyme linked immunosorbent assay (referred to as Endo-ELISA bioassay).

The results showed that BoNT/A bound to anti-heavy chain monoclonal/polyclonal antibody cleaved the SNAP-25 in a time dependent manner, and significant cleavage occurred within the first 20 min. After 60 min incubation about 70% SNAP-25 was cleaved. This set of experiments establish the following points relevant to the use of this approach for designing non-animal based bioassay of botulinum neurotoxins. Anti-BoNT/A heavy chain IgG was able to capture the toxin on the microplate wells, the captured BoNT/A was enzymatically active, and the enzyme activity was strong enough to show significant SNAP-25 cleavage within the first 20 min of reaction.

The Endo-ELISA bioassay was found to be effective for purified as well as native BoNT/A complex, and it worked in a variety of matrices, such as milk, wine, and honey.

Development of High Affinity Monoclonal Antibodies Specific for Botulinum Neurotoxin Type A and a Sensitive Immunoassay with Detection Near that of the Mouse Bioassay

Larry H. Stanker and Paul Merrill
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Clostridium botulinum neurotoxins (BoNT) cause severe and potentially fatal neuroparalytic disease and are considered the most toxic biological agents known. BoNT is synthesized as a single 150 kDa precursor protein, which is cleaved to form two subunit polypeptides, linked by a single disulfide bond. The 'gold standard' for BoNT detection is the mouse bioassay. While the assay it is time consuming (up to 4 days) and lacks specificity, it has a limit of detection in the low picogram range. Most BoNT immunoassays reported appear to have much less sensitive than the mouse bioassay. In this study we describe the development of high affinity monoclonal antibodies (Mab). These are IgG_1 and IgG2b subclass MAb's with kappa light chains. They specifically bind BoNT serotype A and have measured Kd values in the low pM range. Western blot analysis demonstrated that four of the Mabs specifically bind the 100 kDa heavy-chain subunit, in while one of the antibodies specifically binds the 50Kda light-chain. Using a simple sandwich immunoassay format with a heavy-chain specific Mab for capture, a directly labeled anti light-chain Mab for detection and a luminescent substrate, detection of BoNT type A in the low picogram range was observed. Further characterization of these MAb and their application to rapid immunoassay formats will be discussed.

Quantification of the Activity Causing Flaccid Paralysis of Botulinum Neurotoxin by Measuring the Compound Muscle Action Potential (CMAP)

Yasushi Torii[1], Motohide Takahashi[2], Setsuji Ishida[2], Takashi Sakamoto[3], Tetsuhiro Harakawa[1], Akihiro Ginnaga[1], Shunji Kozaki[4], Ryuji Kaji[3]
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[4]Osaka Prefecture University, Japan

Purpose

Quantitative determination of botulinum toxin usually depends on the LD50 method after mouse intraperitoneal injection. The index of this method is the lethal activity of the toxin, and the method requires a number of mice. As the method of direct determination of flaccid paralysis after administration of the toxin, the method for testing quality of toxin by determining compound muscle action potential (CMAP) was examined.

Materials and methods

Type A botulinum toxin (S or LL) solution was diluted serially so as to obtain 9 graded doses. Each dose in 0.1 ml was injected into left hind gastrocnemius muscle of a group of 5 anesthetized mice (SD strain). By use of stimulatory and recording electrodes inserted in the gastrocnemius muscle near the lumbar vertebra, the maximum CMAP amplitude of the injected muscle was determined by electromyography.

Results and discussions

CMAP amplitude was on the decrease toward the fourth day after administration of the toxin, and then recovered gradually. Statistical analysis was made on the data of the 1st , the 2nd, the 4th, the 7th and the 14th day of administration. For example, dose response was found on each dose, and a linear relation was observed between 0.01 and 30 mouse ip LD50/body on the 1st day (and between 0.01 and 1.0 mouse ip LD50/body on the 4th day), indicating the possibility of the use of CMAP method for quantitation of botulinum toxin.

All studies were carried out in accordance with the guidelines of the Japanese Society of Pharmacology on experimental animals and with approval of the Animal Ethics Committee of our institute.

Assays to Detect Active Botulinum Neurotoxin

Jing Xu[1], Jun Hang[1], Jia-Hai Lee[1], Peixuan Zhu[1], Pete Amstutz[1], Cha-Mei Tang[1], Daniel Shelton[2], Mark Poli[3], Victor Rivera[3], and Clifford C. Shone[4]
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Active Botulinum Neurotoxin (BoNT) serotype A cleaves SNAP-25 peptide at a specific location. Detection of the cleaved SNAP-25 provides information on the activity and serotype of the toxin. The assays are based on the use of antibody specific to SNAP-25 cleaved by BoNT serotype A developed by Dr. Shone of Health Protection Agency. A proof-of-principle enzymatic activity assay is demonstrated.

The detection platform is the Integrating Waveguide Biosensor, which uses capillary tube waveguides to detect low level fluorescence signals. In the test, BoNT samples are incubated with a proprietary form of the SNAP-25 substrate, and the enzymatic reaction product is detected by fluorescent immunoassay inside the capillary waveguide. Excitation light impinges on the waveguide at a 90º angle. The emitted fluorescence signal is gathered efficiently by the waveguide and exits at one end through a set of lenses and optical filters to an optical detector. Emission light from the entire waveguide is integrated, thus increasing the detected signal, while background noise is minimized by excitation at a 90º angle.

Preliminary data indicates a detection level of 500 pg/ml in a three hour test. Optimization of the assay is expected to improve the limit of detection in the future.

APPENDIX D

Federal Register Notices and Public Comments

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APPENDIX D1

Federal Register Notices

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DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

National Toxicology Program (NTP), NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Nomination To Hold a Workshop on Alternative Methods To Replace the Mouse LD_{50} Assay for Botulinum Toxin Potency Testing: Request for Comments, Nominations of Experts, and Submission of In Vivo and In Vitro Data

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Request for comments, nominations of scientific experts, and submission of data.

SUMMARY: In October 2005, the Humane Society of the United States (HSUS) submitted a nomination to NICEATM requesting that alternative test methods to the mouse LD_{50} assay for botulinum toxin potency testing be assessed and prioritized for prevalidation and validation efforts. The nomination proposed that an initial key step in this process would be for the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to organize a workshop on this topic. ICCVAM considered the nomination and supports with a high priority the concept of a workshop to discuss alternative methods and approaches that might reduce, refine, or replace the use of animals for botulinum potency testing. The Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) considered the nomination and the ICCVAM proposal at its meeting on December 12, 2005, and agreed that the proposed activity should have a high priority. At this time, NICEATM requests (1) information on development and/or validation activities relevant to reduction, refinement (less pain and distress), and/or replacement alternatives for botulinum toxin potency testing, (2) public comments on the appropriateness and relative priority of proceeding with a workshop on this topic, (3) the nomination of scientific experts who might participate if a workshop occurs, and (4) the submission of data from mouse LD_{50} botulinum potency testing and *ex vivo* and *in vitro* test methods used for botulinum toxin potency testing. The HSUS nomination is available at *http://iccvam.niehs.nih.gov/* see ''Nominations and Submissions.''

DATES: Comments, nominations of expert scientists, and data submissions should be received by March 13, 2006.

ADDRESSES: Correspondence should be sent by mail, fax, or e-mail to Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC 27709, (phone) 919–541–2384, (fax) 919–541–0947, (e-mail) *niceatm@niehs.nih.gov.*

SUPPLEMENTARY INFORMATION:

Background

In October 2005, the HSUS submitted a nomination to NICEATM to organize a workshop to evaluate the state-of-the-science for potential alternatives to the mouse LD_{50} assay for botulinum toxin potency testing. The HSUS nomination is available at *http://iccvam.niehs.nih.gov/* see ''Nominations and Submissions.'' ICCVAM considered the nomination and supports the concept of a workshop to discuss alternative methods and approaches that might reduce, refine, or replace the use of animals for botulinum potency testing with a high priority. The SACATM discussed this nomination at its meeting on December 12, 2005, and advised NICEATM and ICCVAM that they consider the development and validation of alternatives to the mouse LD_{50} assay for botulinum toxin potency testing a high priority. SACATM also suggested that prior to convening a workshop that ICCVAM and NICEATM find out what efforts toward developing or validating alternatives might already be underway by companies that conduct botulinum potency testing. NICEATM now seeks (1) information on any activities directed at the development and/or validation of alternatives to the mouse LD_{50} assay for botulinum toxin potency testing, (2) input from the public on this nomination for a workshop, (3) the nomination of scientific experts who might participate in any future workshop on this topic should it occur, as well as (4) data from mouse LD_{50} botulinum potency testing and *ex vivo* and *in vitro* test methods used for botulinum toxin potency testing. NICEATM and ICCVAM will consider this information and determine how to best move forward with this nomination.

Request for Comments, Nominations of Scientific Experts and Request for Data

NICEATM requests information on the status of any efforts to develop alternatives to the mouse LD_{50} assay for botulinum toxin potency testing, as well as public comments on the appropriateness and relative priority of the proposed workshop activity. In addition, NICEATM requests the nomination of scientists with relevant knowledge and experience to potentially participate in the workshop should it be held. Areas of relevant expertise include, but are not limited to: neurophysiology, neuropharmacology, neurotoxicity, immunology, potency testing of toxins and other biologicals in animals and *in vitro* systems, development and use of *in vitro* methodologies, and biostatistical data analysis. Each nomination should include the person's name, affiliation, contact information (*i.e.*, mailing address, e-mail address, telephone and fax numbers), and a brief summary of relevant experience and qualifications.

NICEATM invites the submission of data from *in vivo* botulinum toxin potency testing, including clinical observations and corresponding time-course information, and information and data from *ex vivo* and *in vitro* test methods being used as potential alternatives to the mouse assay for botulinum toxin potency testing. Submitted data will be used to further evaluate the usefulness and limitations of *in vitro* potency test methods and may be included in future NICEATM and ICCVAM reports and publications as appropriate. The data will also be included in a NICEATM database to support the investigation of alternative test methods for assessing potency of botulinum toxin.

When submitting chemical and protocol information/test data, please reference this **Federal Register** notice and provide appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, as applicable).

NICEATM prefers data to be submitted as copies of pages from study

notebooks and/or study reports, if available. Raw data and analyses available in electronic format may also be submitted. Each submission should preferably include the following information, as appropriate:

- Specific type of botulinum neurotoxin tested (*e.g., Clostridium botulinum* neurotoxin type A)
- *In vivo* potency test protocol used.
- *In vivo* potency test results.
- Individual animal responses, including time of onset of specific clinical signs and death.
- Alternative *ex vivo* or *in vitro* test protocol used.
- Alternative *ex vivo* or *in vitro* test results.
- The extent to which the study complied with national or international Good Laboratory Practice (GLP) guidelines.
- Date of the study.
- The organization that conducted the study.

Although public comments and data can be accepted at any time, information submitted by the deadline listed in this notice would be most useful for determining whether a workshop is the appropriate next step in pursuing an alternative to the mouse LD_{50} assay for botulinum toxin potency testing. In addition, submitting information by this date ensures its availability to workshop participants if a workshop is held.

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (Pub. L. 106–545) establishes ICCVAM as a permanent interagency committee of the NIEHS under the NICEATM. NICEATM administers the ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM can be found at the following Web site: *http://www.iccvam.niehs.nih.gov.*

The SACATM, established January 9, 2002, is a federally chartered advisory committee composed of scientists from the public and private sectors (**Federal Register**: March 13, 2002: Vol. 67, No. 49, page 11358). The SACATM provides advice to the Director of the NIEHS, ICCVAM, and NICEATM regarding statutorily mandated duties of ICCVAM and activities of NICEATM. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at *http://ntp.niehs.nih.gov/*, see ''Advisory Board & Committees.''

Dated: January 17, 2006.

Samuel H. Wilson,

Deputy Director, National Institute of Environmental Health Sciences.

[FR Doc. E6–1019 Filed 1–26–06; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

National Toxicology Program (NTP), NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM): Scientific Workshop on Alternative Methods To Refine, Reduce, or Replace the Mouse LD_{50} Assay for Botulinum Toxin Testing; Request for In Vivo and In Vitro Data

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), Department of Health and Human Services.

ACTION: Workshop announcement and data request.

SUMMARY: The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and NICEATM announce an upcoming ''ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, or Replace the Mouse LD_{50} Assay for Botulinum Toxin Testing.'' The workshop is being co-organized by ICCVAM, NICEATM, and the European Centre for the Validation of Alternative Methods (ECVAM). This workshop is open to the public with attendance limited only by the space available. ICCVAM and NICEATM also invite the submission of (1) data from botulinum toxin test methods and (2) abstracts for scientific posters for display at the workshop (discussed more under ''Supplemental Information'').

DATES: The workshop will be held on November 13 and 14, 2006. Sessions for both days will begin at approximately 8:30 a.m. and end at approximately 5 p.m. The deadline for submission of an abstract is September 29, 2006. The deadline for submission of data is October 20, 2006.

