

# LLNA Peer Review Meeting Summary Minutes

## Introduction

A public meeting of an independent peer review panel was convened on September 17, 1998, in Gaithersburg, Maryland to review the murine local lymph node assay (LLNA), which was proposed as an alternative toxicological test method for assessing contact hypersensitivity (allergic contact dermatitis) potential of chemicals and products. The meeting was coordinated by ICCVAM and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and was sponsored by the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP). The following expert scientists served on the peer review panel:

- Jack Dean, Ph.D., Sanofi Pharmaceuticals, Inc., Malvern, Pennsylvania (Panel Chair)
- Klaus Andersen, M.D., Ph.D., Odense University Hospital, Odense, Denmark
- Paul Bailey, Ph.D., Mobil Oil Corporation, Paulsboro, New Jersey
- Robert G. Hamilton, Ph.D., Johns Hopkins University, Baltimore, Maryland
- Joseph Haseman, Ph.D., National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina
- Masato Hatao, Ph.D., Shiseido Research Center, Yokohama, Japan
- Martinus Lovik, M.D., Ph.D., National Institute of Public Health, Oslo, Norway
- Howard Maibach, M.D., University of California/SF, San Francisco, California
- B. Jean Meade, D.V.M., Ph.D., National Institute of Occupational Safety and Health, Morgantown, West Virginia
- Jean Regal, Ph.D., University of Minnesota, Duluth, Minnesota
- Ralph Smialowicz, Ph.D., US Environmental Protection Agency, Research Triangle Park, North Carolina
- Peter Thorne, Ph.D., University of Iowa, Iowa City, Iowa
- Lorraine E. Twerdok, Ph.D., American Petroleum Institute, Washington, District of Columbia
- Stephen E. Ullrich, Ph.D., MD Anderson Cancer Center, Houston, Texas

## Introductions

Dr. Jack Dean, chair, called the meeting to order at 8:30 a.m., and asked each person in attendance to state their name and affiliation.

## **Welcome from the National Toxicology Program**

Dr. George Lucier, Director of the National Toxicology Program, thanked the ICCVAM participating agencies and stakeholders, the LLNA sponsors, and the peer review panel for their efforts. Dr. Lucier also provided a brief overview of the history of ICCVAM and NICEATM.

## **Introduction to ICCVAM and NICEATM**

Dr. William Stokes, ICCVAM Co-Chair and Director of NICEATM, explained the ICCVAM review process, and the steps that had been undertaken in the review of LLNA. He discussed the role of the ICCVAM committee, its expert subgroup (Immunotoxicology Working Group) and the peer review panel, and the process by which test methods are reviewed and forwarded to agencies for action. Public Law 103-43 directed the NIEHS to develop and validate alternative methods that can reduce or eliminate the use of animals in acute or chronic toxicity testing, to establish criteria for the validation and regulatory acceptance of alternative testing methods, and to recommend a process through which scientifically validated alternative methods can be accepted for regulatory use. Criteria and processes for validation and regulatory acceptance were developed in conjunction with 14 other Federal agencies and programs with broad input from the public. These are described in the document "Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods," NIH Publication 97-3981, March, 1997. This document is available via the internet at <http://ntp-server.niehs.nih.gov/htdocs/ICCVAM.htm>.

ICCVAM was subsequently established in a collaborative effort by NIEHS and 13 other Federal regulatory and research agencies and programs. The Committee's functions include the coordination of interagency reviews of toxicological test methods and communication with stakeholders throughout the process of test method development and validation. The following Federal regulatory and research agencies and organizations are participating in this effort:

- Consumer Product Safety Commission
- Department of Defense
- Department of Energy
- Department of Health and Human Services
- Agency for Toxic Substances and Disease Registry
- 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

- National Institute for Occupational Safety and Health/CDC
- Department of the Interior
- Department of Labor
- Occupational Safety and Health Administration
- Department of Transportation Research and Special Programs Administration
- Environmental Protection Agency

