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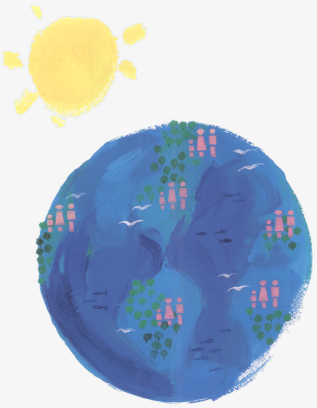

Session 4A Panel Discussion Summary: Refinement Alternatives - Using *Ex Vivo* Assays to Avoid Pain and Distress in Botulinum Toxin Testing

Dr. Elizabeth Shores & Dr. Leonard Smith, Moderators

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

Crowne Plaza Hotel, Silver Spring, MD

National Toxicology Program | NIEHS | National Institutes of Health | U.S. Department of Health and Human Services

Panel Discussion Question # 1

- **Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current e*x 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?**
  - Mouse phrenic nerve bioassay undergoing validation, and may be considered adequate for batch release testing (in Germany)
  - Intercostal NMJ assay also undergoing validation
  - Limitations include:
    - Still require animals for donation of tissues - therefore, not a replacement, rather refinement/reduction alternative
    - Technically challenging

Panel Discussion Question # 2

- **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 e*x 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?**
  - Potentially, but matrix effects have not been completely resolved
    - Sample preparation (e.g., dialysis) may improve
  - Limitations include:
    - lack of speed
    - difficulties in set up
    - complexity of equipment required

Panel Discussion Question # 3

- **Can any of the current e*x 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?**
  - Mouse phrenic diaphragm bioassay is promising
    - Validation will define exact numbers in animal reduction, but anticipated to be reduced by at least 50%
  - Limitations include:
    - These methods are complex and difficult to implement.
    - Results from *ex vivo* methods may not correlate with those from *in vivo* methods
    - Need to show true comparability to the LD50
    - Labor intensive
    - Costly, specialized equipment

Panel Discussion Question # 4

- **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 e*x 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?**
  - Not known at this time given the lack of experience with these types of samples
  - Limitations include:
    - Lack of speed
    - Difficulties in set up
    - Cost
    - Complexity of equipment required - but it is relatively robust, so initial start-up would be the biggest problem
    - Sensitivity may be a problem - but this has not been demonstrated
    - Potential matrix effects - HSA does not appear to pose a significant problem

Panel Discussion Question # 5

- **Should e*x 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?**
  - One possibility would be smooth muscle preparations from larger animals which could yield a greater number of preparations from a single animal
    - Although slaughterhouse animals not likely to be useful or appropriate for this use
  - Intercostal NMJ assay also a possibility
  - Also useful for antibody detection

Panel Discussion Question # 6

- **What are the pros and cons of the different e*x vivo* methods reviewed?**
  - Pros of the MPN assay
    - Not an in vivo animal experiment but resembles mice bioassay (donor animals used in lieu of in vivo assay)
    - Results within 2 hours
    - Experimental conditions can easily be varied
    - Can also be used to quantify neutralizing antibodies
    - Quantitative endpoints
  - Cons of the MPN assay
    - Animals are still required
    - Requires experimental skills
    - Sophisticated and costly equipment
    - Low throughput

Panel Discussion Question # 7

- **What current knowledge gaps with regard to the reviewed e*x 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?**
  - Validation studies are essential
    - Need knowledge about correlation with LD50 test results
  - Given the lack of available information, particular attention should be devoted to excipients in products and their effect on toxin mode of action on *ex vivo* muscle

Panel Discussion Question # 8

- **Of the *ex vivo* methods discussed, which should have the highest priority for further development and validation studies?**
  - All based on similar principles, but priorities likely to be unique to individual laboratories at this time
  - All need validation
    - Intercostal NMJ assay/and mouse hemidiaphragm assay has undergone validation and is now undergoing comparability studies

Panel Discussion Question # 9

- **What is the best way to assess the validation status of these *ex vivo* methods?**
  - Extensive comparability studies needed
    - Precision is likely to be poorer for the ex vivo assays (this is known for the intercostal NMJ assay) than other assays (e.g., endopeptidase assays)
      - Precision for the hemidiaphragm assay hasn't been evaluated
    - Increased precision may not be necessary to meet regulatory requirements (with reference to the LD50 assay)

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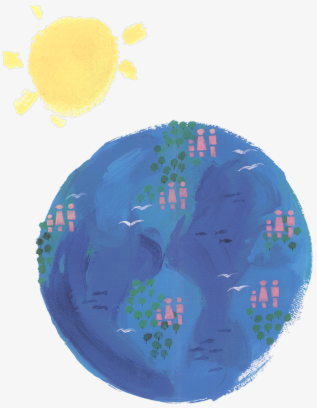

Session 4B Panel Discussion Summary: Refinement Alternatives - *In Vivo* Botulinum Assays that Do Not Require Death as an Endpoint

Dr. Leonard Smith & Dr. William Stokes, Moderators

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

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Panel Discussion Question # 1