Individuals who plan to attend the workshop are strongly encouraged to register in advance (by October 30, 2006) with NICEATM. Registration information, an agenda, and additional information will be available on the workshop Web site (*http://iccvam.niehs.nih.gov/methods/biolodocs/biolowkshp/wkshpinfo.htm*) and upon request from NICEATM (see ''**FOR FURTHER INFORMATION CONTACT**'' above).

ADDRESSES: The workshop will be held at the Crowne Plaza Hotel, 8777 Georgia Avenue, Silver Spring, MD 20910. Persons needing special assistance, such as sign language interpretation or other reasonable accommodation in order to attend, should contact 919–541–2475 (voice), 919–541–4644 TTY (text telephone), through the Federal TTY Relay System at 800–877–8339, or e-mail to *niehsoeeo@niehs.nih.gov*. Requests should be made at least 7 days in advance of the event.

FOR FURTHER INFORMATION CONTACT: Correspondence should be addressed to Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC 27709, (phone) 919–541–2384, (fax) 919–541–0947, (e-mail) *niceatm@niehs.nih.gov.*

SUPPLEMENTARY INFORMATION:

Background

In October 2005, the Humane Society of the United States (HSUS) submitted a nomination to NICEATM to organize a workshop to evaluate the state-of-the-science for potential alternatives to the mouse LD_{50} assay for botulinum toxin potency testing. The HSUS nomination is available at *http://iccvam.niehs.nih.gov*. See ''Nominations and Submissions.'' ICCVAM considered the nomination and supported, with a high priority, the concept of a workshop to discuss alternative methods and approaches that might reduce, refine, or replace the use of animals for botulinum toxin potency testing. The Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) discussed this nomination at its meeting on December 12, 2005, and concurred with ICCVAM. The goals of the workshop are to (1) review the state-of-the-science and current status of alternative methods that may refine (less pain and distress), reduce, or replace the use of mice for botulinum toxin testing and (2) identify priorities for research, development, and validation efforts needed to advance the use of alternative methods for botulinum toxicity testing.

Preliminary Workshop Agenda

Day 1 Monday, November 13, 2006

- Welcome and Introduction of Workshop Goals and Objectives.
- Session 1 Overview of Public Health Needs for Botulinum Toxin Testing and Regulatory Requirements.
- Session 2 Current Understanding and Knowledge Gaps for Botulinum Toxin.
- Session 3 Potential Replacement of Animal Use for Botulinum Toxin Potency Testing.

Day 2 Tuesday, November 14, 2006

- Session 4 Refinement (Less Pain and Distress) of Animal Use for Botulinum Toxin Potency Testing.
- Session 5 Reduction of Animal Use For *In Vivo* Botulinum Testing.
- Session 6 Wrap-up of Panel Discussions.

Call for Abstracts

ICCVAM and NICEATM invite the submission of abstracts for scientific posters to be displayed during the workshop. Posters should address current developments and/or the 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. The body of the abstract is limited to 400 words or less and key references relevant to the abstract may be included after the abstract body. However, the length of the abstract and references should not exceed one page. All submissions should be in at least 12-point font and all margins for the document should be no smaller than one inch. Title information should include the names of all authors and their affiliations. The name and contact

information (i.e., address, phone number, fax number, e-mail address) for the corresponding or senior author should be provided at the end of the abstract.

A statement indicating whether animals or humans were used in studies described in the poster must accompany all abstracts. All abstracts that involve studies using animals or animal tissues should be accompanied by a statement from the senior author certifying that all animal use was carried out in accordance with applicable laws, regulations, and guidelines, and that the appropriate Institutional Animal Care and Use Committee approved the studies. All abstracts that involve studies using humans should be accompanied by a statement from the senior author certifying that all human use was conducted in accordance with applicable laws, regulations, and guidelines, and that the appropriate Institutional Review Board approved the studies.

Abstracts should be submitted by e-mail to *niceatm@niehs.nih.gov.* The deadline for abstract submission is close of business on September 29, 2006. ICCVAM and NICEATM will review the submitted abstracts. The corresponding author will be notified of the abstract's acceptance, along with guidelines for the poster format, approximately five weeks prior to the workshop.

Request for Data

NICEATM invites the submission of data and information from *in vivo* botulinum toxin testing and *ex vivo* and *in vitro* test methods being used or evaluated as potential alternatives to the mouse assay for botulinum toxin testing. The deadline for data submission is October 20, 2006. These data will be provided to the workshop participants and workshop panels for their review and consideration during workshop discussions. A similar request for data was announced previously (**Federal Register**, Vol. 71, No. 18, pp. 4603–4604, January 27, 2006, available at *http://iccvam.niehs.nih.gov/*).

When submitting chemical and protocol information/test data, please reference this **Federal Register** notice and provide appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, as applicable). NICEATM prefers data to be submitted as copies of pages from study notebooks and/or study reports, if available. Raw data and analyses available in electronic format may also be submitted. Each submission should preferably include the following information, as appropriate:

- Specific type of botulinum neurotoxin tested (e.g., Clostridium botulinum neurotoxin type A).
- *In vivo* potency test protocol used and test results.
- Individual animal responses, including time of onset of specific clinical signs and death.
- Alternative *ex vivo* or *in vitro* test protocol used and test results.
- The extent to which the study complied with national or international Good Laboratory Practice guidelines.
- Date of the study.
- The organization that conducted the study

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 U.S. Federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 2851–2, 2851–5 [2000]) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM–related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found at the ICCVAM–NICEATM Web site (*http://iccvam.niehs.nih.gov*).

SACATM provides external advice to the Director of the NIEHS, ICCVAM, and NICEATM regarding statutorily mandated duties of ICCVAM and activities of NICEATM. Additional information about SACATM, including the charter, roster, and records of past meetings can be found at *http://ntp.niehs.nih.gov/go/167.*

Dated: August 7, 2006.

David A. Schwartz,

Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E6–13525 Filed 8–16–06; 8:45 am]

BILLING CODE 4140–01–P

APPENDIX D2

Public Comments Received in Response to *Federal Register* Notice Vol. 71, No. 18, pp. 4603-4, January 27, 2006

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ALLERGAN

2525 Dupont Drive, P.O. Box 19534, Irvine, California, USA 92623-9534 Telephone: (714) 246-4500 Website: www.allergan.com

Timothy G. Terrell, DVM, PhD, DACVP
Vice President, Drug Safety Evaluation
Allergan, Inc.
2525 Dupont Drive
RD2-2A
Irvine, CA 92612

March 31, 2006

Dr. William S. Stokes
NICEATM Director
NIEHS
P.O. Box 12233
MD EC-17
Research Triangle Park, NC 27709

RE: Nomination To Hold a Workshop on Alternative Methods to Replace the Mouse LD_{50} Assay for Botulinum Toxin Potency Testing: Request for Comments, Nominations of Experts, and Submission of In Vivo and In Vitro Data, Federal Register, 71(18):4603, January 27, 2006.

Dear Dr. Stokes:

Pursuant to the request from NICEATM and ICCVAM for information on activities directed at the development of alternatives the mouse LD_{50} assay for botulinum toxin potency testing, I am providing the enclosed information.

Allergan supports the concept of a workshop to discuss alternative methods and approaches that might address the 3 R's for testing botulinum toxin for potency and product release. We have had internal R&D efforts focused on development and assessment of approaches to address each of the areas, reduction, refinement and replacement of the use of animals for botulinum potency testing for more than a decade. Significant progress has been achieved; however, the ultimate goal of replacement of the mouse LD_{50} assay with a non-animal alternative assay has not yet been met. This remains a goal of the company with ongoing efforts focused on research on alternative assay methods.

ICCVAM has requested data from mouse LD_{50} botulinum toxin potency testing and ex vivo and in vitro test methods used for potency testing. The types of data requested include raw data, protocols, test results and study reports. Unfortunately, because of the proprietary nature of that type of data, Allergan is not able to share it with the committee.

Allergan would like to support and contribute to the efforts by NICEATM and ICCVAM in the investigation of alternative test methods. Included with this submission are:

- Nominations of scientific experts who might participate in any future workshop
- Selected Literature References
- Compact Disc containing electronic files of all submitted material

As I mentioned in our discussion earlier this month, Allergan representatives including myself will be willing to give a presentation to the committee via webcast during your planning discussions if it is the wish of ICCVAM.

I will serve as the primary contact for future communications. In addition, I would most definitely like to participate in any future workshop on this topic should it occur. Correspondence can be sent by mail, fax or e-mail.

Sincerely yours,

Timothy G Terrell

Timothy G. Terrell

NOMINATION OF SCIENTIFIC EXPERTS

Lance L. Simpson, PhD
Professor of Medicine, Biochemistry and Molecular Pharmacology
Thomas Jefferson University
1020 Locust Street
Room 314 Jefferson Alumni Hall
Philadelphia, PA 19107-6799

K. Roger Aoki, PhD
Vice President, Neurotoxins
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2525 Dupont Drive
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Irvine, CA 92612

Leonard Smith, PhD
Chief, Department of Immunology and Molecular Biology
Division of Toxinology and Aerobiology
United States Army Medical Research Institute of Infectious
Diseases (USSAMRIID)
Fort Dietrich, MD 21702-5011

Prof. J. Oliver Dolly
International Centre for Neurotherapeutics
Research & Engineering Building
Dublin City University
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Eric A. Johnson, ScD
Professor, Department of Food Microbiology & Toxicology
Food Research Institute
University of Wisconsin-Madison
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Madison, WI 53706-1187

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From: Michael Balls
Sent: Tuesday, March 14, 2006 5:19 AM
To: NIEHS NICEATM
Subject: 2006.01.27 ICCVAM Federal Register Notice

Dear Sirs

I wish to record my concern about the use of LD50 testing in relation to botulinum toxin testing and the urgent need for alternative (non-animal) test procedures.

FRAME was one of the first organisations to draw attention to this issue, and our opinions are summarised in the attached three articles from *ATLA*.

FRAME strongly supports the suggestion that an ICCVAM Workshop should be held on this topic, and we hope that this will given a high priority, so that the most promising alternatives to animal testing for botulinum toxin can be identified, further developed, validated and accepted into regulatory practice as soon as possible.

Sincerely

Michael Balls

Professor Michael Balls
Chairman of the FRAME Trustees
Norfolk, UK

FRAME References

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www.ardf-online.org

Alternatives Research & Development
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Received
NICEATM
3/13/06

March 13, 2006

Dr. William S. Stokes
NICEATM Director
NIEHS
P.O. Box 12233, MD EC-17
Research Triangle Park, North Carolina 27709

RE: Federal Register Doc. E6-1019

Dear Dr. Stokes,

On behalf of both the Alternatives Research & Development Foundation (ARDF) and the American Anti-Vivisection Society (AAVS), I am submitting these comments to support the HSUS nomination for ICCVAM assessment of alternative test methods to the mouse LD50 assay for botulinum toxin potency testing.

Having been aware for some time of the efforts of the Fund for the Replacement of Animals in Medical Experiments (FRAME) in England on this topic, we are pleased to see that ICCVAM has taken the advice of its SACATM and supports with a high priority the concept of a workshop to discuss alternative methods and approaches and we welcome the opportunity to commend this effort.

Specifically, our response to your request for comments is:

1) ARDF and AAVS support and confirm the information already provided to ICCVAM in the HSUS nomination and have nothing further to add at this time.

2.) ARDF and AAVS consider that proceeding with a workshop on this topic is highly relevant to ICCVAM's mission, highly appropriate and should be a high priority. Considering the large numbers of animals affected, the suffering involved and severity of the test, the use of LD50-- a widely discredited test, and the opportunity to complement efforts by counterparts in Europe who are examining the same issue, this is an ideal candidate for prioritization by ICCVAM.

3.) Scientific experts on this topic are probably apparent from the literature citations provided by HSUS. In our experience, individuals well known to you, Coenraad Hendriksen of the Netherlands, and Robert Combes from FRAME in England, would be

excellent workshop panelists, if you could prevail upon them to participate. Of course, ARDF and AAVS have no objection to Martin Stephens from HSUS participating as well, if that is not prohibited by rules excluding nominators. If ICCVAM is interested in the most informed participants, those three could be a great asset. (I am not including contact information since these are prominent individuals; if that information is needed, please advise and I will be happy to obtain it.)

4.) ARDF and AAVS are not in possession of test data from mouse LD50, etc. However, this request affords us the opportunity to urge ICCVAM to utilize whatever tools it may have available to secure the extensive information that Botox™ manufacturer Allergan, Inc. has in its possession. Clearly, this company's documentation would be most valuable in the assessment.

In addition, this request for data affords us the opportunity to emphasize that we would only support collection of existing animal data, and not any use of animals for new data collection or validation experiments.

In closing, we would like to encourage that the workshop focus on expediting the utilization of *in vitro* alternative tests, such as the SNAP-25 assay. It would be unfortunate if the energy expended in pursuing this course resulted in merely a timid modification of protocols as is sometimes seen. We urge ICCVAM to be diligent and bold in its mission to assess and validate and reach beyond refinement.