The LLNA was proposed to ICCVAM for consideration as a stand-alone test to identify chemicals that have the potential to cause contact hypersensitivity (allergic contact dermatitis). The test method submission was prepared by three co-sponsors: Drs. G. Frank Gerberick (Procter & Gamble, US); Ian Kimber (Zeneca, UK); and David A. Basketter (Unilever, UK). Independent peer review is an essential prerequisite for consideration of a method for regulatory acceptance (NIEHS, 1997). The peer review panel (PRP) was charged with developing a scientific consensus on the usefulness of the test method to generate information for human health risk assessment purposes. The proposed test method and results of the peer review will be forwarded by ICCVAM to Federal agencies for consideration. Federal agencies will determine the regulatory acceptability of the method according to their mandates.

### **Overview of the LLNA Peer Review Process**

Ms. Denise Sailstad, IWG Co-Chair, provided an overview of the role of the IWG in the review of the LLNA, outlining the specific accomplishments of the IWG. She reiterated the two main questions that the working group had drafted as the focus of the review. The questions were as follows:

1. Has the LLNA been evaluated sufficiently and is its performance satisfactory to support its adoption as a stand-alone alternative?
2. Does the LLNA offer advantages with respect to animal welfare considerations (refinement, reduction, and replacement)?

### **Summary of Current Agency Requirements**

Dr. David Hattan, IWG Co-Chair, summarized Federal agency and international regulations and recommendations for dermal contact hypersensitivity testing. Several test methods are currently accepted by the EPA. EPA OPPTS and the OECD (Guideline Number 405) both currently accept the LLNA as a screening test for dermal hypersensitivity. If the test results are positive, no further testing is required. However, if the LLNA test is negative, then one of the guinea pig tests must be conducted; FDA currently recommends the use of the Guinea Pig Maximization Test (GPMT) or the Buehler Assay (BA).

## **Overview of the Proposed LLNA Test Method Protocol**

Each of the test method sponsors (Drs. G. Frank Gerberick, David Basketter, and Ian Kimber) gave a brief introduction to the LLNA. Allergic contact dermatitis results from two separate but related sequential immunological events caused by a chemical substance. First, an initial exposure(s) causes a primary immune response known as sensitization. If there is additional exposure following sensitization, then a secondary immune-mediated response occurs, which is characterized by skin erythema, swelling, and pruritis. The scientific basis for the proposed LLNA test is that lymphocytes in draining lymph nodes of ears of mice proliferate as the primary response to topical exposure with chemicals that cause dermal sensitization. This proliferation is detected by measuring the amount of 3H-methyl thymidine incorporated into dividing lymphocytes. Radioactive thymidine incorporation results from increased proliferation of resident or migratory lymphocytes in the lymph node in response to the chemical challenge. The resulting data are measured on an individual lymph node basis and presented as a stimulation index (SI) after comparing the level of radioactive incorporation in treated versus the control mice. The measured lymphocyte response is an essential element in the process of sensitization. In contrast, currently accepted guinea pig assays measure skin reactivity to a secondary challenge with the test substance. Their presentations were followed by assay-related questions from the PRP.

## **Review of the LLNA submission**

The PRP then proceeded to present and discuss the various sections that they were asked to evaluate. The conclusions for each of the sections are summarized below.

## **Test Method Description**

Dr. J. Meade, the section coordinator, presented the analysis and conclusions reached by the test method description section reviewers, which included Drs. P. Bailey, M. Lovik, H. Maibach, and J. Regal.

The panel concluded that the proposed test method protocol (Local Lymph Node Assay ICCVAM Submission, April, 1998) was generally adequate, but recommended the following additions and/or changes:

1. Until a systematic comparison of data between (a) mouse strains, and (b) male and female mice are conducted, the protocol should specify the use of female CBA mice only.
2. Animals should be individually identified.
3. Body weight data should be collected at the start and end of the assay.
4. Lymphocyte proliferation data should be collected at the level of the individual animal.
5. Statistical analysis should be performed.
6. A single dose of a moderate sensitizer should be included as a concurrent positive control in each study.