- **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?**
  - This is a refinement alternative, but not a replacement
    - They have the potential to replace the severe LD50 endpoint with a considerably less severe procedure from which mice typically recover.
  - Limitations include:
    - Still require use of animals
    - Calibration in terms of LD50 is required since product units are in LD50s
    - Qualitative endpoints (photodocumentation) may support findings
    - Transferability/Training issues (reference photographs and a training video would help)

Panel Discussion Question # 2

- **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?**
  - Again, not a replacement but a refinement
  - Limitations include:
    - Still require use of animals
    - Materials often not well defined
      - Need to known level of toxin
    - Tolerated dose often unknown
    - Labor intensive

Panel Discussion Question # 3

- **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?**
  - May actually use more animals depending on the assay used
    - Refinement, but not reduction
  - Need validation prior to making a definitive determination

Panel Discussion Question # 4

- **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?**
  - Further validation is needed prior to deciding
  - Need to know the range of toxin concentration in the sample

Panel Discussion Question # 5

- **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?**
  - Several variations on *in vivo* models based on muscular paralysis exist in addition to flaccid paralysis or abdominal ptosis in mouse.
    - Some approaches were developed primarily to study toxin duration of action and muscle weakness.
    - Some 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 to toxin, essential for potency testing.
  - However, at present most are considered only as research tools

Panel Discussion Question # 6

What are the pros and cons of the different non-lethal *in vivo* methods reviewed?

- Pros of the Hind-Limb Assay
  - Clinical relevant measure of activity
    - local weakness
    - non-lethal endpoint
  - Applicable to other products
  - Dose response
  - Repeated measures
  - Local vs distal effects
- Pros of the Abdominal Ptosis Assay
  - Fully functional assay with dosing more similar to clinical use
  - Animals normally exhibit no signs of stress or pain and therefore requires minimal monitoring of animals
  - More humane, ethical and economical
  - Fast compared to LD50 (48 h vs 72 or 96 h)
  - No specialized equipment or reagents required
  - Robust and easily transferred to other laboratories
- Cons of Both Assays
  - Animals used (no reduction)
  - Subjective/Qualitative scoring

Panel Discussion Question # 7

- **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?**
  - Demonstrate correlation with LD50
    - Selection of suitable samples to be included in such studies will be critical
  - Demonstrate transferability and robustness.
  - Need a method published as a test guideline

Panel Discussion Question # 8

- **Of the non-lethal *in vivo* methods discussed, which should have the highest priority for further development and validation studies?**
  - Those that are fast (≤48h), user friendly, easily transferable to a quality control setting, and cause the least adverse effects on animals

Panel Discussion Question # 9

- **What is the best way to assess the validation status of these non-lethal *in vivo* methods?**
  - Need validation and comparability studies

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

Dr. William Stokes & Dr. Leonard Smith, Moderators

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

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National Toxicology Program NIEHS

Panel Discussion Question # 1

- **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?**
  - Moribund animals (i.e., those in a pre-death condition) should be euthanized
    - Caution: some animals in a moribund state may still be alive at study termination
  - Health Canada has validated and has been using an earlier non-lethal endpoint for a number of years.
    - A collaborative 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).
  - Need studies that would demonstrate the predictivity of each potential non-lethal endpoint for death within the observation period.
    - Increased frequency of observations may identify moribund animals and decrease the number of spontaneous deaths

Panel Discussion Question # 2

- **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?**
  - What is needed is clear documentation of the clinical signs and their severity that occur throughout the progression of toxicity.
    - There may be a difference in clinical symptoms (progression and timeline) among serotypes
    - Then evaluate each clinical sign (or a battery of clinical signs) and severity for potentially being used as a predictive humane endpoint
    - Identifying the clinical signs that indicate the lack of reversibility is essential to accurately predict death
    - Further investigate the signs summarized by Dr. Calvert (i.e., respiratory distress, hiccough, bulging eyes)

Panel Discussion Question # 3

- **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?**
  - They have not been investigated for their predictive value, but should be further investigated
  - 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.
  - Monitor activity levels through a subcutaneous computer chip and derive a computer algorithm to quantify
  - Telemetry could be used to collect objective data
    - Recognizing cost issues that may be associated
  - Respiratory changes could be monitored

Panel Discussion Question # 4

- **What current knowledge gaps regarding predictive humane endpoints should be addressed in research, development, and validation studies? What additional studies are needed?**
  - Collection of complete clinical signs and other objective data to identify predictive early endpoints during routine studies
  - Validation studies to identify endpoints that are not reversible and that are predictive of eventual death
  - Verify their accuracy in multiple laboratories

Panel Discussion Question # 5

- **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?**
  - Telemetric measurements
  - Daily body weights to measure systemic effects
  - Videography/photography to record observations
  - For all LD50 assays, all available scores, objective measures (such as temperature), mouse characteristics (such as weight, sex, etc.) should be collected and appropriately linked in databases
    - Used for correlation of the various measures with lethality
    - Evaluate clinical signs in the dark cycle when behaviors typically exhibited