Further, we would like to caution ICCVAM against following any tendency to validate against the existing animal test, since in this case, the LD50 is so famously unreliable and this may prove the ironic obstacle in validating a superior *in vitro* test alternative.

Thank you for this opportunity to comment on ICCVAM's consideration of the HSUS nomination and next steps. In summary, ARDF and AAVS urge ICCVAM to proceed with scheduling the expert workshop and move forward to reduce the tremendous animal suffering resulting from botulinum toxin potency testing in mice as soon as possible.

Sincerely,

Sue A. Leary
President

From: Peggy Cunniff
Sent: Monday, March 13, 2006 12:28 PM
To: NIEHS NICEATM
Subject: Response to NICEATM nomination for workshop to replace Botulinum Toxin Potency Testing

Dr. William S. Stokes
NICEATM Director
NIEHS
P.O. Box 12233, MD EC-17,
Research Triangle Park, NC 27709

Dear Dr. Stokes,

As president of the International Foundation for Ethical Research (IFER) and as executive director of the National Anti-Vivisection Society (NAVS), I am writing to convey our endorsement for the proposed workshop to evaluate the state-of-the-science for potential alternatives to the mouse LD50 assay for botulinum toxin potency testing. We agree with the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the proposal of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that the development, validation and regulatory acceptance of alternatives to the mouse LD50 assay for the testing of Botox-type products is a high priority. IFER and NAVS support the immediate formation of a workshop comprised of experts to consider scientifically viable alternatives that would replace the use of animals. This would be an advancement to the current testing regime that relies on an inhumane methodology for botulinum potency testing that has never been validated.

The National Anti-Vivisection Society is a non-profit educational organization that promotes the advancement of science without harming animals. The International Foundation for Ethical Research supports the development, validation and implementation of alternatives to the use of animals in research, education and testing. Again, thank you in advance for your thoughtful consideration of these comments on behalf of IFER and NAVS in support of the proposed workshop as an appropriate undertaking and as a priority.

Sincerely,

Mary Margaret Cunniff
President, International Foundation for Ethical Research
Executive Director, National Anti-Vivisection Society

From: David Morton
Sent: Friday, March 10, 2006 11:23 AM
To: NIEHS NICEATM
Subject: Safety and potency testing of Botulinum toxin

Dear Sirs,

I am a veterinarian with experience of devising humane endpoints in research with a specific interest in vaccines and toxin testing. Some 20 years ago I developed a clinical endpoint for tetanus toxin and since then I have been involved in various HEP assessments. The UK Joint Working Group on Refinement (supported by the BVA Animal Welfare Fdn, UFAW, RSPCA and FRAME) is going to be looking at the issue of HEPs and I shall probably chair it. I attach some references that may help you decide if I am able to help.

Thanks

David Morton

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David B. Morton,
Professor of Biomedical Science & Ethics,
School of Biosciences
University of Birmingham, UK

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From: Thea Sesardic
Sent: Friday, March 10, 2006 1:44 PM
To: NIEHS NICEATM
Subject: Botulinum toxin - Alternative methods

Dear Dr William S Stokes,

I have sent today by currier delivery information related to proposed workshop on Alternative Methods to Refine, Reduce and Replace the Mouse LD50 Assay for Botulinum Toxin Potency Testing.

After working on this topic for nearly 15 years I am happy to be able to share experience with NTP/NICEATM and ICCVAM in view of eliminating LD50 test.

SOP's for methods used at NIBSC are included as pdf files in this Email together with short summary of information that was sent by currier. Copies of all published documents together with example of data was included in delivery package. It is not possible to provide raw data and details of all assays we have accumulated over the last 15 years - however we have provided examples of what we can do and further discuss on workshop. Please do not copy the data provided unless we agree on how it will be used.

After review of data you could suggest to focus on a particular assay for which we than could provide more information if required.

Kind regards and I look forward to hearing from you.

Thea Sesardic

Dorothea (Thea) Sesardic PhD
Principal Scientist
Division of Bacteriology
NIBSC
Hertfordshire, UK

Response to

Department of Health and Human Services
National Institute of Health

NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEAM)

Nomination to hold Workshop on Alternative Methods to Replace the Mouse LD50 Assay for Botulinum Toxin Potency Testing

Experts:

Dr Dorothea (Thea) Sesardic
Principal Scientist
Division of Bacteriology
National Institute for Biological Standards and Control (NIBSC)
Hertfordshire, UK

Position: Group leader, bacterial toxin laboratory with >15 years experience on testing of therapeutic formulations of Botulinum toxins.

Dr Rose Gaines Das
Head of Biostatistics
NIBSC

Position: Head of Biostatistics with >25 years experience on validation of assay methods

Dr Russell G A Jones
Senior Scientist
Division of Bacteriology
NIBSC

Position: Expert on botulinum toxins and botulinum anti-toxins with 5 years experience.

List of publication on assay methods for reduction, refinement and replacement for potency assays of Botulinum toxins in support of nomination for NICEATM

In vivo LD50 (reduction/standardisation)

McLellan K, Gaines Das R, Ekong TAN and Sesardic D. (1996). Therapeutic botulinum type A toxin: factors affecting potency. *Toxicon*, **34,** 975-985.

Sesardic D, Gaines Das RE and Corbel MJ (1994). Botulinum toxin: How to define biological activity. *Journal of the Royal Society of Medicine (letter)* **87,** 307.

Sesardic D, Leung T, Gaines Das R (2003). Role of standards in assays of botulinum toxins: international collaborative study of three preparations of botulinum type A toxin. *Biologicals,* 31: 265-276.

Jones RGS, Corbel MJ and Sesardic D. (2006). A review of WHO International Standards for Botulinum Antitoxins. *Biologicals,* (in press).

In vivo non-LD50 (refinement)

Sesardic D, McLellan K, Ekong TAN and Gaines Das R. (1996). Refinement and validation of an alternative bioassay for potency testing of therapeutic botulinum type A toxin. *Pharmacology & Toxicology* 78, 283 - 288.

Sesardic D, Jones RGA, Leung T, Alsop T, Tirney R. (2004) Detection of antibodies against botulinum toxins. *Movement Disorders*, 19: 85-91.

In vitro (replacement)

Ekong TAN, McLellan K and Sesardic D. (1995). Immunological detection of *Clostridium botulinum* toxin type A in therapeutic preparations. *Journal of Immunological Methods* **180**, 181-191.

Ekong TAN, Feavers I and Sesardic D. (1997). Recombinant SNAP-25 is an effective substrate for *Clostridium botulinum* type A endopeptidase activity. *Microbiology*, **143,** 3337-3347.

Ekong TAN, Gee C, Blasi J and Sesardic D. (1997). An alternative bioassay for botulinum neurotoxin type A based on its andopeptidase activity. In Animal Alternatives, Welfare and Ethics (Eds: LFM van Zutphen and Balls M). Developments in Animal and veterinary Sciences 27, 1039-1044.

Sesardic D, Corran PH, McLellan K, Feavers I and Ekong TAN (1997*). In vitro* assays for estimating the activity of therapeutic preparations of botulinum toxin. In: Animal Alternatives, Welfare and Ethics (Eds: LFM van Zutphen and Balls M). Developments in Animal and Veterinary Sciences 27, 1033-1038.

Gaines-Das RE, Heath AB, Martin H and Sesardic D. (1999). Validation of in vitro assays for Botulinum Toxin: A case study. In Alternatives to Animals in the

Development and Control of Biological Products for Human and Veterinary Use. (Eds Brown F, Hendriksen C and Sesardic D). *Developments in Biological Standardization* Vol 101. pp267-276.

Sesardic D, Corran P, Gee C and Ekong TAN. (2000). *In vitro* approaches for estimating activity of tetanus toxin as an alternative assay for specific toxicity. In: "*Progress in the reduction refinement and replacement of animal experimentation*" (Balls M, van Zeller A-M and Halder M Eds.,) pp 969-974.

Sesardic D, Martin H, Tierney R and Bigalke H. (2000). An *in vitro* assay for testing of neutralising antibodies to botulinum toxins. In*: "Progress in the reduction refinement and replacement of animal experimentation*" (Balls M, van Zeller A-M and Halder M Eds.,) pp 1001-1008.

General : reviews and related relevant publications

Corran PH and Sesardic D. (1994). Meeting Report: International Workshop On Bacterial Toxins in Medical Use. *Biologicals* **22**, 83-84.

Sesardic D. (1996). Requirements for valid alternative assays for testing of biological therapeutic agents. *Developments in Biological Standardization* **86,** 311-318.

Brown F, Hendriksen CFM and Sesardic D (Eds). (1999) Alternatives to Animals in the Development and Control of Biological Products for Human and Veterinary Use. *Developments in Biological Standardization* Vol 101.

Sesardic D. (1999). Alternatives to the use of animals for bacterial toxins and antitoxins. In : Celebration of 50 years of progress in biological standardization and control at WHO. *Developments in Biological Standardization*,100:75-82.

Brown F. Hendriksen CFM, Cussler C and Sesardic D. (Eds). (2002). Advancing Science and Elimination of the Use of Laboratory Animals for Development and Control of Vaccines and Hormones. *Developments in Biological Standardization* Vol 111, Karger Press, Basel. Switzerland.

Leung T, Corran P, Gee C, Ekong TAN and Sesardic D. (2002). Application of an in vitro endopeptidase assay for detection of residual toxin activity in tetanus toxoids. In Brown F, Hendriksen CFM, Sesardic D & Cussler K (Eds). Advancing Science and Elimination of the Use of Laboratory Animals for Development and Control of Vaccines and Hormones. *Developments in Biological Standardisation,* 111: 335-340.

Meunier FA, Lisk G, Sesardic D and Dolly OJ. (2003). Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. *Molecular and Cellular Neuroscience*, 22: 454-466.

European Pharmacopoeia Monograph

Botulinum ToxinType A for injection. European Pharmacopoeia 01/2005:2113.

Standard operating procedures

1. Non-lethal mouse local muscular paralysis assay: In vivo assessment of botulinum type A toxin
2. Phrenic nerve hemidiaphragm assay: *In vitro (ex vivo)* assessment of botulinum neurotoxicity.
3. *In vitro* SNAP-25 endopeptidase immunoassay for potency testing of botulinum toxin A preparations.

Data to be presented at the workshop or included in surface mail:

1. **Mouse LD50:** method as in relevant publications. This test was performed at NIBSC from 1991-1996 after which it was replaced by in vivo mouse paralysis test. It is no longer performed at NIBSC for potency testing of therapeutic products since 1996. LD50 data used in comparisons with in vitro SNAP-25 assay were generated by marketing authorisation holders and therefore confidential. Collaborative study compared LD50 assays in 10 laboratories (published data).

2. **Mouse paralysis**: method as in published literature and in electronic version of SOP. Test performed routinely for type A botulinum toxin at NIBSC from 1996-2000 (>10 assays per year) and thereof only for annual re-calibration of product specific reference standards for use in vitro batch release test. Example data with statistical evaluation provided from one recent assay and described in memo from Rose E Gaines Das to D Sesardic 01 March 2006 to be sent in package by surface mail. In house experience also with type B therapeutic toxin. Examples of calibration of product specific reference – confidential information.

3. **Mouse isolated phrenic nerve hemidiaphragm**: method as in electronic version of SOP. At present under in house validation for confirming potency of bulk active toxin and product specific reference standards. Limited and only unpublished data. Example of dose response curve for type A toxin on poster presented on the 5th International Conference on Basic and Theraeutic Aspects of Botulinum and Tetanus Toxins, Botulinum, Denver Colorado, USA June 2005.

4. **SNAP-25**: method as in relevant literature and electronic version of SOP. In routine use at NIBSC since 1999 for batch release of type A botulinum toxin products. Data compared with LD50 using MAH LD50 data. Examples provided for information taking out actual lot numbers but should not be copied without prior consent.

5. **Rat primary spinal cord cell**: preliminary unpublished data on dose response of inhibition of 3H glycine release following incubation with botulinum tpxin A.

5

NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

Phrenic Nerve Hemidiaphragm Assay

In vitro (ex *vivo*) assessment of botulinum neurotoxicity

Written by: Dr R G A Jones

Authorized by: Dr D Sesardic

Introduction

Botulinum toxin type A induces muscular paralysis following specific binding and uptake by the pre-synaptic nerve terminal, and subsequent cleavage of SNAP25, a protein essential for the release of neurotransmitter at the synaptic junction. There are three separate functional regions of the toxin responsible for specific binding, translocation and enzymatic activities respectively. Loss of any one of these activities will result in an inactive toxin molecule, as assessed by a fully functional model such as the mouse LD_{50} test. Following the three R's principle, alternative fully functional assays are required. Use of the ex-*vivo* phrenic nerve hemidiaphragm assay therefore, full-fills this criteria.