7.  $^3\text{H}$ -methyl thymidine or  $^{125}\text{I}$ -iododeoxyuridine may be used in the LLNA.
8. The decision process to identify a positive response should include an  $\text{SI} \geq 3$ , statistical significance, and dose response information.
9. An illustration should be added to the protocol, indicating the nodes draining the exposure site that are to be harvested.

### **Test Method Data Quality**

Dr. L. Twerdok, the section coordinator, presented the analysis and conclusions reached by the test method data quality section reviewers, which included Drs. M. Lovik, R. Smialowicz, and S. Ullrich. The PRP recommended that retrospective data audits be conducted on at least three of the intra- and inter-laboratory LLNA validation studies conducted by the Sponsors.

### **Test Method Performance**

Dr. P. Thorne, the section coordinator, presented the analysis and conclusions reached by the test method performance section reviewers, which included Drs. K. Andersen, P. Bailey, J. Meade, and J. Haseman. The panel concluded that the LLNA performed at least as well as the currently accepted guinea pig methods (GPMT/BA) for the hazard identification of chemical sensitizing agents. The review involved the evaluation of LLNA data on 203 chemicals, of which both LLNA and guinea pig data were provided for 126 chemicals. Both LLNA and human (Human Maximization Test [HMT]/ Human Patch Test Allergen [HPTA]) data were provided for 74 of the 203 chemicals. From the analysis generated during the review process, the accuracy<sup>1</sup> of the LLNA when compared to the GPMT/BA was 89% (N = 97), and when compared to all guinea pig tests (GPT) was 86% (N = 126). The accuracy of the LLNA when compared to human tests was 72% (N = 74). The accuracy of the GPMT/BA when compared to human tests was 72% (N = 57), and the accuracy of the GPT when compared to human tests was 73% (N = 62).

Additionally, when the analysis was limited to only those compounds for which there was LLNA, guinea pig, and human data, the accuracy of the LLNA when compared to human tests and the accuracy of the GPMT/BA when compared to human tests was 72% (N = 57) in both comparisons. In terms of accuracy, sensitivity, specificity, and positive and negative predictivity, the PRP found the performance of the LLNA to be similar to that of the GPMT/BA. Equally important, the performance of the LLNA and GPMT/BA were similar in regard to human data (HMT/HPMT).

### **Test Method Reliability**

Dr. R. Smialowicz, the section coordinator, presented the analysis and conclusions reached by the test method reliability section reviewers, which included Drs. R. Hamilton, M. Hatao, J. Haseman, and P. Thorne.

The panel concluded that the data submitted for review demonstrated that the LLNA has adequate repeatability and reproducibility, and that the qualitative data demonstrated good inter- and intra-laboratory reliability.

## **Other Literature and Scientific Reviews**

Dr. S. Ullrich, the section coordinator, presented the analysis and conclusions reached by the reviewers for the other literature and scientific reviews section, which included Drs. K. Andersen, H. Maibach, and J. Regal.

This section evaluated the published literature on the LLNA that was not generated by the test sponsors. The results presented in the literature support the use of the LLNA for testing the sensitization potential of chemicals. Future protocol modifications may allow for the assay to more accurately predict the sensitizing potential of metal salts and irritants; these groups of chemicals appear to have high false positive and false negative rates, respectively, when evaluated using the submitted protocol.

## **Other Considerations**

Dr. J. Regal, the section coordinator, presented the analysis and conclusions reached by the other considerations section reviewers, which included Drs. R. Hamilton and M. Hatao.

The panel discussed the transferability of the test method, and issues relating to cost and time effectiveness. It was concluded that the test method was transferable among labs and that there is potential for the method to be more cost effective than the guinea pig assays.

## **Related Issues**

Dr. M. Hatao, the section coordinator, presented the analysis and conclusions reached by the related issues section reviewers, which included Drs. H. Maibach, J. Meade, and S. Ullrich.