Electrical stimulation of the phrenic nerve causes twitch responses in the focally innervated mouse diaphragm muscle. The diaphragm muscle consists of a thin sheet which remains viable for hours in an organ bath, allowing the inhibitory effects of botulinum neurotoxin to be directly measured. Toxin potency is directly measured by the degree of muscular paralysis induced by the toxin acting on the pre-synaptic nerve terminal. Activity of a toxin batch is estimated relative to a product specific toxin reference material of defined activity.

Materials / Methods

Equipment

Computer: Dell Optiplex with 1.25GB RAM

PowerLab/4SP 4 channel recorder (ADInstruments, UK)

Bridge Amp ML110 (ADInstruments, UK)

4 Channel Dual Impedence Stimulators (ADI, Digitimer, UK)

Isometric force transducers, GM 2 type (FMI, Germany)

Hemidiaphragm Equipment / tissue baths / holders (FMI, Germany)

Thermocirculator (Harvard Apparatus, UK)

95% Oxygen/5% Carbon dioxide Gas cylinder (BOC, UK)

Gas Regulator (BOC, UK)

Extractor Fan / filter Kit 2000 with hood and lamp (Nederman/ VWR International)

Low energy light bulb 20w (equivalent to 100w) fitted to lamp (Various)

Small tissue clips / green braided polyester suture 2 metric 3-0 UPS (FMI, Germany)

Calibration weight 5g (Ohaus).

Black thread (Various)

Krebs Gelatine Ringer solution

Gelatin / NaCl / KCl / KH_2PO_4 stock (4X store sterile at 4-8°C, warm to RT before use)
27.6g/L or 55.2g/2L NaCl
1.44g/L or 2.88g/2L KCl
0.64g/L or 1.28g/2L KH_2PO_4
8g/L or 16g/2L gelatin
Heat until all the gelatin is completely dissolved, adjust volume with ion-exchanged, distilled water to exactly 1 or 2L, mix, aliquot 4X 500ml and autoclave.

Sodium hydrogen carbonate stock / $NaHCO_3$ (833mM) – 21g per 300ml

Magnesium sulphate heptahydrate stock / $MgSO_4.7H_2O$ (120mM) – 2.96g per 100ml

Calcium chloride 2-hydrate stock / $CaCl_2$ $.2H_2O$ (250mM) – 3.68g per 100ml

D-glucose / $C_6H_{12}O_6$ (2.22M) – 39.6g per 100ml

Store all stock solutions at 4-8°C.

Make up 1L of fresh Krebs solution daily from the stock solutions:

To exactly 250ml of the gelatin stock add approximately 600ml distilled water whilst mixing on a magnetic stirrer, then add 30ml of the Sodium hydrogen carbonate stock solution and 10ml of each of the Magnesium sulphate heptahydrate stock and the Calcium chloride 2-hydrate stock solutions and 5ml of the D-glucose stock (2.22 M) or 1.98g and adjust the pH to 7.5 if required and finally the volume to exactly 1L.

Krebs with the final composition should be produced:

Gelatine (0.2%), NaCl (118mM), KCl (4.83mM), KH_2PO_4 (1.19mM), $NaHCO_3$ (25mM), $MgSO_4.7H_2O$ (1.2mM), D-glucose / $C_6H_{12}O_6$ (11.1 mM), $CaCl_2$ $.2H_2O$ (2.54mM).

This solution is continuously oxygenated with carbogen (95% Oxygen/5% Carbon dioxide) gas.

Method

(i) Turn on PowerLab - The left most indicator on the front panel, the Power indicator, should glow green. The second indicator from the left of the front panel, the Status indicator, should flash yellow 4 times (indicating a SCSI ID number of 4). The status indicator will then turn off (If not consult Powerlab manual). Turn on computer and monitor.

(ii) Access chart recorder

Double click the mouse on; 4 channels icon, Chart v5 should open. Settings saved should be: 400 samples/sec. High pass = DC, Low pass = 200Hz, Range = lmV. Digital filter = Low pass / 40Hz Cut off frequency.

Data pad settings: Time, Comment text, Ch1 freq, Ch2 freq, Ch1 Avg, Ch2 Avg, Ch3 freq, Ch4 freq, Ch3 Avg, Ch4 Avg, Comment time.

(iii) Calibration

Click mouse on (set up) and go to (zero all inputs). Start chart. Using the 5g Ohaus calibration weight, attach for a short time to each transducer. Stop the trace. Select the area of trace for channel 1 to include the 5g weight area. Click on (channel 1) and go to (units conversion). Select area of the base line and click the boxed arrow pointing to zero units. Select area representing the 5g displacement and click on the boxed arrow pointing to 5 units. Click on (apply), then (O.K.)

Repeat the procedure for all remaining channels.

(iv) Dissection / set up.

Wrap an ice block with blue roll. Place some Krebs (gassed with 95% O_2 / 5% CO_2) on a separate ice block. Obtain 20-30g (male) MF1 mice from the B.S.S. (order at ~ 20-25g). Transfer the mice in a spare cage to the dissection area. Kill the mice by cervical dislocation (A schedule 1 method). Avoid over stretching the mice as this may damage the diaphragms neuromuscular junction / nerve or severe the phrenic nerves. Place the mice under the lamp/extractor hood on the ice block.

Lay the animal on its back and remove the fur and skin covering the upper part of the abdomen and thorax. Remove the muscle layers covering the chest and expose the rib

cage. Lift the rib cage along the midline with a pair of forceps and make an incision half way between the xiphisternum and the neck. Examine inside the thoracic cavity to ensure that the phrenic nerves are not adhering to the chest wall as this occasionally happens. If this is the case, gently dislodge the nerves. Make lateral cuts on either side of the midline incision, parallel to the ribs, and fill the cavity with cold gassed Krebs solution. Lift the caudal edge of the incised rib cage and remove all the ribs except the one attached to the diaphragm on the animals left side. Carefully free the left phrenic nerve of connective tissue / fat and tie a short length of dark coloured thread around the uppermost part of the nerve and cut the nerve above the attached thread. Cut the wall of the abdomen to free the rib cage. The diaphragm and phrenic nerve should be irrigated regularly with cold gassed Krebs. Holding the xiphisternum with forceps cut straight down through the diaphragm to the spine, taking care not to cut or stretch the phrenic nerve. Cut the diaphragm laterally from its attachment to the body wall so freeing the preparation from the animal. Place the nerve and muscle in a petri dish containing gassed Krebs and trim the ribs / diaphragm to a suitable size to fit in the tissue bath. Attach a small tissue clip with an attached loop of thread to the tip of the tendonous tissue at the apex of the diaphragm preparation for later attachment to the transducer.

Quickly but gently locate the centre of the rib on the platinum spike electrode of the tissue holder and impale to secure the preparation. Gently pull the thread attached to the nerve through the ring electrode. Hook the loop from the tissue clip / diaphragm onto the force transducer and lower the mounted preparation into the organ bath containing gassed warmed Krebs. Adjust the tension to give a baseline tension of 1 - 1.5g (15mN). Gently pull the thread attached to the nerve until the tip of nerve is pulled through the ring electrodes. The nerve may be held in place by either suspending a small weight or attaching the thread with Blue tack. Start recording and stimulation of the nerve (~3V, 1Hz, 0.2ms). Regularly adjust the baseline tension to ~ 1.5g (15mN) as required. Ensure that the preparation is adequately aerated, but over aeration will produce excessive frothing. Nerve stimulation should be maintained throughout the experiment, except during washing or direct stimulation. Tissue preparations are stimulated indirectly (via the nerve) using a supramaximal voltage

(~3V). A short burst of direct (muscle) stimulation (~50V, 1Hz, lms) may also help the tissue to stabilise.

Wash the preparation with ~6ml fresh Krebs/gelatine at least twice. Adjust the tension as required until the baseline tension is consistent and no longer needs adjustment. Once a consistent twitch and baseline tension are produced without change over a period of greater than 30min the tissue is ready for toxin addition. No more adjustments should be made to the baseline tension for the rest of the experiment. If the twitch response is decreasing after this time or the preparation is producing responses of less than 0.5 gram tension then the preparation should either be given longer to stabilise or be discarded. The twitch response should be regular and consistent, if not the tissue should again either be given longer to stabilise or be discarded. No visible change to the baseline tension or twitch size should be found 30min prior to adding toxin.

Tissue baths are maintained at 37°C and contain 6ml of Krebs / gelatine oxygenated with 95% O_2 + 5% CO_2. A short burst of direct (muscle) stimulation (~50V, 1Hz, lms) is applied shortly (5-10min) before toxin addition and at the end of the experiment as an added control.

(v) Experimental protocol

Reconstitute toxin vials (therapeutic or reference preparations) typically with 1ml of gassed Krebs / gelatine. Dilute to the desired concentration (typically between 1 – 20 LD_{50} U/ml) to give a total volume of 6ml Krebs / gelatine in a labelled bijou, mix gently and incubate at 37°C for 30 min. Stop the chart and drain the bathing Krebs solution, quickly but carefully add the toxin solution (pouring the toxin solution down the side of the tissue bath and not directly onto the tissue or thread connected with the force transducer) and immediately start the chart (Stimulation is automatically stopped when controlled by the PowerLab if the chart is not recording). Appropriately label the toxin addition on the chart. Click on the *, located at the bottom left of the chart, if a single channel is to be labelled then type the channel number and click add. If all channels are to be labelled then enter * and label the chart appropriately and click on add.

Typically after up to three and a half hours after adding the toxin, directly (muscle) stimulate the tissue and stop the chart. Click the mouse onto the (save) icon and appropriately label the file and save.

Discard or remove tissue and store for further studies. Discard remaining Krebs. Lower tissue holders back into the tissue baths and wash out thoroughly with tap water followed by distilled water. Once every month or as necessary clean the system with dilute (10%) sodium hypochlorite solution. Ensure all hypochlorite is thoroughly washed out of the system with several washes of distilled water and spills have been thoroughly washed off (as it will kill the tissue and is highly corrosive). The gas should not be turned off during cleaning or washing to prevent the back flow of liquid into the gas pipe. Tissue baths and tissue holders should be bathed and washed with fresh Krebs/gelatine thoroughly before further use.

Antibody neutralisation of toxin is assessed by premixing a fixed toxin concentration with the antitoxin and incubating for 30 minutes at 37°C before addition to the tissue at T0. An identical dose cycle is used to that shown above.

(vi) Data analysis:

Measure the average twitch height (10-20 twitches) before toxin addition and at 10 min intervals following toxin addition. Express as a percentage of twitch height before toxin addition. Plot the percentage twitch height against time and calculate the time to 50% inhibition.

Following toxin addition the twitch size should vary by less than 10% over the first 30 min. Any data falling outside these limits should be either excluded or if the decrease occurs in the first 10 min due to tissue movement but then remains stable, the data may be recalculated with 100% contraction set at 30min post toxin addition. (It is, however, preferable to have 5% or less deviation if possible.).

Using a reference toxin preparation with a known potency perform a dose response curve using at least 4-6 replicates per dose. Unknown toxin concentrations can then be calculated by repeatedly testing (at least 4 times) at a suitable dilution which can be

read off the linear part of the dose response curve. Other suitable methods of calculating the relative potency such as parallel line analysis may also be considered.

NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

In vitro SNAP-25 endopeptidase Immunoassay for potency testing of botulinum type A toxin preparations

Written by: Y Liu

Authorised by: D Sesardic

INTRODUCTION

The L-chain of Botulinum neurotoxin type-A is known to contain a zinc-dependent endopeptidase enzyme whose toxic action results from cleavage of the critical synaptic protein SNAP-25. The assay is based on the immunochemical estimation of the cleavage of the SNAP-25 substrate by BoNT/A. A fragment of SNAP-25 spanning the toxin cleavage site (137-206) is immobilized onto wells of a microtitre plate and subsequently treated with BoNT/A. This results in a new epitope ($SNAP\text{-}25_{190\text{-}197}$) being exposed which was previously concealed. This new epitope is then measured with a specific antibody (anti-$SNAP\text{-}25_{190\text{-}197}$) to obtain an estimate of toxin endopeptidase activity.

MATERIALS AND REAGENTS

Materials

NUNC Maxisorb ELISA plates
Graduated pipettes (25ml, 10ml, 5ml)
Multichannel pipette and tips
Gilson (P1000, P200, P100, P20) (in calibration)
Measuring cylinder (1L)
Multiscan ELISA plate reader running Genesis software
pH meter (in calibration)
Electronic balance (in calibration)

Reagents

Coating Buffer (0.1M $NaHCO_3/CO_3$, pH 9.6)
Phosphate Buffer Saline (PBS)
PBS + 0.05% Tween-20 (PBST)
Marvel (skimmed milk powder)
HEPES (Sigma, H-3375, FW: 238.3)
Bovine Serum Albumin (BSA, Sigma, A0281, fatty acid free, globulin free)
DL-Dithiothreitol (DTT, Sigma, D5545)
Synthetic peptide substrate $SNAP\text{-}25_{137\text{-}206}$

Toxin:
Botulinum Toxin A (in house product specific reference, product batch)

Pre-reduction buffer: 50mM HEPES-NaOH, pH 7.0, + 20µM $ZnCl_2$, + 10mM DTT

Reaction Buffer: 50mM HEPES-NaOH, pH 7.0, + 20µM $ZnCl_2$, + 5mM DTT + 1mg/ml BSA

Substrate: Synthetic SNAP-25 peptide ($SNAP\text{-}25_{137\text{-}206}$):

70aa substrate (GGFIRRVTND ARENEMDENL EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT KMLGSG) [Ekong *et al*, 1997]. Synthesized and purified (>80% pure).

Stock solution @10mg/ml aliquoted and stored at -20C.

Detecting antibody: Primary Antibody: Site-specific anti-peptide (CTRIDEANQ) antibody raised in New Zealand White rabbits. Specificity as reported in Ekong *et al*, 1997, Ekong *et al*, 1995.

Secondary Antibody: Goat anti-rabbit HRP conjugate (Sigma, A0545)

Substrate: 2, 2'-Azino-bis (3-ethylbenzthiozoline 6 sulfonic acid) (ABST) (Sigma, A9941)

Calibration checks and equipment checks related to this SOP

Before commencement of assay the following equipment calibrations must be carried out:

1. Calibrations of balance according to Bacteriology (NIBSC SOP QM005)
2. Calibrations of pH meter to pH 4 and 7
3. Calibrations of Gilson pipettes annually by qualified technician and a four monthly validation check laboratory personnel (NIBSC SOP PIP)

Unless otherwise stated in the SOP there is no requirement to use volumetric glassware in traceable calibration of the preparation of reagents, solutions or dilutions used in this SOP. Semi-automated pipettes, disposable plastic graduated pipettes, syringes, measuring cylinders and glassware are appropriate to the volumes being used, are adequate for this purpose. Volumes less than 1ml are dispensed using Gilson pipettes in calibration. All reagents used in the preparation of solutions should be General Purpose Reagent grade, unless otherwise stated.

PROCEDURE FOR ENDOPEPTIDASE ASSAY

Immobilisation of SNAP-25 substrate

1. Prepare solution of 2µg/ml synthetic SNAP-25 substrate in coating buffer.
2. Add 100µl/well of SNAP-25 substrate solution to 96-well plates
3. Incubate at 4^{o}C overnight.
4. Next day, wash plates 3x in PBST.
5. Add 150µl/well of 5% Marvel in PBST (M-PBST) to block.
6. Incubate for 1h at 37^{o}C in a humidified box
7. Wash plates 3x with dH_2O and blot dry for immediate use

N.B Sealed plates can be stored at -20^{o}C for up to 2 months.

Treatment of immobilised substrate with toxin

1. For toxin samples with 100U/vial: 8 vials of toxin reference, 6 vials of toxin test sample are needed
   For toxin samples with 500U/vial: 4 vials of toxin reference, 3 vials of toxin test sample are needed
   (NB. 2 Samples can be tested in 1 assay)

2. Label toxin sample vials as follows:
   S1, S2, S3
   T1, T2, T3 (if more than 1 sample is to be assay)
   Label product specific reference vials as R1, R2, R3, R4, A (plate control and main reference)

3. Reconstitute test sample(s) and product specific reference toxin sample in Pre-reduction buffer as follows:

Carefully remove caps and stoppers ensuring that none of the material is lost.

Sample A (100U/vial): Add 100µl/vial of Pre-reduction buffer.
Pool vials of the same preparation to give a total of 3 samples vials and 4 reference vials.

Sample B (500U/vial): Add 300µl/vial of Pre-reduction buffer

4. Gently mix to ensure all the material is dissolved and incubate vials for 20mins at 37°C.

5. Place SNAP-25 coated plates on ice. (if plates have been stored, wash plates 3x in dH_2O and blot dry prior to placing on ice)

6. Remove vials from incubator and place on ice.

7. Add 180µl/well of Reaction buffer to row A
   Add 100µl/well of Reaction buffer to the remaining wells (rows B-H)

8. Add 20µl reduced toxin per well to Row A according to the plate layouts show below.

   The plate layout is designed to minimize the edge effect and the main reference (A) is located in the centre of the plate to avoid any variations in the absorbance.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
PLATE 1	S1	S1	R1	R1	T1	T1	A	A	T2	T2	S2	S2
PLATE 2	T2	T2	S2	S2	R2	R2	A	A	T3	T3	S3	S3
PLATE 3	T3	T3	S3	S3	T1	T1	A	A	R3	R3	S1	S1
PLATE 4	T2	T2	S2	S2	R2	R2	A	A	R3	R3	S1	S1

9. Perform doubling dilutions down each plates straight after adding sample/reference to row A

10. Seal the plates individually with self-adhesive tape and incubate plates in a humidified container for 60mins at 37°C (do not stack).

Estimation of immobilized intact and cleaved SNAP-25 substrate

1. Wash toxin treated plates 3x in PBST. Blot dry.
2. Add 100µl/well of 5µg/ml R-14 in 2.5% M-PBST (Primary-Ab)
3. Incubate for 90mins at 37^{o}C
4. Wash plates 3x in PBST. Blot dry.
5. Add 100µl/well of 1/2000 goat anti-rabbit-HRP conjugate in 2.5% M-PBST
6. Incubate for 90mins at 37^{o}C
7. Add 100µl/well ABTS substrate solution
8. Allow colour to develop at room temperature (leave for ~30-45mins)
9. Shake and read absorbance at 405nm using the ELISA plate reader.

Statistical Analysis

1. Absorbance reading obtained from Multiscan plate reader is transferred to word document with the appropriate plate layout and dilutions.

2. Using the bioassay program RANDOM, three points are chosen that are liner and parallel to the plate control reference (reference). The potency of the test samples is calculated relative to the main reference (A) by multiply the potency value of the test sample (not the log form) by the assigned unit/vial value of the main reference. Thus expressing the potency relative to the reference.

3. The potency values of the test samples is entered into Excel work sheet to calculate the overall mean of the test samples (± S.D) [see example]

4. The mean potency value of the test samples obtained from the *in vitro* endopeptidase assay needs to fall within the Manufacturer's Specifications and limits (±15%) or the European Pharmacopoeia specifications and limits (±20%) in order to be released by NIBSC.

   If test sample fails to meet either specification the sample is assayed in vivo using the mouse local flaccid paralysis assay.

Example:

Layout

	1	2	3	4	5	6	7	8	9	10	11	12
Plate1	S1	S1	R1	R1	T1	T1	A	A	T2	T2	S2	S2
Plate2	T2	T2	S2	S2	R2	R2	A	A	T3	T3	S3	S3
Plate3	T3	T3	S3	S3	T1	T1	A	A	R3	R3	S1	S1
Plate4	T2	T2	S2	S2	R2	R2	A	A	R3	R3	S1	S1

Vial	**Replicate 1**	**Replicate 2**	**Replicate 3**	**mean**		**plate control (REF)**	
S1	711	598	532	613.7		650	
S2	732	499	433	554.7		605	
S3	502	515		508.5		597	
						537	
						593	
						596.4	**40.27**
		Overall mean (S.D.) of # Test Sample =		558.9	52.71		

BUFFERS FOR ELISA

A. Phosphate Buffered Saline pH.7.4 (20x)

CHEMICAL	AMOUNT
Sodium chloride	800g
Potassium dihydrogen orthophosphate	20g
Di-Na-H-orthophosphate (dihydrate)	143g
Potassium chloride	20g
Distilled Water	Made up to 5 Litres

B. PBS/0.05% Tween

CHEMICALS	VOLUME
20x PBS solution	250ml
Tween 20	2.5ml
Distilled Water	Made up to 5 Litres

C. 0.05M Citric Acid, pH 4.0 (ABTS Substrate buffer)

CHEMICALS	AMOUNT	AMOUNT (2.5L)
Citrate monohydrate	10.51g	26.28g
Distilled Water	Made up to 1 Litre	Made up to 2.5 Litres

D. Carbonate buffer, pH 9.6 (ELISA plate coating buffer)

CHEMICALS	VOLUME	VOLUME (2.5L)
Sodium carbonate, 0.015M	1.59g	3.98g
Sodium Hydrogen carbonate,0.035M	2.93g	7.33
Distilled Water	Made up to 1 Litre	Made up to 2.5 Litres

- Store at 4^{o}C for up to 2 weeks. For long term storage, autoclave (121^{o}C for 15mins), store up to at 4^{o}C

E. 50mM HEPES-NaOH buffer, pH 7.0 + 20µM $ZnCl_2$

CHEMICALS	AMOUNT	AMOUNT (2.5L)
50mM HEPES	2 litre	2.5 Litres
10mM $ZnCl_2$	4ml	5ml

- 50mM HEPES: 11.915g in 1 litre dH_2O
- 10mM $ZnCl_2$ (FW: 136.3): 0.0136g in 10ml dH_2O
- Dilute $ZnCl_2$ to 20µM in HEPES buffer (1/500 dil). Adjust pH to 7.0 using 10M NaOH and Store at 4^{o}C.

NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

Non lethal mouse local muscular paralysis assay:

In vivo assessment of botulinum type A toxin

Written by: Dr R G A Jones

Authorized by: Dr D Sesardic

Introduction

Botulinum toxin type A induces muscular paralysis following specific binding and uptake by the pre-synaptic nerve terminal, and subsequent cleavage of SNAP25, a protein essential for the release of neurotransmitter at the synaptic junction. The non-lethal muscular paralysis assay relies on the measurement of the local flaccid muscular paralysis at the inguinocrural region of mice injected with a low dose of botulinum toxin (Takahashi *et al.* 1990; Sesardic *et al.* 1996). Toxin potency is reflected by the degree of muscular paralysis induced by the toxin. Activity of a toxin batch is estimated relative to a product specific toxin reference material of defined activity. The method is regulated under the UK Animal (Scientific procedures Act) 1986. It is the responsibility of the operator to ensure that the procedures they carry out are performed strictly in accordance with the method stated on the project licence and that their personal licence covers the techniques they are going to undertake.

Equipment:

class II EPC safety cabinet
sterile syringes 1ml
sterile needles 27G x 0.5" (0.4 x 12mm), and 21G x 1.5" (0.8 x 40mm)
pH meter
heater / stirrer
electronic balance
racks for tubes

Reagents and Chemicals:

Gelatin Phosphate Buffer, pH 6.5 (GPB)	
0.2% Gelatin	2g
0.05M Disodium hydrogen orthophosphate (2 hydrate)	8.9g
Water (Milli Q)	up to 1L

- Warm and stir the mixture until all the solids have dissolved.
- Allow to cool to room temperature and adjust the pH to 6.5 with 50% orthophosphoric acid and make up to 1L.
- Decant solution into 150ml glass bottles and sterilize by autoclaving at 121°C for 25 minutes. Store solution at +4°C.

Sample storage
Samples of botulinum type A toxin are stored according to the manufacturer's instructions. E.g. either in the fridge 4 to 8°C or freezer -15 to -45°C, as appropriate.

Animals:

Female mice, strain MF1 (Harlan) weighing 17-22g, are allowed to acclimatise according to in house standard procedures. Groups of 4 mice per cage are used for testing and each mouse is colour coded for identification. Animals are housed as indicated in the in house SOP for animal husbandry with free access to food and water.

Procedure:

Note – all work should be done in a class II EPC safety cabinet or a clean working area as appropriate.

Preparation of dilutions:

Using a 1ml syringe and needle (21G x 1.5") vials are carefully reconstituted with 1ml of GPB (Add GPB so that it strikes the wall of the vial first, to avoid vigorous agitation of the toxin) to give nominal potencies of 500U/ml assuming 500U/vial or 100U/ml assuming 100U/vial. Mix gently making sure that all the powder is dissolved and allow to stand at room for approximately 10 minutes.

Combine contents of duplicate vials using a fresh syringe and needle or after removing the vial stoppers and mix and dilute to 50U/ml (e.g. 100µl of 500U/ml nominal potency + 900µl GPB or 200µl of 100U/ml nominal potency + 200µl GPB).

A suitable range of dilutions should be made, e.g.:

Concentration U/ml	**Amount (ii) toxin µl**	**Amount GPB µl**	**Sample**
2.1	210	4790	REF, sample 1 (and 2)
1.5	150	4850	REF, sample 1 (and 2)
1.0	100	4900	REF, sample 1 (and 2)
0.5	50	4950	REF, sample 1 (and 2)

or

Concentration U/ml	**Amount (ii) toxin µl**	**Amount GPB µl**	**Sample**
1.5	150	4850	REF, sample 1 (and 2)
0.8	80	4920	REF, sample 1 (and 2)
0.4	40	4960	REF, sample 1 (and 2)
0.25	25	4975	REF, sample 1 (and 2)

Syringes (1ml with 27G x 0.5") are numbered and samples randomised before injection in two blocks of cages so that each dose of each preparation occurs once in each of the two blocks. Randomisation is carried out for each assay, and two examples of this randomization are shown below using one or two test samples.