This section reviewed other potential endpoints and modifications that could be considered in the future. The following workshops were recommended:

1. A workshop on the ICCVAM evaluation process focusing on providing guidance for individuals planning on making future assay submissions as well as for individuals that may be involved in the evaluation process; A workshop on the use of the LLNA for detecting the photosensitization potential in conjunction with UVA irradiation;
  2. A workshop to identify the most predictive methods for detecting immediate-type hypersensitivity following oral exposure to chemicals and drugs;
  3. A workshop to explore alternative endpoints of the LLNA; and
  4. A workshop to consider the potential of the ex vivo LLNA as well as other possible refinements.
- It was concluded by the PRP that more research is needed before such a workshop should be planned.

## **Public Comments**

Several individuals from Federal regulatory agencies made comments at the meeting with respect to issues that would be important from a regulatory standpoint. Dr. Ken Hastings, FDA/CDER, stated that their agency would want individual animal data collected in order to consider the data.

Dr. John Langone, FDA/CDRH, stated that the dataset definitely supports the use of the LLNA for detecting the sensitization potential of moderate and potent sensitizers, but that the data was not as conclusive for weak sensitizers. Because of this point, Dr. Langone recommended using statistics as part of the criteria for identifying sensitization hazard potential. He further stated that established reference statistical data would help in future refinements to the assay.

Dr. Al Munson, NIOSH, encouraged the PRP to accept the 3-fold index as the method for determining contact hypersensitivity potential. He added that this method of determination came about as a judgement factor, and that to this point, the use of this index has been adequate. Further, Dr. Munson felt that as further knowledge of the assay is collected, it may be appropriate to consider other factors, such as statistical analysis. He reiterated that the test was designed and validated using the 3-fold index, and that there was no data to support the use of a different measurement as the predictive endpoint.

Dr. Lynnda Reid, FDA/CDER, stated that her agency would like to see the use of concurrent positive controls when testing using the LLNA. Dr. Reid stated that without such controls, it would be difficult for her agency to accept negative results.

Other public comments were also offered. A representative from the Institute for In Vitro Sciences requested caution in adding items to the existing validation model. He stated that to adequately address the use of statistics instead of the 3-fold index, the data would need to be entirely reevaluated.

A representative from Eli Lilly stated that for determining if a compound is immunotoxic, a review of incidences would be important. Thus, he stated that he would want the lymph nodes to be collected at the level of the individual animal, and statistics to be used in decisionmaking.

Dr. Martin Stephens, Humane Society of the United States (HSUS), stated that HSUS is pleased with the ICCVAM process since it allows for consideration of animal welfare in new assay development. Dr. Andrew Rowan, HSUS, further stated that the HSUS would like to see alternative tests approved when they are at least as good as current animal tests; he felt that it is unnecessary (and inappropriate from an animal welfare perspective) to wait until enough data is gathered to show that the alternative method is better than the animal test.

## **Peer Review Panel Conclusions**

The peer review panel conclusions were summarized by Drs. J. Dean and L. Twerdok.

The PRP unanimously concluded to recommend the LLNA as a stand-alone alternative for contact sensitization hazard assessment, provided that the protocol modifications discussed under the test method description (above) were made.

The PRP also agreed that the LLNA had several advantages over guinea pig methods in that it provided quantitative data, allowed dose-response assessment, reduced animal distress, potentially reduced animal numbers, was potentially more cost effective, required much less time, involved the induction phase of sensitization, and will allow future refinement and mechanistic studies. Possible assay weaknesses (e.g., false negative results with some metals and weak sensitizing agents, false positive results with some strong irritants) were identified; it was concluded that these should be addressed in future workshops. Also, data to support the testing in the LLNA of mixtures was not provided and the evaluation of pharmaceuticals was limited.

### **Adjournment**

The meeting was adjourned by Dr. Jack Dean at 5:30 p.m.