Cage / syringe number	**Sample**	**Dose**	**Number of Mice**
1	-ve control GPB	0	4 per cage
2	REF	2.1 U/ml	4 per cage
6	REF	1.5 U/ml	4 per cage
9	REF	1 U/ml	4 per cage
7	REF	0.5 U/ml	4 per cage
4	Test Sample 1	2.1 U/ml	4 per cage
8	Test Sample 1	1.5 U/ml	4 per cage
5	Test Sample 1	1 U/ml	4 per cage
3	Test Sample 1	0.5 U/ml	4 per cage
17	REF	2.1 U/ml	4 per cage
12	REF	1.5 U/ml	4 per cage
16	REF	1 U/ml	4 per cage
14	REF	0.5 U/ml	4 per cage
10	Test Sample 1	2.1 U/ml	4 per cage
11	Test Sample 1	1.5 U/ml	4 per cage
13	Test Sample 1	1 U/ml	4 per cage
15	Test Sample 1	0.5 U/ml	4 per cage

Cage / syringe number	Sample	Dose	Number of Mice
1	-ve control GPB	0	4 per cage
2	REF	2.1 U/ml	4 per cage
12	REF	1.5 U/ml	4 per cage
6	REF	1 U/ml	4 per cage
7	REF	0.5 U/ml	4 per cage
4	Test Sample 1	2.1 U/ml	4 per cage
11	Test Sample 1	1.5 U/ml	4 per cage
5	Test Sample 1	1 U/ml	4 per cage
13	Test Sample 1	0.5 U/ml	4 per cage
3	Test Sample 2	2.1 U/ml	4 per cage
8	Test Sample 2	1.5 U/ml	4 per cage
9	Test Sample 2	1 U/ml	4 per cage
10	Test Sample 2	0.5 U/ml	4 per cage
17	REF	2.1 U/ml	4 per cage
25	REF	1.5 U/ml	4 per cage
20	REF	1 U/ml	4 per cage
21	REF	0.5 U/ml	4 per cage
18	Test Sample 1	2.1 U/ml	4 per cage
19	Test Sample 1	1.5 U/ml	4 per cage
22	Test Sample 1	1 U/ml	4 per cage
15	Test Sample 1	0.5 U/ml	4 per cage
24	Test Sample 2	2.1 U/ml	4 per cage
16	Test Sample 2	1.5 U/ml	4 per cage
14	Test Sample 2	1 U/ml	4 per cage
23	Test Sample 2	0.5 U/ml	4 per cage

Dosing and monitoring of animals:

Eight mice (2 cages of 4) receive an injection (s.c.) of 0.1ml volume of each dilution in the left inguinocrural region. Two operators are required to administer the injections, one person to hold the animal while the other carefully injects 0.1ml taking care not to inject too deeply.

Following injection of botulinum toxin, the animals are checked for signs of muscular paralysis at the inguinocrural / abdominal region at 24h and 48h post-injection (sometimes at 30h if required). Any animals showing slight signs of systemic toxicity should be more regularly monitored and culled immediately if moderate or severe signs of toxicity develop.

Scoring:

Scoring should be independently performed by trained individuals, preferably blind to the randomisation. Colour coded mice are picked up individually by their tail and scored independently by each observer.

0 : No signs, normal
1: Just detectable (slight bulge at injection site). E.g. Covering an area of approximately 0.5cm diameter or less (or less than two nipples).

2 : More pronounced bulge. E.g. Covering an area of greater than 0.5cm diameter (or greater than or equal to two nipples), but less than the maximum radius of the hind leg heal.
3: More extensive bulge extending over a larger area. Extending below hips / top of thigh when viewed from the side and beyond the maximum radius of the hind leg heal.
4: Maximal local effect. More extensive bulge extending over a larger area will often extend as far as the bottom of the rib cage, or over a large area with extensive distension or bulging.

'Beyond a 4'.: When high concentrations of toxin are used a No4 bulge may flatten out with time (e.g. at 48h) or go 'beyond a 4'. This may be an early (e.g. 24h) indicator of subsequent systemic signs of toxicity.

Examples of scoring forms are shown below. Additional sheets are used if additional times or other characteristics of the mice, such as weight, are recorded.

Title: Botulinum toxin (type A) Non lethal assay (PPL 80/_____) Date of assay: _____. Test ID number:_____
M1=Blue head, M2= Blue tail, M3=Pink head, M4=Pink tail.

Time Scored: _____ Observer Initials:_____

Time	Cage	M1 Blue H	M2 Blue T	M3 Pink H	M4 Pink T	Total score	Comment
24hr	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						

Time Scored: _____ Observer Initials:_____

Time	Cage	M1 Blue H	M2 Blue T	M3 Pink H	M4 Pink T	Total score	Comment
48hr	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						

Data analysis for the consistency of scorers:

The scores from independent inspectors for each dilution are collected. Consistency between inspectors is assessed. If less than 60% of mice are scored identically and / or more than 4% of the mice have a score difference of 2 or more then the data should be referred to a statistician for a more detailed consideration of the differences between inspectors.

Data analysis for potency:

Reference concentrations should be adjusted for any difference between the actual assigned value and the assumed value of 500 or 100 U/vial (e.g. Actual assigned value divided by assumed value of 500 or 100U X Assumed dilution concentration).

The mean score for each cage is calculated and dose response curves plotted. Activity of the test sample is estimated by comparing with the response obtained with the reference standard. Using the linear region of the dose response curve, a parallel line analysis is performed and potency of test sample calculated relative to the reference standard. Product specific standard of defined activity is included in each assay.

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March 10, 2006

Dr. William Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

Via email to: niceatm@niehs.nih.gov

Dear Dr. Stokes,

On behalf of The Humane Society of the United States (HSUS) and our 9.6 million members and constituents, I am writing in response to NICEATM's *Federal Register* notice of January 27 [FR Doc. E6-1019], which announced a proposed expert workshop on potential alternatives to the LD_{50} assay for assessing the potency of botulinum toxin-based products, to be conducted as a high priority. We appreciate ICCVAM/NICEATM's responsiveness to The HSUS' Test Method Nomination, "Nomination of Alternative Methods to Replace the Mouse LD_{50} Assay for Botulinum Toxin Potency Testing." The HSUS fully supports the decision to hold the expert workshop as a high priority.

The HSUS views the proposed workshop as an important first step in replacing animal use in the manufacture of Botox® Cosmetic and similar products. There are a number of potential alternatives to the LD_{50} assay for this purpose, and these are in various stages of development by different laboratories around the world. What is needed now is a comprehensive assessment of the existing non-animal methods and their associated data, to determine which show the most promise and what needs to be done to complete the development and validation of the most promising test methods. Also needed is an assessment of what interim steps should be taken to reduce and refine animal use for this purpose, while promising non-animal methods are being brought to fruition. The planned workshop is a logical and efficient approach to carrying out these assessments. Of course, ICCVAM/NICEATM's assessment should be coordinated with, and informed by, related efforts in the United Kingdom and the European Union.

The HSUS was pleased with the positive comments made by Food and Drug Administration (FDA) staff at the December 12, 2005 meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), in response to ICCVAM/NICEATM's preliminary announcement of its planned expert workshop.

The FDA has an important role to play as ICCVAM/NICEATM moves forward with the workshop and subsequent activity, given that the FDA has regulatory authority over botulinum toxin-based products used for cosmetic and therapeutic purposes. The FDA's active involvement in this process would be consistent with the agency's stated commitment to alternative methods in general, and to alternatives to the LD_{50} assay in particular.

The HSUS notes that other federal agencies also have an interest in testing methods for botulinum toxin, including the Department of Defense, the Centers for Disease Control and Prevention, the Department of Agriculture, the Department of Homeland Security, and the Environmental Protection Agency. The HSUS also encourages these entities to play an active role in ICCVAM/NICEATM's planned workshop and subsequent activities.

Apart from government agencies, The HSUS believes that manufactures of botulinum toxin-based products, including Botox manufacturer Allergan, Inc., can and should make an enormous contribution to the expert workshop and follow-up activities. Allergan should have considerable data on the unspecified alternatives that the company claims to be working on, as well as years of data on LD_{50} testing of Botox products. ICCVAM/NICEATM should encourage Allergan and other manufacturers to submit their data promptly, to allow for prompt scheduling and preparation of the workshop.

The HSUS would also like to caution against an over-reliance on LD_{50} data for assessing and validating the proposed alternative methods. Mouse LD_{50} data will undoubtedly correlate poorly with biological events in humans, especially in this case where the animal test is not even assessing the clinically-relevant endpoint of muscle paralysis. With respect to the botulinum toxin, unlike in so many areas previously investigated by ICCVAM/NICEATM, the human mechanism is well characterized and serves as the basis for some of the non-animal methods, such as the SNAP-25 assay. The panel should be able to determine approaches for the validation of the proposed alternative methods that will not require additional animal studies be carried out, or that—in the short-term—can be coordinated with routine testing currently required for regulatory approvals.

We encourage NICEATM to look for experts for the workshop that will bring the essential expertise in neurotoxin mechanisms and neural cell culture to the panel. Based on our review of the technical literature, The HSUS would like to nominate the following people as experts for the workshop: Dorothea Sesardic, Elaine Neale, Edwin Chapman, and Lance Simpson. Attached, you will find further details on their expertise and contact information. We have not contacted them to ask whether they would be willing to participate in the workshop, but we recommend that they be invited to do so. We would also like to nominate Dr. Sherry Ward, an in vitro toxicologist. Dr. Ward has a comprehensive perspective on potential 3R alternatives for assessing the potency of botulinum toxin-based products, having taken the lead in drafting The HSUS' Test Method Nomination mentioned above. As a consultant to The HSUS, Dr. Ward's participation in the expert workshop would give representation to the animal protection community. I would be happy to forward her CV and contact information.

The HSUS believes that ICCVAM/NICEATM can play a crucial role in eliminating the animal suffering associated with the testing of Botox-like products, and in doing so contribute further to the worldwide effort to move beyond the heavily criticized LD_{50} assay. The consensus of the December 2005 SACATM meeting was that the use of the LD_{50} assay for assessing the potency of Botox-like products is an excellent example of the crude, outdated, and inhumane types of animal tests that ICCVAM/NICEATM should be targeting. We urge ICCVAM/NICEATM to fast track this endeavor.

Sincerely,

Martin L. Stephens, Ph.D.
Vice President, Animal Research Issues

Promoting the protection of all animals
2100 L Street, NW, Washington, DC 20037 ▪ 202-452-1100 ▪ Fax: 202-778-6132 ▪ www.hsus.org

HSUS recommendations for alternatives to mouse LD_{50} expert panel:

Name: Dorothea Sesardic
Address: Division of Bacteriology, National Institute for Biological Standards and Control, Hertfordshire, UK [2006]
Expertise: SNAP-25 assay; mouse LD50 assay

Name: Elaine A. Neale
Address: Chief of the Section on Cell Biology, National Institute of Child Health and Human Development, NIH, Bethesda, Maryland
Expertise: neural cell-based assays

Name: Edwin R. Chapman
Address: Departments of Physiology and Food Microbiology and Toxicology, and the Neuroscience Training Program, University of Wisconsin, Madison, WI 53706
Expertise: mechanisms, sensors, neural cell-based assays

Name: **Lance L. Simpson**
Address: Department of Medicine, Jefferson Medical College, Philadelphia, PA
Expertise: Cellular mechanisms research and isolated phrenic nerve-hemidiaphragm preparations used in some expts.

From: Frank Gessler
Date: Fri, 10 Mar 2006 12:00:41 +0100
To: <niceatm@niehs.nih.gov>
Subject: Comments on Federal Register January 27, 2006: Vol. 71, No. 18, page 4603; Botox-workshop

Dear Dr. Stokes,

in reply to the nomination to hold a workshop on alternative methods to replace the mouse LD50 assay for Botulinum Toxin Potency testing, published in the Federal Register (January 27, 2006: Vol. 71, No. 18, page 4603), I would like to submit the attached files:

GesslerCov.pdf Cover letter of the submission
gessler.pdf Comments on the workshop

Thank you very much,
Best Regards

Frank Gessler

_____

Frank Gessler, Dr. med. vet.
Institute of Agronomy and Animal Production in the Tropics
University of Goettingen
Germany

IBT

Institut für angewandte
Biotechnologie der Tropen
an der Georg-August-Universität
Göttingen

Institute for Applied
Biotechnology in the Tropics
at the Georg August University
Goettingen

Dr. Wiiliam S. Stokes
Director NICEATM
NIEHS
P.O. Box 12233
MD EC-17
Research Triangle Park, NC 27709
USA

by e-mail niceatm@niehs.nih.gov

Göttingen, March 9th, 2006

Federal Register January 27, 2006: Vol. 71, No. 18, page 4603
Comments on nomination of Workshop on Alternative Methods to replace the mouse LD_{50} assay for Botulinum toxin potency testing

Dear Dr. Stokes,

in-process control and batch release testing of therapeutic/cosmetic preparations of Botulinum neurotoxins (BoNT) show the need for refining or replacing the currently used mouse bioassay. Potency testing of the toxins, however, is not limited to this application. The biological activity of the toxins needs to be quantified in various clinical samples as well as in food/feed and environmental matrices. For more than ten years the Institute for Applied Biotechnology in the Tropics has focused on the lab detection and quantification of Botulinum neurotoxins type A to F: During the recent years approx. 3000 samples per year have been submitted for BoNT detection and have been examined in the mouse bioassay. Serological in vitro assays were successfully developed, established and include an immunoaffinity column and a magnetic beads assay for BoNT/C and D.

Most current in vitro methods for potency testing are limited to the quantification of the biological activity of the light chain of the toxins. We are about to focus our research efforts on the development of BoNT potency tests, e.g. a cell culture based assay, which

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Amtsgericht
Göttingen 1668

should allow for the quantification of the BoNT biological activity of the heavy and light chain as well as for the detection of neutralizing antibodies.

For your information I have attached my short CV and my list of publications on Botulinum neurotoxins and neurotoxin detection.

I would appreciate if you will consider the comments, which you will also find attached.

Sincerely Yours,

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Comments on the

Nomination to hold a workshop on Alternative Methods to replace the mouse LD_{50} assay for Botulinum toxin potency testing issued by NICEATM

Federal Register January 27, 2006: Vol. 71, No. 18, page 4603

ad (1) Information on development and/or validation activities

The Institute for Applied Biotechnology is currently involved in two developments for botulinum neurotoxin potency testing: One method aimes to quantify the peptide cleavage activity in all liquid laser desorption ionization, the second approach is a cell culture based assay, which measures the biological activity of the neurotoxins as a whole (heavy and light chain).

ad (2) Comments on the appropriateness and priority of a workshop

Currently several approaches for BoNT potency testing are under development, which have the potential to replace or at least refine the mouse bioassay. Urgent action is needed to identify the most promising techniques and the applications for them. R&D activities, in-process control and batch release testing of BoNT in therapy and cosmetics do not necessarily need the same methods as testing of clinical, food or environmental samples. However, alternative methods suitable for a variety of applications would merit the validation work with BoNT products, but with detection/diagnostic evaluations of other sample matrices as well. To conclude, a workshop would offer the unique opportunity to move forward in refining/replacing the mouse bioassay and should be given high priority.

ad (4) Submission of data from mouse LD_{50} botulinum potency testing

With approx. 3000 mouse bioassays per year, the Institute for Applied Biotechnology has gained experience in BoNT potency testing with human, veterinary, food and environmental samples, but also with BoNT preparations of various purities (culture supernatant, toxin complex, 150 kD toxin) and of almost all types (A to F). A

considerable effort is needed to carefully check and analyse the data. Thus the evaluation and the raw data can not be supplied by March, 13th, but would be available at a later stage.

PETA
PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

HEADQUARTERS
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NORFOLK, VA 23510
TEL 757-622-PETA
FAX 757-622-0457

March 10, 2006

Dr. William Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

Via electronic transmission to: niceatm@niehs.nih.gov

Dear Dr. Stokes:

These comments are submitted on behalf of People for the Ethical Treatment of Animals and our more than 1 million members and supporters in response to a January 27, 2006 notice in the *Federal Register* inviting public comment on the appropriateness and relative priority of convening a workshop addressing replacement of the mouse lethal dose 50 percent (LD50) test for botulinum neurotoxin (BoNT) potency testing. PETA supports this Humane Society of the United States (HSUS) nomination and agrees that this activity is important and appropriate and that the replacement of lethal BoNT potency tests in animals should be urgently pursued[1].

Lot release testing of biologicals consumes 10-20% of all animals used in laboratories and should be a high priority area for replacement efforts. The lot release testing of BoNT products presents an opportunity to replace a great deal of ongoing and readily avoidable animal testing. The HSUS nomination highlights the fact that an extremely cruel and outdated test (involving death by paralysis-related suffocation) is currently conducted on mice despite the fact that mechanistic human biology-based *in vitro* tests exist. The replacement of the BoNT LD50 by alternative tests could and should have happened years ago as it is both feasible and compelling. ICCVAM should build upon the momentum of the considerable efforts already expended towards this goal, and expeditiously work towards validation of the SNAP-25 assay and other *in vitro* tests.

A common barrier to *in vitro* test method development is a lack of mechanistic understanding, leading to the traditional reliance on experiments based on an unvalidated assumption of animal

[1] It should be noted that BoNT potency testing is most relevant to the U.S. Food and Drug Administration (FDA) and that HSUS apparently tried unsuccessfully to engage the FDA on this issue prior to submission to ICCVAM. ICCVAM's mandate is to provide a forum for replacing those tests which are most commonly required or used across many Agencies. However, in the absence of individual Agencies having their own formal and transparent mechanisms for validation of novel methods, increasingly, Agency-specific test methods are landing on ICCVAM's doorstep as the only means of achieving official validation in the US (of the last three ICCVAM submissions or nominations, two are mainly FDA-specific and one is mainly EPA-specific). To address this problem, Agencies should develop processes for Agency-specific method validation enabling ICCVAM to more proactively address ubiquitous and challenging endpoints such as carcinogenicity, developmental toxicity, and target organ toxicities, efforts towards which have stagnated. However, given the current lack of Agency-specific validation procedures, the current dearth of non-Agency-specific ICCVAM nominations/submissions, and the urgent imperative to replace the BoNT LD50 potency test with an available *in vitro* test, we support the use of the ICCVAM forum for this activity.

surrogacy. However, in this case, the mechanism by which BoNT leads to its paralytic and poisonous effects (inhibiting acetylcholine release through the cleavage of vesicle targeting proteins in pre-synaptic neurons) has been well studied and the relevant proteins and interactions have been thoroughly characterized. This enabled the development of functional *in vitro* assays (first published a decade ago) based on assessing the cleavage by BoNT proteins of human target peptides. One of these *in vitro* tests (the SNAP-25 assay) is already routinely used by the UK national control agency (NISBC). These well-established mechanistic human biology-based *in vitro* tests represent the best possible means of assessing BoNT product potency.

Thus, the proposed workshop should focus on how to most rapidly achieve the validation, adoption, and regulatory acceptance of the available *in vitro* tests as they give every indication of having excellent specificity, sensitivity, and speed but have not been the subject of a formal validation effort. The SNAP-25 assay should be validated for use as a standalone test wherever possible but even if is not deemed a complete replacement, this should not hold up efforts to validate and adopt it for the conditions for which it is appropriate. If there are any circumstances under which a follow-up test is necessary or the use of molecular *in vitro* tests may not be appropriate, a cell-based assay (or at worst, an *ex vivo* test) should be validated as an alternative. However, it is important not to create a system in which the majority of *in vitro* tests are followed up with another assay, especially one *in vivo* or *ex vivo*. While the alternative *in vivo* and *ex vivo* mouse assays described in the HSUS nomination involve protocols that are more clinically relevant and humane than the LD50, they should not be the focus of the proposed workshop when excellent *in vitro* tests are available. **The proposed ICCVAM workshop should strongly prioritize realizing validation of *in vitro* tests over the *in vivo* or *ex vivo* assays as the *in vitro* tests do not involve the use of animals and are likely to be more sensitive, specific, human-relevant, consistent, cost-effective, and quicker.**

As *in vitro* BoNT potency tests are assessed and especially as validation efforts are planned, it is crucial that the highly variable, less sensitive, and unvalidated mouse LD50 assay not be viewed as the gold standard. Other problems with the mouse LD50 assay include the fact that there are numerous biological differences between mice and humans which would clearly affect the nature of a mouse vs. human response to BoNT sample exposure and that it does not assess a clinically relevant endpoint (death instead of local paralysis). Thus, results from mechanistic human biology-based *in vitro* tests may not correlate with those from mouse LD50 assays. **Using LD50 results as reference data may make it more challenging or impossible to validate the *in vitro* tests.** The accuracy of validation efforts is always highest when using reference data relevant to the species of interest and biological endpoint. When human data is available or could be safely generated (e.g., through human foot method described in HSUS nomination), it should always be utilized as reference data. If this is not possible, a production consistency approach could be taken: The *in vitro* test could be run alongside the current method for a set number of batches in order to prove that its overall potency prediction aligns. **Regardless of what reference data is utilized, in no case should animals be subjected to LD50 tests solely to obtain reference data for a validation exercise.**

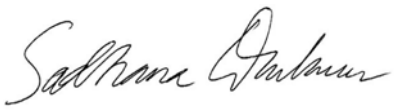
Dr. William Stokes
March 10, 2006
Page 3

A few words of caution regarding the practicalities of the proposed ICCVAM workshop: The focus should be on prioritizing the potential tests and identifying the *most expedient means* of achieving their validation. Although this workshop would constitute ICCVAM's first official effort to address this issue, much groundwork has already been laid so the process should be hastened to the greatest extent possible. The SNAP-25 assay in particular is a test based on the relevant human mechanism and has been proven to work well, and it need not take years of prolonged study, additional meetings, and new studies in order to validate it and start using it for appropriate applications. If at all possible, it should be quickly and cost-effectively validated based on retrospective data (especially if data can be obtained from the NIBSC who has used this test for years). Another concern is that the HSUS nomination mentions several types of BoNT-related tests (BoNT product tests, antitoxin tests, diagnostic tests, and so on) which have related but distinct protocols. PETA recommends that the workshop address only BoNT product potency testing in order to provide focus to the effort; once clearly validated for one use, *in vitro* BoNT-related tests can more easily be adapted and validated for other uses. In any case, it is important that the scope of the workshop be clearly defined. Lastly, any ICCVAM efforts on this topic should of course continue to be closely coordinated with ongoing or imminent efforts in Europe.

In conclusion, the submitted nomination represents an opportunity to conduct an expeditious review and work towards rapidly replacing an outdated animal test with improved alternatives. We strongly urge ICCVAM to move ahead quickly to convene a panel of experts who can make the necessary scientific judgments regarding the proposed alternative tests with a view towards a speedy affirmation of their value in assessing BoNT potency. Consumer safety, scientific rigor, and animal welfare concerns will all be best served by promoting the use of human-relevant mechanistic *in vitro* assays for botulinum toxin related testing.

Thank you for your attention and responsiveness to these comments.

Sincerely,



Sadhana Dhruvakumar
Director, Medical Testing Issues
People for the Ethical Treatment of Animals

From: Gill Langley
Date: Tue, 07 Mar 2006 09:34:16 +0000
To: <niceatm@niehs.nih.gov>
Subject: Botulinum toxin testing

Dear Madam or Sir,

I write on behalf of an English medical research charity, the Dr Hadwen Trust for Humane Research. We have 36 years' experience in funding the development of non-animal alternatives to replace animal experiments in medical research and testing (www.drhadwentrust.org.uk).

We understand that ICCVAM/NICEATM is planning to co-ordinate an expert workshop on potential alternatives to the LD50 testing of Botulinum toxin products. We support the concept of an expert workshop as an important first step in the process of implementing non-animal alternatives, and we believe that this should be a high priority. There is enormous inertia within the regulatory system and a workshop of this kind could make a big difference.

The Dr Hadwen Trust funded developmental work carried out by Dr Dorothea Sesardic at UK's NIBSC, to develop the SNAP-25 assay several years ago. We understand that that Dr Sesardic is interested in attending the proposed workshop and we believe strongly that she would make very important contributions to such a group.

Thank you for your attention.

Yours sincerely,

(Dr) Gill Langley MA PhD MIBiol
Scientific Adviser
Dr Hadwen Trust for Humane Research
Hitchin, England.

From: Andre Menache
Sent: Tuesday, February 21, 2006 11:03 AM
To: NIEHS NICEATM
Subject: Comment on the Mouse LD50 Assay for Botulinum Toxin PotencyTesting

Animal Aid UK fully supports the Humane Society of the United States campaign to replace the Mouse LD50 assay for botulinum toxin potency testing and welcomes the opportunity afforded by ICCVAM to receive submissions.

In a letter dated 13 February and signed by Mr Andy Burnham, parliamentary under secretary of state, The British Home Office indicated to Animal Aid that 'significant progress' is expected in the near future with respect to the validation of the non animal SNAP-25 assay.

Although a purely cell-based method (neural cells) to replace the use of animals at the purified bulk toxin stage is not yet available, there is no scientific need to use living animals at any stage of botulinum production.

If ICCVAM is considering a validation study of the SNAP-25, it would make sense to also validate the ex vivo mouse diaphragm method, as a replacement for the in vivo tests (the LD50 and the non-lethal endpoint mouse test). The mouse diaphragm method typically requires four (killed) mice for each purified bulk toxin, compared with up to 50 live mice using the non-lethal end point, and considerably more using the LD50.

Please do not miss this important and timely opportunity to eliminate the use of live animals in the production and testing of botulinum toxin.

Yours sincerely,

Andre Menache BSc(Hons) BVSc MRCVS FRSH
Scientific Consultant
Animal Aid
Kent / UK

From: Lockley Hannah
Sent: Monday, February 20, 2006 7:48 AM
To: NIEHS NICEATM
Subject: Nomination for work Workshop participant

Dear Dr William Stokes,

We received an e-mail on Friday 3rd February, inviting the APC to nominate participants for a possible workshop on alternative methods to refine, reduce and replace the mouse LD50 assay for Botulinum Toxin potency testing.

By way of background, the APC (Animal Procedures Committee) is an advisory, Non-Departmental Public Body established and appointed under the terms of sections 19 and 20 of the Animals (Scientific Procedures) Act 1986. Its role is to advise the Home Secretary on matters concerned with the Act and his functions under it, relating to any experimental or other scientific procedures applied to a protected animal which may have the effect of causing that animal pain, suffering, distress or lasting harm; and also, to examine other related subjects we consider worthy of further study. There are currently 21 members of the committee. Further information about the APC can be found at our website http://www.apc.gov.uk

At a recent committee meeting, the secretariat informed members of your request and the committee agreed to endorse the nomination of Dr Sesardic. Dr Sesardic is a researcher who has previously received funding from the Home Office for research into this area. Her work included progressing development of an in vitro test -

"Synthetic and recombinant technology was used to prepare the natural substrate for the botulinum type A toxin. This was used to develop an endopeptidase assay, more sensitive than the mouse bioassay, for the detection of toxin in therapeutic preparations. This has the potential ultimately to replace entirely the in vivo bioassay systems"

Dr Sesardic has recently been invited to a meeting in Strasbourg (5 April 2006) as a potential study leader of an initiative to review botulinum toxin assay methods and review the need for validation studies. Dr Sesardic is happy to be nominated as a possible workshop participant for your initiative.

Finally, in discussing this nomination at the last meeting, the committee emphasised that they view the possible workshop to be appropriate and that a high priority should be placed on progressing alternative methods to replace the mouse LD50 assay for Botulinum Toxin potency testing.

Please contact me if you require any further information.

Kind regards,

Hannah Lockley
Deputy Secretary
Animal Procedures Committee Secretariat

Dr. William S. Stokes
NICEATM Director, NIEHS
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

Dear Dr. Stokes,

I would like to thank ICCVAM for its positive response to The HSUS proposal to investigate alternatives to LD50 testing of Botox Cosmetic and similar products. I agree that holding an expert workshop on the subject is an excellent first step.

The LD50 test is an inhumane and antiquated test that should no longer be used in product testing, especially for vanity products such as Botox Cosmetic. I encourage you to follow through on your plans, so that no animal needs suffer in the manufacturing of such products.

Sincerely,

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APPENDIX E

Registered Participants

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Participant	Affiliation
Michael Adler	U.S. Army Medical Research Aberdeen Proving Ground, MD
David Allen	ILS, Inc./NICEATM RTP, NC
Pete Amstutz	Creatv Microtech, Inc. Potomac, MD
Karl Andrutis	Tufts University Cummings School of Veterinary Medicine North Grafton, MA
K. Roger Aoki	Allergan, Inc. Irvine, CA
Stephen Arnon	CA Department of Health Services Richmond, CA
Jason Barash	CA Department of Health Services Richmond, CA
John Barr	CDC/National Center for Environmental Health Atlanta, GA
Christopher Bishop	Wickham Laboratories, Ltd. Fareham U.K.
Meta Bonner	EPA Washington, DC
June Bradlaw	International Foundation for Ethical Research Rockville, MD
So-ching Brazer	Emergent BioSolutions Gaithersburg, MD
Richard Buhr	USDA-ARS Athens, GA
Thomas A. Burns, Jr.	ILS, Inc./NICEATM RTP, NC
Jerry Calver	Calver Biologics Consulting Ontario, Canada
Cheng Cao	U.S. Army Center for Health Promotion & Preventive Medicine Aberdeen Proving Ground, MD
Eric Carrier	Mentor Corp. Santa Barbara, CA
Grantley Charles	Allergan, Inc. Irvine, CA
Luisa Wai Wai Cheng	USDA Albany, CA
Neepa Choksi	ILS, Inc./NICEATM RTP, NC
Kenneth G. Clarke	Allergan, Inc. Irvine, CA

Participant	Affiliation
William Cover	Allergan, Inc. Irvine, CA
Martin Crumrine	National Institutes of Health Bethesda, MD
Elias Dahlsten	University of Helsinki Helsinki, Finland
Dario DeMedici	Instituto Superiore di Sanitá Rome, Italy
J. Oliver Dolly	Dublin City University Dublin Ireland
Shokooh Dorchehzadeh	NRCGEB Tehran, Iran
Dirk Dressler	Rostock University Rostock Germany
Joseph Eifert	Virginia Polytechnic Institute and State University Blacksburg, VA
Elena Enache	Food Products Association Washington, DC
Christine Escargueil	NIBSC Potters Bar, UK
Lucia Fenicia	Instituto Superiore di Sanitá Rome, Italy
Joan Fitzgerald	Allergan, Inc. Westport, Ireland
Pam Ford	Health Protection Agency Salisbury, Wiltshire, UK
Richard France	RegenTec Ltd. Nottingham, UK
Joseph Francis	Allergan, Inc. Irvine, CA
Christina Furseth	Mentor Biologics, Inc. Madison, WI
Rose Gaines-Das	National Institute for Biological Standards & Control Herfordshire, UK
Frank Gessler	University of Goettingen Goettingen, Germany
Bruce Gingras	IITRI Chicago, IL
Mike Goodnough	Metabiologics Madison, WI
Heather Green	Association of Public Health Laboratories Silver Spring, MD
Guenter Gross	University of North Texas Denton, TX

Participant	Affiliation
Marlies Halder	ECVAM Ispra, Italy
Mark Hallett	NIH/NINDS Bethesda, MD
Joseph Hanig	FDA/DAPR Silver Spring, MD
Coenraad Hendriksen	Netherlands Vaccine Institute AL Bilthoven, Netherlands
Alexander Huber	University of Nottingham Nottingham, UK
Abigail Jacobs	FDA/CDER Silver Spring, MD
Alan Jennings	Health Protection Agency Salisbury, Wiltshire, UK
Eric Johnson	University of Wisconsin-Madison Madison, WI
Virginia Johnson	Emergent BioSolutions Gaithersburg, MD
Alicia Karas	Tufts Cummings School of Veterinary Medicine North Graffton, MA
James Keller	FDA/CBER Bethesda, MD
Lynda Kelley	USDA Athens, GA
Renecca Kerns	United Vaccines, Inc. Madison, WI
Donald Kolbe	USDA/APHIS Ames, IA
Son Kuan	Solstice Neurosciences South San Francisco, CA
Jodie Kulpa-Eddy	USDA/APHIS Riverdale, MD
Christopher Le	Department of Defense Frederick, MD
Sue Leary	Alternatives Research & Development Foundation Jenkintown, PA
Ross LeClaire	Battelle Columbus, OH
James Lindsay	USDA-ARS Beltsvlle, MD
Miia Lindstrom	University of Helsinki Helsinki, Finland
Liz Lipscomb	ILS, Inc./NICEATM RTP, NC

Participant	Affiliation
Linda Litchfield	ILS, Inc./NICEATM RTP, NC
Mary Losikoff	FDA College Park, MD
Catherine Lyons	Allergan, Inc. Westport, Ireland
Susan Maslanka	CDC Atlanta, GA
Debbie McCarley	NIEHS RTP, NC
Richard McFarland	FDA/CBER Rockville, MD
Leslie McKinney	FDA/CDER Silver Spring, MD
Jill Merrill	FDA/CDER Silver Spring, MD
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Takahaski Motohide	National. Institute of Infectious Diseases Tokyo, Japan
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Ada Hangfong O'Donnell	UMUC Silver Spring, MD
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Barry Perry	PA Dept. of Health Lionville, PA
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Ram Ramabhadran	EPA RTP, NC

Participant	Affiliation
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Randall Ruble	Lake Immunogenics, Inc. Ontario, NY
Andreas Rummel	Institute of Toxicology Medizinische Hochschule Hannover Hannover, Germany
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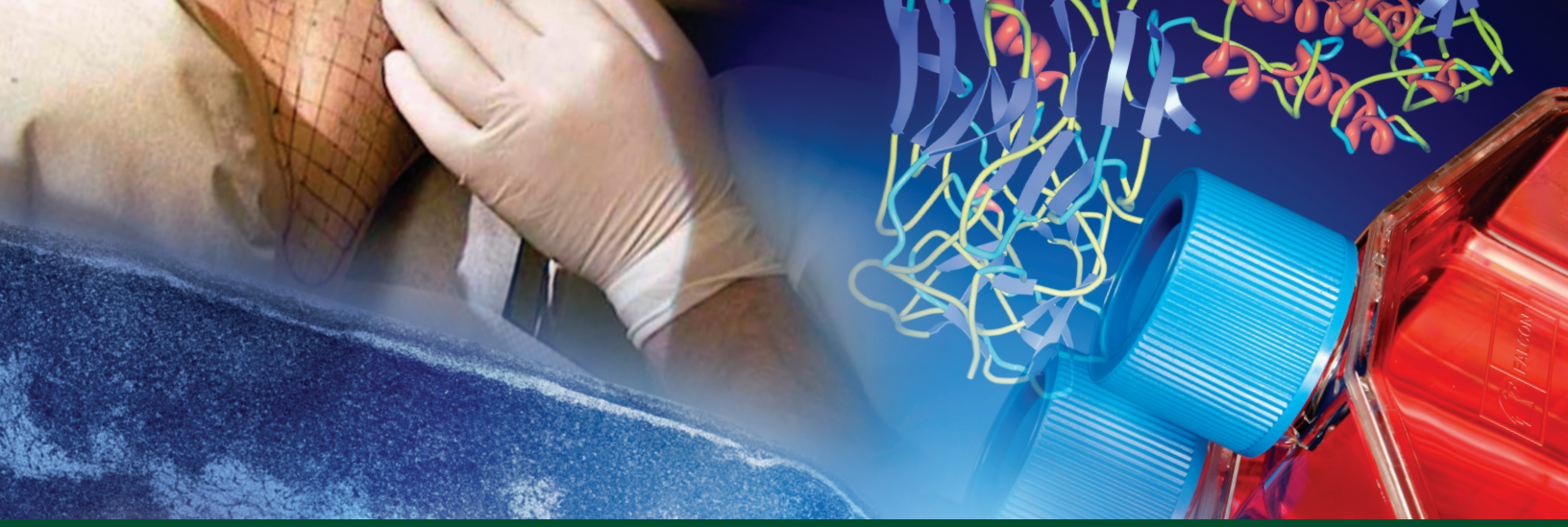
Shashi Sharma	FDA/CFSAN College Park, MD
Clifford Shone	Health Protection Agency Wiltshire, UK
Wendy Elizabeth Shores	FDA/CDER Silver Spring, MD
Paul Simon	HHS/OPHEMC Washington, DC
Lance Simpson	Thomas Jefferson University Philadelphia, PA
Bal Ram Singh	University of MA-Dartmouth N. Dartmouth, MA
Dale Slavin	FDA/OVRR Rockville, MD
Leonard Smith	U.S. Army Research Institute of Infectious Diseases Ft. Detrick, MD
Andrea Stahl	U.S. Army Research Institute of Infectious Diseases Ft. Detrick, MD
Rebekah Starner	Battelle Columbus, OH
Martin Stephens	Humane Society of the U.S. Washington, DC
Kristie Stoick	Physicians Committee for Responsible Medicine Washington, DC
William S. Stokes	Director, NICEATM NIEHS RTP, NC

Participant	Affiliation
Sandra Stringer	Institute of Food Research Norwich, Norfolk, UK
Loree Talley	The Humane Society of the U.S. Gaithersburg, MD
Cha-Mei Tang	Creatv MicroTech, Inc. Potomac, MD
Harold Taylor	Merz Pharmaceuticals, GmbH Frankfurt, Germany
Tim Terrell	Allergan, Inc. Irvine, CA
George Tin	Allergan, Inc. Irvine, CA
Xiaomi Tong	Emrgent BioSolutions, Inc. Gaithersburg, MD
Yasushi Torii	The Chemo-Sero-Therapeutic Research Institute Kumamato, Japan
Michelle Vassar	Battelle Columbus, OH
Lillian Van DeVerg	National Institute of Allergy and Infectious Diseases Bethesda, MD
Lucy Vereshchagina	Morgan Lewis Washington, DC
Maria Virata-Theimer	FDA/CBER Rockville, MD
Sherry Ward	The Humane Society of the U.S. New Market, MD
Teresa Washington	NIH Bethesda, MD
Richard Whiting	FDA/CFSAN College Park, MD
Phan Winter	Booz, Allen, Hamilton Arlington, VA
Doug Winters	ILS, Inc./NICEATM RTP, NC
Dongxiang Xia	Virginia Dept. of Health Norfolk, VA
Jing Xu	Creatv MicroTech, Inc. Potomac, MD
Haifeng Yu	Solstice Neurosciences, Inc. South San Francisco, CA
Mei-ying Yu	FDA/CBER Bethesda, MD



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NIEHS
National Institute of
Environmental Health Sciences

NTP